ACS SYMPOSIUM SERIES **636**

Veterinary Drug Residues

Food Safety

William A. Moats, EDITOR
Agricultural Research Service,
U.S. Department of Agriculture

Marjorie B. Medina, EDITOR
Agricultural Research Service,
U.S. Department of Agriculture

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.fw001

Developed from a symposium sponsored
by the Division of Agricultural and Food Chemistry,
at the 209th National Meeting
of the American Chemical Society,
Anaheim, California,
April 2–7, 1995

American Chemical Society, Washington, DC

RA 1270 .V47V48 1996 Copy 1

Veterinary drug residues

Library of Congress Cataloging-in-Publication Data

Veterinary drug residues: food safety / William A. Moats, editor, Marjorie B. Medina, editor.

p. cm.—(ACS symposium series, ISSN 0097–6156; 636)

"Developed from a symposium sponsored by the Division of Agricultural and Food Chemistry at the 209th National Meeting of the American Chemical Society, Anaheim, California, April 2–7, 1995."

Includes bibliographical references and indexes.

ISBN 0–8412–3419–1

1. Veterinary drug residues—Toxicology—Congresses.

I. Moats, William A. (William Alden), 1928– . II. Medina, Marjorie B., 1945– . III. American Chemical Society. Division of Agricultural and Food Chemistry. IV. American Chemical Society. Meeting (209th: 1995: Anaheim, Calif.) V. Series.

RA1270.V47V48 1996
615.9'54—dc20 96–26216
CIP

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.fw001

This book is printed on acid-free, recycled paper.

Copyright © 1996

American Chemical Society

All Rights Reserved. The appearance of the code at the bottom of the first page of each chapter in this volume indicates the copyright owner's consent that reprographic copies of the chapter may be made for personal or internal use or for the personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per-copy fee through the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. This consent does not extend to copying or transmission by any means—graphic or electronic—for any other purpose, such as for general distribution, for advertising or promotional purposes, for creating a new collective work, for resale, or for information storage and retrieval systems. The copying fee for each chapter is indicated in the code at the bottom of the first page of the chapter.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

American Chemical Society
Library
1155 16th St., N.W.
Washington, D.C. 20036

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.fw001

Advisory Board

ACS Symposium Series

Robert J. Alaimo
Procter & Gamble Pharmaceuticals

Mark Arnold
University of Iowa

David Baker
University of Tennessee

Arindam Bose
Pfizer Central Research

Robert F. Brady, Jr.
Naval Research Laboratory

Mary E. Castellion
ChemEdit Company

Margaret A. Cavanaugh
National Science Foundation

Arthur B. Ellis
University of Wisconsin at Madison

Gunda I. Georg
University of Kansas

Madeleine M. Joullie
University of Pennsylvania

Lawrence P. Klemann
Nabisco Foods Group

Douglas R. Lloyd
The University of Texas at Austin

Cynthia A. Maryanoff
R. W. Johnson Pharmaceutical Research Institute

Roger A. Minear
University of Illinois at Urbana–Champaign

Omkaram Nalamasu
AT&T Bell Laboratories

Vincent Pecoraro
University of Michigan

George W. Roberts
North Carolina State University

John R. Shapley
University of Illinois at Urbana–Champaign

Douglas A. Smith
Concurrent Technologies Corporation

L. Somasundaram
DuPont

Michael D. Taylor
Parke-Davis Pharmaceutical Research

William C. Walker
DuPont

Peter Willett
University of Sheffield (England)

Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of this series is to publish comprehensive books developed from symposia, which are usually "snapshots in time" of the current research being done on a topic, plus some review material on the topic. For this reason, it is necessary that the papers be published as quickly as possible.

Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according to the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS BOOKS DEPARTMENT

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.fw001

Preface

VETERINARY DRUGS are compounds administered to animals therapeutically to promote growth or otherwise to improve animal health. When these compounds are administered to food-producing animals, residues may be present in milk, eggs, or tissues. These drug residues are of concern because they may be toxic in food products, they may produce their pharmacologic effects on consumers, or they may cause allergic reactions in sensitive individuals. Drug residues in animal products can be avoided by using prescribed treatment protocols and allowing sufficient withdrawal times after treatment for the compounds to be depleted from the animals. There is nonetheless a potential for abuse from failure to adhere to prescribed dosages and withdrawal times and use of unapproved compounds.

Concerns about residues, whether warranted or not, can affect consumer behavior. Some stores advertise that their products are free of things like "growth hormones, steroids, and antibiotics." As pointed out by Christine Bruhn in this book, a widespread consumer perception is that poultry meat is loaded with growth hormones, although these are never in fact used in poultry production. Faulty testing procedures may create an impression that residues are more prevalent than is actually the case. It has been suggested, without supporting evidence, that cases of premature menarche observed in Puerto Rico were a result of growth hormone residues in chickens. Residues or the potential for residues can also be used as an international trade barrier.

The use of accurate and reliable testing procedures for residues provides a means of detecting misuse and abuse of veterinary drugs, and is essential to protecting the public against such misuse. At the same time, it is important to reliably establish that products are residue-free. The U.S. Department of Agriculture Food Safety and Inspection Service has estimated that as many as 400 compounds, not all of which are veterinary drugs, have the potential to produce residues in animal products. Obviously, surveillance for all these compounds is impractical. Residue monitoring must therefore be limited to spot checks unless serious residue problems are present.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.pr001

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

This volume covers real and perceived problems of residues associated with veterinary drugs, some general approaches to surveillance, problems encountered with testing for residues, and some recent advances in methods for measurement of residues of various types of drugs.

WILLIAM A. MOATS
Meat Science Research Laboratory
Agricultural Research Service
U.S. Department of Agriculture
Beltsville, MD 20705

MARJORIE B. MEDINA
Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
Wyndmoor, PA 19038

April 26, 1996

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.pr001

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

Chapter 1

Good Animal Husbandry Practice and Residues in the United States

Lester M. Crawford

Association of American Veterinary Medical Colleges, 1101 Vermont Avenue, Northwest, Suite 710, Washington, DC 20005

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch001

Proper utilization of drugs and chemicals in food animals provides economic benefits to both consumer and producer while still protecting the public from hazards. In the United States of America it is the responsibility of state and federal authorities to provide this protection. Food animals on the hoof are considered to be food subject to the regulatory provisions of the U.S. Food and Drug Administration (FDA) because the only reason for existence of these animals is to serve as a source of food for the consumer.

The use of drugs to control and treat animal disease and to promote faster, more efficient growth of livestock is a common practice. About 80 percent of livestock and poultry in the United States receive such animal drugs. However, if animal drugs are misused, the resulting residues in the edible tissues of slaughtered animals threaten the health of humans. FDA and the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA/FSIS) work cooperatively to monitor the use of these animal drugs.

Animal drug manufacturers are required by FDA to show that each new animal drug is safe and effective before it is approved for marketing. Manufacturers also must submit for review by both FDA and FSIS a reliable assay method for detecting drug residues in slaughtered animals. FSIS regularly monitors on a random basis tissue samples of slaughtered animals.

FDA sets tolerances for acceptable levels of residues of a drug in animal tissue after first determining the level at which the drug does not produce any measurable physiological effect in laboratory animals.

0097–6156/96/0636–0001$15.00/0
© 1996 American Chemical Society

The Environmental Protection Agency (EPA) also cooperates in the residue monitoring program where pesticide residues are determined to be due apparently to direct applications of environmental contamination. In summary, FSIS assumes primary responsibility for the wholesomeness of the meat supply, EPA has responsibility to assure that pesticides are used according to label directions, and FDA has final authority for enforcement of the laws governing use of animal drugs and acceptability of medicated feeds.

(1) Epidemiology

Violative residues in edible tissues, milk and eggs can result from misuse of animal drugs in feed, the presence of pesticides and industrial chemicals, and from natural toxicants such as aflatoxin. Accidental inclusion of pesticides or industrial waste materials in feed formulation, improper storage of feed and pesticides, and application of pesticides to forage or grain crops are among the causes of contamination. Animals also can be exposed when allowed access to rubbish piles with discarded chemicals or pesticides, drinking water contaminated with pesticide run-off or industrial pollution, and when exposed to insecticides either on their bodies on in their housing.

Persistent chemicals of accidental or unanticipated origin caused a number of incidents of violative residues in food animals in recent years. These incidents ranged in size from situations that involved one producer to those that involved a large number of producers over a large area. Residues of persistent chemicals probably do not pose as large a public health threat, or as large an economic threat to producers, as violative residues of natural toxicants, feed additives, or animal drugs. Nevertheless, the presence of these chemicals is serious because they are insidious. First, the residues are unanticipated by all parties concerned because the compounds do not have a role in normal agricultural production.
Second, the chemicals are persistent which means that although they can be detected earlier, because of their nature they will be harder to remove.

In the United States the animal production industry is extensive and the use of drugs to promote growth and prevent disease in confinement rearing is common. Most of the animals raised n this country for slaughter are raised in confinement rather than being allowed to roam on large acreage.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch001

This confinement increases the possibility of disease, making it necessary to use drugs subtherapeutically. However, these drugs are subject to withdrawal times prior to slaughter which allow the animal to excrete any residue before marketing. The problem occurs when the withdrawal times are not adequately followed by producers and feedlot operators.

USDA/FSIS routinely takes random samples of meat carcasses at the slaughtering plants to check for detectable residues. Where residues are found USDA notifies the FDA which makes a follow-up investigation at the animal producer level to determine the source of the contamination. Not following label directions and failure to observe the required withdrawal times are the common reasons for the contamination. Other types of contamination, such as those caused by chemicals and pesticides, are more readily detectable at earlier stages.

(2) Technology

If producers and feedlot operators follow the label directions and observe proper withdrawal times for animal drugs, the environment should be free of this form of contamination. Producers and feedlot operators should always be alert for the possibility of the accidental introduction of industrial hazards such as PCBs, PBB, TCDD and other halogenated hydrocarbons. The nature of persistent chemicals is such that they often are viewed by the public as a more serious threat than other potential chemical residues. Assuming that producers and feedlot operators have observed the label directions and withdrawal times, meat products are then processed in facilities which are subject to continuous inspection by the USDA. At the retail sales level, the product should be free of contamination and if properly refrigerated and properly prepared should remain free of contamination.

The United States is undertaking these project activities to attain anti-contamination goals:

For illegal residues in animal-derived food FDA will

> Utilize contacts with officials of the national producer groups;
>
> Conduct an epidemiological study of currently available residue reports;

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch001

Coordinate voluntary compliance plans and operations with industry and other agencies; and

Develop educational and informational materials for regulated groups.

Key animal food producer groups will be provided with informational material to help them set up quality assurance programs to prevent unintentional or accidental contamination of animal feedstuffs. In addition, enforcement of all laws designed to prevent illegal distribution of veterinary prescription drugs are being strengthened.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch001

Chapter 2

Human Health Risks Associated with Drug Residues in Animal-Derived Foods

S. F. Sundlof and J. Cooper

Center for Veterinary Medicine, U.S. Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

Adulteration of the food supply by agricultural chemicals has gained national attention as a potential health hazard. This paper examines the risk to human health from consumption of drug residues in animal-derived foods. In particular, it focuses on antimicrobial residues and residues of natural and synthetic hormones used to enhance the growth of livestock. In addition, it discusses the issue of bacterial resistance and the effects on human health from the use of antimicrobial drugs in livestock.

Food safety has become one of the most visible and emotional issues confronting affluent societies. In a national survey conducted by the Food Marketing Institute, the first concern of consumers pertained to residues in meat. Other health-related issues such as cholesterol and saturated fat content were perceived by the public as less threatening than chemical residues (*1*).

The public, including the medical profession, is repeatedly presented with information that is conflicting, often times misleading, and usually critical of food regulatory agencies. The occurrence of potentially hazardous drug residues in food is a major concern to the Food and Drug Administration (FDA). In the United States, the drug approval, food safety, and surveillance programs of the FDA, in concert with the animal drug residue monitoring programs of the U.S. Department of Agriculture's (USDA's) Food Safety and Inspection Service and the milk safety programs of the States, provide assurance that the incidence of illegal drug residues will remain low. While the FDA, USDA, and the States strive to prevent illegal drug residues, it is recognized that no incidence rate of illegal drug residue is acceptable to the consumer. FDA is concerned that consumers not be exposed to illegal or hazardous animal drug residues, and that any food product that contains such residues be removed from the

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

market. In addition to acute incidents related to residues, FDA is also concerned about the long-term effects on public health from chronic exposure to illegal residues.

FDA serves an important role in helping prevent illegal and hazardous drug residues in animal-derived foods. The success of FDA's residue control programs is evidenced, in part, by the low number of reported cases of residue-related diseases in the human population. Another indication that FDA's residue reduction programs have been effective are reductions in illegal residues of sulfamethazine. In 1979, the level of illegal sulfamethazine residues in pork was 13%, and in the 1980s there were illegal sulfamethazine residues in milk (*2*). Today, sulfamethazine residue levels have been reduced to less than 1% in pork, and since the launch of the National Drug Residue Milk Monitoring Program in 1991, there has only been one finding of a violative residue of sulfamethazine in a milk sample (*3*). FDA believes that a continuing compliance presence by the FDA, USDA, and the states is necessary to help ensure a safe food supply.

Effect of Food-Borne Drug Residues on Human Health

There are few reports documenting adverse reactions in persons consuming drug residue-contaminated foods, and the overwhelming majority of these pertain to allergic (not toxic) reactions to penicillin. In reference to the number of reported allergic reactions, Burgat-Sacaze and coworkers (*4*) offer the following explanations:

1. A small percentage of all adverse reactions are attributed to drug residues.
2. A rash is the most frequently reported symptom and seldom, if ever, are these rashes life-threatening.
3. A rash as typically seen by a physician is transient, caused by a reaction to food, and requires tests to identify the specific cause. After a series of tests to food groups, the physician might discount food and attribute the cause to a residue without further checking of the hypotheses.

Beta-lactam Antibiotics. Nearly all reports of adverse reactions from food-borne residues implicate penicillin as the offending agent, and the source of penicillin residues is most often milk or dairy products. These milk residues most likely originated from intramammary infusion of penicillin used for the treatment of mastitis (*5*). Although a substantial number of farm milk samples have been found to contain small amounts of penicillin, there have been relatively few published reports of adverse reactions from milk residues (*6,7,8, 9, 10*). In all instances, the victims reported a history of penicillin allergy or skin disease unrelated to penicillin allergy. Symptoms varied in intensity from mild skin rashes to exfoliative dermatitis. In an investigation of 252 patients with chronic recurrent urticaria, 70 (27.8 %) were determined to be allergic to penicillin by dermal testing. When 52 of these penicillin-allergic patients were restricted to a diet containing no milk or dairy products, 30 (58%) experienced remission of symptoms. Conversely, changing to a milk-free diet caused remission of symptoms in only 2 patients out of a group of 40 (5%) with chronic urticaria but testing negative for

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

allergies to penicillin (*11*). Many drugs other than penicillin including other beta-lactams, streptomycin (and other aminoglycosides), sulfonamides, and to a lesser extent, novobiocin and the tetracyclines are known to cause allergic reactions in sensitive persons; however, only a single report of a reaction to meat suspected of containing streptomycin residues (*12*), appears in the literature.

Drug allergies are generally considered to be type I immune responses (*13*). These reactions are mediated through IgE, and symptoms include anaphylaxis, urticaria, and angioedema. For a type I allergic response to occur, the subject must first be sensitized to the drug. The dose of a drug necessary to produce this primary sensitization is considerably higher than the dose required to elicit an allergic response (*14*). Both epidemiologic and experimental data indicate that food-borne residues of penicillin as low as 5 to 10 IU are capable of producing allergic reactions in previously sensitized persons (*9, 15*).

Chloramphenicol. Chloramphenicol has never been approved in the U.S. for use in food-producing animals, although in the past it has been approved for food animal use in Canada and Europe. Chloramphenicol is approved for use in dogs, but the labeling of all products containing chloramphenicol must bear the label statement: "....the product is not to be used in animals which are raised for food production" (*16*). Nevertheless, chloramphenicol gained wide popularity among food animal veterinary practitioners in the U.S. because of its effectiveness in treating bacterial infections.

Despite its virtues as an antimicrobial agent, chloramphenicol has some inherent properties which can threaten human health. One toxic effect, aplastic anemia in man, appears to be dose-independent and potentially could be induced by low concentrations of chloramphenicol in foods. Aplastic anemia in man represents a true drug idiosyncrasy affecting 1 in 20,000 to 1 in 50,000 patients receiving a typical course of chloramphenicol therapy. The resulting disease is fatal in approximately 70 percent of the cases and those who recover experience a high incidence of acute leukemia (*16*).

By 1985, the U.S. Food and Drug Administration (FDA) had accumulated enough data to establish that most chloramphenicol oral solution marketed (approved only for use in dogs), was being used to treat food-producing animals, usually by injection or infusion. The Agency also determined that withdrawal of approval of chloramphenicol oral solution would have little or no effect on canine practitioners or dog owners because other dosage forms were available which were more convenient to administer. Since the labeling directions for use had not been followed in practice and were not likely to be followed in the future, the FDA withdrew approval of all oral solutions containing chloramphenicol (*17*).

Although there have been no reported cases of aplastic anemia which were attributable to consumption of chloramphenicol residues in food, the possibility of such an event occurring is not remote. Use of chloramphenicol in cattle is thought to be responsible for the death of a Kansas rancher. The rancher was diagnosed as having aplastic anemia 4 months after he began treating his cattle with chloramphenicol (*16*).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

In 1983 approximately 0.5 percent of all calves contained residues of chloramphenicol, but by 1984, the violation rate declined to 0.09 percent and no violations were detected under the U.S. Department of Agriculture Food Safety Inspection Service (FSIS) monitoring program in 1985 or 1986 (*18, 19, 20, 21*). Veterinarians are forbidden from using chloramphenicol for any purpose that would result in the presence of residues in food for consumption by humans. Use of chloramphenicol in food-producing animals is specifically proscribed in the FDA's Extra-Label Drug Use policy (*22*), and FDA has prosecuted veterinarians who have disregarded this policy regarding chloramphenicol.

Bacterial Resistance

Antibacterial drugs are among the most valuable and the most utilized drugs in veterinary and human medicine. Because of the diverse usefulness of this group of compounds, contemporary society is dependent upon antibiotics. Not only have we come to rely on antibiotics to maintain a cost efficient food supply through their growth promoting benefits, we also expect antibiotics to protect the public from lethal human epidemics such as plague.

Unfortunately, bacteria are developing the ability to survive the chemicals we have developed against them. The development of and spread of resistance factors within an ecosystem continues to evolve. It is known that antibiotic survival can be passed to progeny by cellular division, by bacterial mating via gene segments called plasmids, and through "jumping" chromosomal segments called transposons. Bacterial viruses (phages) also have been demonstrated to ferry resistance factors between hosts. In many cases, resistance factors are promiscuously shared within and between bacterial species. For example, resistance factors can be transferred to resident bacteria from a foreign bacterial species even when the donor cannot survive in that environmental niche (*23*). A single resistance factor can protect bacteria from the effects of many types of antibiotics such that the use of one drug, like erythromycin, can select for resistance to other unrelated compounds such as streptomycin (*24*). Conditions favoring the development and selection of bacteria carrying resistance factors are thought to be associated with repeated or prolonged use, and low-level dosing (*25*).

Impact of Resistance on Therapy. Bacterial resistance can affect therapy by reducing the ability of an antibiotic to eliminate or control infection. In the worst case, infection can overcome the victim before appropriate therapy can be instituted. In some cases, resistance renders an infection immune to every antibiotic available. More often, bacterial resistance increases therapeutic costs because inappropriate drug choice prolongs disease. Furthermore, newer, more costly and sometimes more toxic drugs are needed; more diagnostics are needed; and hospitalization may be extended. In addition, diseases such as salmonellosis are more likely to produce clinical illness in persons treated for another condition if the choice of therapy happens to coincide with the resistance pattern carried by the *Salmonella* (*26*).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

Impact of Resistance on Public Health. The impact of transmissible drug resistance on public health and our economy has been controversial since the early days of antimicrobial use in agricultural. The controversy is fueled by the amount of drug used in animal agriculture. The bulk of animal drugs are administered for growth promoting purposes. Growth promoting drugs are given to animals for much of their life at levels which are considered too low to inhibit the growth of most pathogens. This type of dosing is thought to favor drug resistance. If resistance factors carried in animal isolates are transmitted to humans by ingestion or by contact, it is not clear how much effect on human therapy is enough to outweigh the benefits gained by the use of antibiotics in animal feed to maintain a cost effective food supply.

Certain bacterial pathogens such as *Salmonella* have animal reservoirs which serve as a source of human exposure. Numerous episodes have occurred in which humans have developed drug resistant *Salmonella* infections that have been traced to animal sources (*26*). The Swan Committee was formed after such an epidemic in occurred in England. Studies such as the 1988 Institute of Medicine Report (IOM) attempted to designate the impact of growth promotion use of penicillin and tetracycline on *Salmonella* as a model. The authors of this study attempted to describe the extent to which transfer of resistance factors occurs between humans and animals and to define when the risk to human therapy is enough to outweigh the benefits of a cost effective food supply. The authors of the IOM Report agreed with reports from previous studies that there was insufficient information available to answer the question. The FDA Center for Veterinary Medicine (CVM) has determined that there is sufficient reason for concern that sponsors of all antimicrobial products administered in feed in excess of 14 days are required to demonstrate that their product does not increase *Salmonella* shedding or select for bacterial resistance in the intestinal microflora of treated animals (*27*).

The administration of antimicrobials for growth promotion remains controversial with some scientists suggesting that the recently developed vancomycin resistance is community acquired and might be the result of the growth promotion use of drugs such as avoparcin (*28*). Avoparcin is a glycopeptide, like vancomycin, which is approved for animal use in Europe.

Therapeutic Animal Drugs. A relatively small portion of total antibiotics used in the U.S. are administered to animals for therapeutic purposes (*26*). The use of antimicrobials to relieve pain and suffering in animals has generally been accepted as necessary. In contrast to the risk associated with low-level, long-term use of antimicrobials for growth promotion, the risk of the development of resistance due to therapeutic use in animals has been deemed to be within acceptable limits. With therapeutic uses, the time of drug exposure is generally limited
to days, the doses administered are considered sufficient to kill pathogens, and the number of animals undergoing treatment is relatively small compared to those receiving production drugs.

Over the years, the animal pharmaceutical companies (with help from FDA) have been moving away from long-term, low-level dosed products with prophylactic

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

claims and moving toward disease prevention/control products which involve short-term (3 to 21 days) and high dose administration. This has become necessary because of the dense concentration of animals under current food animal production systems. It is generally not economical to treat with the intention to cure all individuals in a herd or flock. Therefore, new products are intended to control the spread of disease through mass treatment. These newer drugs are directed toward specific at-risk populations of animals usually confined as discrete subsets of animals within a production unit. CVM has taken an active role in furthering science-based decisions regarding the therapeutic use of antimicrobials by restricting the marketing of drugs with prophylactic or other disease related claims to prescription rather than over-the-counter status.

As veterinary medicine expands its therapeutic armamentarium into more powerful classes of antimicrobials, including those considered secondary human therapeutic drugs such as fluoroquinolones, and as concern over human drug resistance increases, physicians and microbiologists are voicing concern. The willingness to accept risks associated with therapeutic use of newer antibacterials in animals is lessening. The concern is particularly directed toward mass treatment of flocks or herds. However, since resistance factors have not yet been demonstrated to transfer beyond mutations passed to progeny, the public health effects of fluoroquinolone resistance is limited to zoonotic pathogens such as *Salmonella* and *Campylobacter* spp. Reports from the Netherlands, Spain, and England suggest that human populations in those countries may have acquired fluoroquinolone resistant zoonosis by consuming treated animals (*29, 30, 31*). The Veterinary Advisory Committee from CVM and the Anti-Infective Advisory Committee from the Center for Drug Evaluation and Research met jointly in May, 1994 to discuss the benefits and risks associated with the use of fluoroquinolones in animals. CVM is currently determining how to implement the recommendations from that meeting.

Hormonal Growth Promoting Drugs

Of all of the drugs used in food animal production, none have evoked greater emotional response among the public than the hormonal growth promoting drugs. Consumer apprehension in Europe over these drugs has led in large part to a European Community (EC) directive banning the use of these drugs. Several recent incidents involving precocious sexual development in infants and young children have been blamed on meat containing residues of these substances.

In an effort to improve the efficiency of livestock production, producers have become reliant on the judicious and timely use of growth promoting agents. These production (as opposed to therapeutic) drugs are used to increase the rate of weight gain and/or improve feed efficiency in livestock. All of the hormonal growth promoting drugs are available for over the counter purchase in the U.S. and are generally administered by the livestock producer at specific stages in the production cycle. Growth stimulation by these drugs appears to be somewhat unique to ruminants

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

and, therefore, the hormonal growth promoting agents are approved for use only in cattle and sheep.

Naturally Occurring Hormones Used as Growth Promoting Agents

The natural hormones used in food animal production include estradiol, progesterone, and testosterone (*32*). All products marketed in the U.S. contain estradiol alone or in combination with either progesterone or testosterone. All are formulated as pellets and all are designed to deliver the hormones at a constant sustained rate when injected subcutaneously under the skin of the ear.

Despite public apprehensions concerning the use of these hormones, numerous scientific studies have demonstrated that, when these drugs are used in accordance with good husbandry practices, concentrations of the hormones in meat remain within the normal physiological range that has been established for untreated cattle of the same age and sex. Because the rate of hormone release from the implant is slow and the half-life of these endogenous hormones extremely short (<10 min), no preslaughter withdrawal time is necessary to protect the public health. Although hormone concentrations may be slightly greater in treated vs. untreated cattle, meat from treated animals contains progesterone, estradiol and testosterone at concentrations well within the physiologic range for untreated cattle.

Despite the small increase in estradiol, progesterone, and testosterone in meat from treated animals, the concentrations of these hormones are far less than those naturally found in meat from sexually mature animals. Concentrations of estradiol in muscle from cattle in late pregnancy is 3 to 80 times greater than those found in the muscle of estradiol-treated heifers. Similarly, the concentration of progesterone in muscle from pregnant cattle is more than 20 times that which occurs in progesterone-treated steers, and muscle from mature bulls contains approximately 8 times the concentration of testosterone found in testosterone-treated heifers (*33, 34*). Furthermore, a number of other foods contain naturally occurring hormonally active substances at concentrations far exceeding those found in meat from treated cattle. Some of these foods include milk and milk products, eggs, cabbage and soybean oil. Because the endogenous hormones are naturally occurring in people and in food-producing animals, individuals are exposed throughout their lifetime to large quantities of these substances by endogenous daily synthesis and to much lesser quantities from consumption of unmedicated food animal products. Based on these facts, the FDA has concluded that no harmful effects will occur in persons who daily consume animal tissues that contain an incremental increase of endogenous hormone equal to 1 percent or less of the amount produced daily by the segment of the population with the lowest daily production rate. For estradiol and progesterone, prepubertal boys synthesize the least whereas prepubertal girls synthesize the least amount of testosterone per day. Prepubertal boys, on average per day, produce 100 to 3,000 times the amount of estradiol and more than 500 times the amount of progesterone that would be expected to occur in 500 g of meat from treated animals. Similarly, prepubertal girls produce approximately 600 to 900 times the amount of

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

testosterone that would be expected to be present in 500 g of meat from treated animals (*33, 34*).

The opinion of the FDA that the 3 naturally occurring hormones do not present any harmful effects to the health of the consumer when used as growth promoting agents in cattle has been supported by 2 independent scientific review committees comprised of internationally recognized experts in the area of human food safety. The first committee, chaired by Professor G.E. Lamming, was commissioned by the EC and was composed of 22 scientists from 10 European countries (*35*). The second committee was conducted under the joint auspices of the World Health Organization and the Food and Agriculture Organization (WHO/FAO) and was composed of 11 scientists from 7 countries on 5 continents (*36*).

Synthetic Hormonal Growth Promoting Agents

Trenbolone acetate, a synthetic anabolic hormone with androgenic properties, and zeranol, a product with weak estrogenic activity formed by the reduction of the mycotoxin zearalenone, are approved in the U.S. as growth promoting implants for use in cattle.

Unlike the naturally occurring hormones for which human food safety assessment is based on a ratio of the amount of these substances consumed in food compared to the amount produced endogenously by the consumer, safety assessment for the synthetic hormones must be based on alternative strategies. The major impediment to establishing safe tissue concentrations of the synthetic hormones results primarily from their ability to produce tumors in laboratory animals when these compounds are fed at high concentrations. These tumors occur only in endocrine-sensitive tissues and are similar to those produced by high doses of the naturally occurring hormones. The Delaney Amendment of the U.S. Food, Drug, and Cosmetic Act (FD&C Act) prohibits the addition of any carcinogenic substance to the food supply. Considerable research has gone into assessing the risk of cancer to the consumer from the use of synthetic hormones in food-producing animals. The establishment of hormonal no-effect levels (HNEL) as a basis for assessing human food safety and setting tolerances for residues is scientifically sound because it has been shown that the hormonally active compounds cause no increased incidence of tumors when administered to laboratory animals in amounts below those required to produce detectable hormonal activity (*35*). Numerous other biological models have been developed for the purpose of establishing HNELs for androgenic, estrogenic, and progestogenic compounds.

The same EC directive which banned the use of the naturally occurring hormones also prohibits the use of zeranol and trenbolone acetate. As a result of the EC ban on the hormonal growth promotants, trade embargoes presently exist against U.S. beef and beef by-products. There is little doubt that the EC hormone ban was developed, in large part, in response to pressure by consumer advocacy groups which strongly oppose the use of these hormonal growth promotant drugs in food-producing animals. The exact cause for the profound "anti-hormone" sentiment among the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

European public is unclear, but one event more than others may have been responsible for precipitating the present consumer movement. In 1980, high concentrations of diethylstilbestrol (DES) were found in baby food in Europe (*34*). Although it was later surmised that the extreme concentration of DES in the baby food could have only resulted from severe misuse or intentional and malicious adulteration, the incident instilled in the European public a fear of all hormonal growth promoting agents.

Adding to consumer fears has been the occurrence of 2 outbreaks of premature thelarche (development of the breasts before the age of 8 years). The first outbreak occurred in children attending an Italian school and the second outbreak occurred in Puerto Rico. In both outbreaks, meat containing estrogenic substances was implicated as the cause of the precocious sexual development. Of the two outbreaks, the one in Puerto Rico was by far the more significant in terms of the number of infants and children affected. In 1980 through 1981 pediatric endocrinologists in Puerto Rico reported a three fold increase in the number of premature thelarche cases compared with 1978. The outbreak was originally alleged to be caused by consumption of poultry or meat containing residues of naturally occurring or synthetic hormonal agents (*37*). In response to the possibility of a massive food-borne epidemic, an extensive coordinated investigation was launched by the Centers for Disease Control, the USDA, and the FDA. Beef, pork, milk, water, and blood and urine samples from affected and unaffected children were analyzed for the synthetic and naturally occurring hormones as well as the chlorinated hydrocarbon insecticides (*38*). Surveys were conducted to determine whether exogenous exposure or familial or prenatal factors could be responsible for the reported increase in premature thelarche cases (*39*). New, highly sensitive assays for detecting DES and zeranol in meat and poultry were developed and during 1985 and 1986, FSIS analyzed nearly 700 samples of domestic and imported animal products for these substances (*40*). Altogether, more than 6,500 analyses for drugs and other chemicals were performed on the samples making it the most intense examination of food-borne residues ever conducted for a given area.

Although the amount of data generated during the epidemiologic study was substantial, no underlying cause for the increase in premature thelarche cases could be determined. Of the nearly 700 meat and poultry samples analyzed for estrogenic substances, none contained violative residues. It was finally concluded that the increased number of cases might be attributed to better diagnosis and reporting of premature thelarche by physicians, or to the presence of entirely new, unexpected factors, but not to residues of veterinary drugs in food (*40*).

Although the European hormone ban was intended to eliminate hormonally active residues in foods, it appears to have fostered a black market for illegal and unregulated veterinary drugs. DES residues in baby food were found only in those European countries which had exerted a total ban on the use of anabolic sex hormones (*41*). Since the EC-wide ban, numerous cases have been reported involving the illegal use of growth promoting drugs in food animals. In Europe, the illegal use of an injectable solution containing estradiol and testosterone cyprionate in German veal calves resulted in the seizure or impoundment of 74,000 animals by authorities (*42*).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

In addition to illegal hormone residues, clenbuterol, a beta-adrenergic agonist, has been detected in cattle from a number of European countries and Canada. Clenbuterol has been illegally incorporated into animal feeds in these countries for the purpose of enhancing animal growth rates. In the past 3 years, hundreds of people in Spain have required hospitalization after eating liver from beef cattle treated with clenbuterol (*43*). Clinical signs of poisoning included muscle tremors and tachycardia frequently accompanied by nervousness, headaches, and myalgia (*44*). Two farmers in Ireland are reported to have died while preparing clenbuterol for feeding to livestock (*45*).

Recently, it was reported that the antithyroid drug methimazole (methylimidazole) is illegally being added to cattle feeds along with clenbuterol to increase animal weight and water retention. Maternal use of methimazole during pregnancy for the treatment of hyperthyroidism is associated with a high incidence of congenital aplasia cutis (a characteristic scalp defect) in children. In Spain there has been a significant ($p<0.005$) increasing trend in the prevalence of aplasia cutis from 1984 through 1991. This trend was not related to maternal treatment of hyperthyroidism, but was closely associated with outbreaks of clenbuterol poisonings (*46*). It was postulated that the increase in congenital scalp defects in Spain were caused by consumption of beef containing illegal residues of methimazole. Black market sale and use of growth-promoting drugs has become widespread in Europe since the initiation of the hormone ban (*47*).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

Somatotropic Hormones

The newest member of the anabolic hormonal growth promoting drugs is somatotropic hormone also called growth hormone, somatotropin, bovine somatotropin (bST) or porcine somatotropin (pST). Recent advances in genetic engineering and biotechnology have resulted in the economic production of somatotropic hormone on a commercial scale. Somatotropic hormone administered to livestock is reported to produce dramatic increases in animal growth, milk production and altered body composition favoring the development of lean muscle mass and a decrease in body fat. Residues of bovine somatotropic hormones in meat or milk are not a risk to the human consumer (*48*).

Literature Cited

1. *Consumer Attitudes & the Supermarket Update*. Food Marketing Institute. Trends 1988.

2. *Domestic Residue Data Book*, National Residue Program 1992, United States Department of Agriculture, Food Safety and Inspection Service. **1992**, December, pp. 3-4.

3. Food and Drug Administration, Center for Veterinary Medicine, National Drug Residue Milk Monitoring Program (7371.008) Quarterly Report. **1992**, October 20.

4. Burgat-Sacaze, V.; Rico, A.; Panisset, J.C. Toxicological significance of bound residues. In *Drug Residues in Animals*; Rico, A., Ed. Academic Press: Orlando, FL, **1986**, pp. 1-31.
5. Siegel, B. Hidden contacts with penicillin. *Bull. WHO*. **1959**, *Vol. 21*, pp. 703-713.
6. Erskine, D. Dermatitis Caused by penicillin in milk. *Lancet*. **1958**, *Vol. 1*, pp. 431-432.
7. Vickers, H.R.; Bagratuni, L.; Alexander, S. Dermatitis caused by penicillin in milk. *Lancet*. **1958**, *Vol. 1*, pp. 351-352.
8. Zimmerman, M.C. Chronic penicillin urticaria from dairy products proved by penicillinase cures. *AMA. Arch. Dermatol.* **1959**, *Vol. 79*, pp. 1-6.
9. Borrie, P.; Barrett, J. Dermatitis caused by penicillin in bulked milk supplies. *Brit. J. Med.* **1961**, *Vol. 2*, p. 1267.
10. Wicher, K.; Reisman, R.E.; Abresman CE. Allergic reaction to penicillin. *J. Am. Med. Assoc.* **1969**, *Vol. 208*, pp. 143-145.
11. Boonk, W.J.; Van Ketel, W.G. The role of penicillin in the pathogenesis of chronic urticaria. *Brit. J. Dermatol.* **1982**, *Vol. 106*, pp. 183-190.
12. Tinkleman, D.G.; Bock, S.A. Anaphylaxis presumed to be caused by beef containing streptomycin. *Annl. Allergy*. **1984**, *Vol. 53*, pp. 243-244.
13. Coombs, R.R.A.; Gell, P.G.M. Classification of allergic reactions responsible for clinical hypersensitivity and disease. In *Clinical Aspects of Immunology*; Gell, P.G.M., Ed.; Oxford, Blackwell Scientific: **1975**, pp. 761-781.
14. Huber, W.G. Allergenicity of antibacterial drug residues. In *Drug Residues in Animals*, Rico, A., Ed.; Academic Press: Orlando, FL, **1986**, pp. 33-49.
15. Lindemayr, H.; Knobler, R.; Kraft, D.; Baumgartner, W. Challenge of penicillin-allergic volunteers with penicillin-contaminated meat. *Allergy*. **1981**, *Vol. 36*, pp. 471-478.
16. Settapani, J.A. The Hazard of Using Chloramphenicol in Food Animals. *J. Am. Vet. Med. Assoc.* **1984**, *Vol. 8*, pp. 930-931.
17. Chloramphenicol Oral Solution; Opportunity for Hearing. *Federal Register*. **1985**, *Vol. 50*, pp. 27059-27064.
18. *Domestic Residue Data Book, National Residue Program 1983*, United States Department of Agriculture, Food Safety and Inspection Service, **1986**, July 24, p. 71.
19. *Domestic Residue Data Book, National Residue Program 1984*, United States Department of Agriculture, Food Safety and Inspection Service, **1986**, October 24, p. 70.
20. *Domestic Residue Data Book, National Residue Program 1985*, United States Department of Agriculture, Food Safety and Inspection Service, **1987**, May, p. 53.
21. *Domestic Residue Data Book, National Residue Program 1986*, United States Department of Agriculture, Food Safety and Inspection Service, **1987**, October, p. 69.
22. *Extra-Label Use of New Animal Drugs in Food-Producing Animals*. Compliance Policy Guide 7126.06. Food and Drug Administration: Rockville, MD, **1986**.
23. Roberts, M.C. In *Gene Transfer in the Environment*; Levy, S.B.; and Miller, R.V., Eds.: McGraw-Hill Book Co.: New York, NY, **1989**.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

24. Rollins, L.D.; Lee, L.N.; LeBlanc, D.L. *Antimicrob. Agents Chemother.* **1985**; *Vol. 27*, pp. 439-444.
25. Levy, S.B. *In the Antibiotic Paradox.* Plenum Press: New York, NY, **1992**.
26. Institute of Medicine. *Human Health Risks with the Subtherapeutic Use of Penicillin or Tetracycline in Animal Feed.* Washington, D.C.: National Academy Press, **1989**.
27. *Code of Federal Regulations*, 21 Part 558.15. Office of the Federal Register, National Archives and Records Administration: Washington, DC, **1994**.
28. Bates, J.; Jordens, J.Z.; Griffiths, D.T. *Antimicrob. Agents Chemother.* **1994**, *Vol. 34*, pp. 507-514.
29. Endtz, H.; Ruijs, G.; van Klingeren, G.J.; Jansen, B.; van der Reyden; W.H.; Mouton, T.J. *Antimicrob. Chemotherapy.* **1991**, *Vol. 27*, pp. 199-208.
30. Griggs, D.J.; Hall, M.C.; Jin, Y.F.; Piddock, J.V. *J. Antimicrob. Chemotherapy.* **1994**, *Vol. 33*, pp. 1173-1189.
31. Perez-Trallero, E.; Urbleta, M.; Lopategui, C.L; Zigorraga, C.; Ayestaran, I. *Lancet.* **1993**; *Vol. 342.*, pp. 1371-1372.
32. Sundlof, S.F.; Riviere, J.E.; Craigmill, A.L. *FARAD Trade Name File: A Comprehensive Compendium of Food Animal Drugs*; 8th ed. Institute of Food and Agricultural Science: Gainesville, FL, **1992**.
33. Foxcroft, G.R. Hormonal No-Effect Studies: Concepts Versus Realities. In *Drug Residues in Animals*:; Rico, A., Ed.; Academic Press: Orlando, FL, **1986**, pp. 147-174.
34. Hoffman, B. The Use of Hormones in Food-Producing Animals. *Proceedings of the Health Aspects of Residues of Anabolics in Meat.* **1982**, *Vol. 59*, pp. 16-27.
35. Lamming, G.E. Scientific Report on Anabolic Agents in Animal Production. *Vet. Rec. 1987*; **1987**, *Vol. 121*, pp. 389-392.
36. *Residues of Some Veterinary Drugs in Animals and Foods.* Thirty-Second Meeting of the Joint FAO/WHO Expert Committee on Food Additives, **1988**; FAO Food and Nutrition Paper 41, pp. 1-49.
37. Bongiovanni, A.M. An Epidemic of Premature Thelarche in Puerto Rico. *J. Pediatr.* **1983**, *Vol. 103*, pp. 245-246.
38. Hannon, W.H.; Hill, R.H.; Bernet, J.T.; Haddock, L; Lebron, G.; Cordero, J.F. Premature Thelarche in Puerto Rico: A Search for Environmental Contamination. *Arch. Environ. Contam. Toxicol.*, **1987**; *Vol. 16*, pp. 255-262.
39. Freni-Titulaer, L.W.; Cordero, J.F.; Haddock, L.; Lebron, G.; Martinez, R.; Mills, J.L. Premature Thelarche in Puerto Rico: A Search for Environmental Factors. *Am. J. Dis. Child*. **1986**, *Vol. 140*, pp. 1263-1267.
40. Anonymous. *1985 Special Meat and Poultry Sampling Program Conducted in Puerto Rico by the Food Safety and Inspection Service of the U.S. Department of Agriculture.* **1986**, May, pp. 1-7.
41. Hoffmann, B.; Evers, P. Anabolic Agents with Sex Hormone-Like Activities: Problems of Residues, In *Drug Residues in Animals*; Rico, A., Ed.; Academic Press: Orlando, FL; **1986**, pp. 111-146.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

42. Anonymous. Biggest-Ever Hormone Scandal Uncovered in FRG. *Animal Pharm.* **1988**, *Vol. 161*, p. 2.

43. Anonymous. Spain confronts "extremely serious" clenbuterol abuse. *Animal Pharm* . **1992**, *Vol. 246*, p. 6.

44. Martinez-Navarro, J.F. Food poisoning related to consumption of illicit beta-agonist in liver. *Lancet.* **1990**, *Vol. 336*, p. 1131.

45. Keller, W.C. Illegal use of clenbuterol in food animals. *FDA Veterinarian.* **1991**, *Vol. 6*, No. 4; pp. 9-11.

46. Martinez-Frias, M.L.; Cereijo, A.; Rodriguez-Pinilla, E.; Urioste, M. Methimazole in animal feed and congenital aplasia cutis. *Lancet.* **1992**, *Vol. 339*, pp. 742-743.

47. Anonymous. Europe's Black Market Problem. *Animal Pharm.* **1992**, *Vol. 254*, No. 18; pp. 1-4.

48. Juskevich, J.C.; Guyer, C.G. Bovine growth hormone: Human food safety evaluation. *Science*. **1990**, *Vol. 249*, pp. 875-884

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch002

Chapter 3

Consumer Perceptions and Concerns About Veterinary Drug Residues

Christine M. Bruhn

Center for Consumer Research, University of California, Davis, CA 95616

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch003

Antibiotic and hormone residues are not a major concern of most consumers. Where concern exists, it relates to lack of awareness or trust in regulatory procedures and is fueled by marketing practices of some producers and information dissemination of special interest groups. Concern centers on development of antibiotic resistant strains of bacteria, allergic or physiological response when products are consumed, appropriate animal care, and product wholesomeness. Concern can be addressed by continued vigorous safety testing, monitoring, and enforcement, expanded information dissemination, and responsible advertising.

Antibiotic and hormone residues are currently not a major concern of most consumers, however they have the potential to become so. Only 1% of consumers surveyed volunteered concern about animal drug residues, but when specifically asked, half said they were a serious hazard (*1*). The area of greatest consumer concern is microbiological. In unaided responses, 52% of consumers mention spoilage or bacterial-related problems. This percentage is greater than those mentioning pesticide residues or any other food safety issue.

Concern about residues relates to lack of awareness or trust in regulatory procedures. Focus group discussions with consumers indicates some are concerned with what they see as "changing science" (*2*). It seems to be human nature to remember and focus on those areas where recommended practices change. Some see it as a weakness when dietary or safety recommendations change. For example, people say if you trace back far enough, people were once advised to have a breakfast of bacon and eggs; then egg consumption was discouraged because of cholesterol and bacon because of high fat. Now some are saying eggs are not so bad after all. DDT and Agent Orange are also cited as chemicals scientists said were okay, but are now considered hazardous. The fact that DDT is a concern for wildlife, not people, or Agent Orange was used in warfare is lost in these discussions as are the multitude of regulatory decisions which still stand.

Concern with antibiotic use centers on potential development of antibiotic resistant strains of bacteria, and allergic or physiological response. Consumer

0097–6156/96/0636–0018$15.00/0
© 1996 American Chemical Society

information is needed regarding residue testing and the similarity of antibiotics used for animals and those used by people.

Some industry and special interest groups fuel consumer concern through their marketing practices, news conferences, and promotional material. Some poultry producers advertise their products as not injected with hormones. Since growth hormones are not used in poultry production, this advertisement creates an impression of difference when none exists. This could be considered deceptive advertising, and it contributes to consumer anxiety about the safety of animal production practices. In a California consumer survey, 10% said they were eating less poultry because of concern about hormone residues (*3*).

Some special interest groups use the potential of health concerns to promote their organization and the organization's agenda. The response to Bovine Somatotropin (BST) illustrates this practice. Leading medical and health groups have endorsed the safety of milk produced from cows receiving supplemental (BST); however the Foundation on Economic Trends launched a vigorous campaign questioning milk's safety and other more moderate groups have echoed concern. Research indicates that consumer concerns were reduced when they heard health groups endorsed milk safety (*4*). With the exception of Wisconsin where the BST debate became highly politicized, the effectiveness of this fear campaign has been limited (*5*). Milk sales across the nation are up and products labeled from non-supplemented cows represent a very small market share.

The health and environmental benefits of use of BST in cattle is rarely told. Consumers seldom hear that BST allows the animals to use feed more efficiently--an environmental benefit since less resources will be used and less manure produced. Neither are they told that use of growth hormones in beef cattle has an insignificant effect on muscle estrogen level but increases the ratio of lean muscle to fat. Dietary fat is consumers' primary food-related nutritional concern (*1*). Since people are striving to reduce dietary fat while continuing to enjoy traditional food, being able to choose leaner meat is a good thing.

Animal care and management must be addressed with sensitivity. People want animals treated with compassion. Industry advertisements are not always sensitive to this perspective. A product advertised in a trade publication that promised to turn a cow "into a factory" is not well received by a public that brings children to the petting area of a fair.

Inappropriate animal care contributes to a declining confidence in the production and regulatory system. People are very distressed by pictures of "downer animals," animals that are quite ill or injured. One instance of apparent mistreatment captured by video and aired at community meetings or over television can tarnish an industry's reputation of integrity and responsibility.

Consumers believe product safety could be improved by the government developing and enforcing strict regulations, producers and processors following strict standards, and consumers adopting safe handling practices (*1*) . Enforcement of safety standards should be rigorous and fines significant. Consumers recognize that regulators can not be everywhere, but they expect high compliance from a regulatory and checking system with enforcement that hits the pocketbook. Producers might rethink standards based on ***voluntary*** compliance. If standards are not followed universally, the entire industry may suffer from lost public confidence.

A paperwork trail that verifies antibiotic use will contribute to public confidence. The controversy over use of the growth regulator Alar on red apples created the major food safety controversy of the decade (*6*). It is likely that consumer concern was increased by the apparent lack of data on the amount of Alar used. A senior EPA administrator acknowledged on national television that he had no idea how much Alar was used. Apple growers cited one use level, while chemical analysis of apple juice suggested another. Should a crisis arise in the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch003

veterinary drug area, regulators, veterinarians, and producers need data to quantify product use and industry practices.

Illness and death due to *E. coli* O157:H7 has placed cattle under increased scrutiny. The cattle industry and university researchers will be looking closely at animal production practices which lead to the presence of this human pathogen. Special interest groups will be ready to make accusations and establish blame wherever they can. The industry's best defense and the approach which promises the greatest public health advantage for the consumer is information. Therefore record keeping as to antibiotic use and factors correlated with the presence *of E. coli 0157:H7* should be established.

Consumers increasingly rely on themselves to assure foods are safe (*1*). Therefore information to consumers on the purpose of various animal production practices and each person's role in assuring food safety is essential to create an informed public. Programs should emphasize that microorganisms are a ***natural*** part of the ecosystem. All members of the food production network from producer to consumer need to assure the microbes do not become harmful. This includes sound hygienic practices, refrigeration, and proper cooking. Microbiological levels of raw products could be reduced and safety enhanced by irradiation (pasteurization by gamma rays), steam chemical rinses, or other treatments (*7*).

People get most of their information from the media, with television used most frequently, followed by newspapers, other people and magazines (*6,8*). On issues of agricultural production and health, they place the greater confidence in information from health authorities, scientists, and farmers than that from activists. Our country has become more urbanized and people have less knowledge of farm practices and challenges. A modern U.S. oriented television program with the warmth and humanity of James Harriot's "All Creatures Great and Small" would be an appealing way to increase the public's familiarity with agriculture production. Until Hollywood comes up with such a blockbuster, person-to-person contacts offer a significant opportunity to increase appreciation and understanding of today's agricultural production.

When communicating about the safety of veterinary drug residues, it is appropriate to first identify the nature of the consumer's concern. A concern related to animal treatment would be addressed differently than one related to the safety of an animal product. Next identify and note a shared value system. Veterinarians, dairy and poultry farmers and cattle ranchers care about animal health and want to produce safe food. People need to be updated on progress toward this goal. Consumer concern should be addressed by continued vigorous safety testing, monitoring and enforcement, responsible advertising, and sustained information dissemination.

Literature Cited

1. *Opinion Research,* Trends 95: Consumer attitudes and the supermarket 1994, Food Marketing Institute, Washington, DC, 1995.
2. Bruhn, C.; Peterson, S.; Phillips, P,; Sakovich, N. Consumer response to information on integrated pest management. *Journal of Food Safety* 12 (1992) 315-326.
3. Bruhn, C., University of California Davis, unpublished data.
4. Hoban, T.J. Consumer awareness and acceptance of bovine somatotropin (BST), North Carolina State University, conducted for the Grocery Manufacturers of America, 1994.
5. Bradford, L. B.; Buttel, F.H.; Jackson-Smith, D.B. The political economy of rBST adoption in America's dairyland. Paper presented at the American Association for the Advancement of Science Annual Meeting, Atlanta, GA, 1995.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch003

6. *Center for Produce Quality*, Fading scares-future concerns: Trends in consumer attitudes toward food safety, Produce Marketing Association, Alexandria, VA, 1992.
7. Diehl, J.F. Safety of Irradiated Foods. Marcel Dekker, Inc., N.Y., NY, 1995.
8. Hoban, T.J.; Kendall, P.A., Consumer attitudes about the use of biotechnology in agriculture and food production, North Carolina State University, Raleigh, NC, 1992.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch003

Chapter 4

European Union Regulatory Residue Analysis of Veterinary Drugs: A Strategic Approach

R. W. Stephany and L. A. van Ginkel

European Community Reference Laboratory, RIVM/ARO, P.O. Box 1, NL–3720 BA, Bilthoven, Netherlands

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

After an introduction into the field of anabolic agents, with some "new" illegal steroids and beta-agonists as examples, an overview is given of the recent developments within the European Union (EU) for regulatory residue analysis of food animals treated with veterinary drugs, banned anabolic agents inclusive. Four supporting corner stones are implemented (minimum quality criteria, reference materials, manuals of methods and workshops) and a series of hierarchically linked laboratories (routine or field laboratories, national reference laboratories and EU reference laboratories) are operational. The most relevant difference between the traditional quality assuring approach of harmonizing methods of analysis and the alternative EU approach is that not methods but quality criteria and critical control points are harmonized. With this approach analytical strategies are applied which are defendable in Court of Justice and are complying explicitly with the objectives of the EU regulatory investigations.

Introduction

For the promotion of muscle growth (so-called "fattening") in slaughter animals like cattle, veal calves, sheep, swine and also to a lesser extent in poultry, the use of hormonal anabolic agents has proven to be very effective and its applications and residues have been reviewed extensively (1-5). For fattening purposes in the past 40 years estrogenic, androgenic and (pro)gestagenic compounds have been used as such or in combination. Most of these compounds are steroids. Of the numerous different steroids known to be illegally used in the European Union (EU), the former European Communities, up to the present, the androgen nandrolone (17-beta-19-nortestosterone) is found most frequently, as such or as fatty acid esters in injection sites. This doping agent is very challenging in residue control because for some species of slaughter animals like horses and male, non castrated pigs (boars) the steroid is endogenous. In young boars average natural levels of nandrolone (in µg/kg) have been observed of 1 in muscle, 23 in liver, 55 in urine and 88 in bile. Highest levels observed for these matrices are 13, 200, 132 and 212, respectively (6). Nandrolone and its esters is also known as a contaminant of feed, especially milk replacers for

0097–6156/96/0636–0022$15.00/0
© 1996 American Chemical Society

veal calves (7,8). In such cases its contaminating origin is most likely rendered fat of illegally injected slaughter animals, a common ingredient of milk replacers.To make it even more complex for the meat inspections, recently the natural occurrence of the nandrolone metabolite 17-alpha- or epi-19-nortestosterone has been confirmed in pregnant cows and new born calves (9,10). Furthermore a whole series of "new" steroids are detected in illegal applications like algestone and boldenone (11).
Although the growth promoting effect of the adrenal hormone adrenaline already was known in the early sixties, the systematic misuse of adrenaline derivatives, the so-called beta-agonists, for fattening purposes became wide spread within the EU about 1988 (12-14). Again the compounds are used as such or in combination with steroids (15-20), in this case especially corticosteroids. A typical list of the beta-agonists found within the EU and used illegally for fattening purposes, is given in Table 1.
Most of the "black market designer agents" have not been given names suggested by their chemical structures. While these names have been assigned in a chemically rigorous way, they do provide a practical taxonomy in maintaining a catalog of this rapidly growing list of relatively simple "synthesize them yourself" effective growth promoters (Figure 1).
Although clenbuterol still is the favoured drug of use, its bromo-analogue "brombuterol" is now also observed as well as the t-pentyl-analogue of mabuterol, the so-called "mapenterol".

Table 1 :Examples of N-alkylphenyl (or pyridyl) ethanolamines illegally used in the EU for growth promoting purposes.*

Name	Y_2	Y_3	Y_4	Y_5	X	R	Illegally used
clenbuterol	H	Cl	NH_2	Cl	C	t-butyl	yes[$]
"clenproperol"	H	Cl	NH_2	Cl	C	isopropyl	yes
"clenpenterol"	H	Cl	NH_2	Cl	C	pentyl	yes
"clencyclohexerol"	H	Cl	NH_2	Cl	C	c-hexyl	yes
"brombuterol"	H	Br	NH_2	Br	C	t-butyl	yes
salbutamol	H	CH_2OH	OH	H	C	t-butyl	yes[$]
mabuterol	H	Cl	NH_2	CF_3	C	t-butyl	yes
"mapenterol"	H	Cl	NH_2	CF_3	C	t-pentyl	yes[$]
pirbuterol	-	CH_2OH	OH	H	N	t-butyl	unknown
terbutaline	H	OH	H	OH	C	t-butyl	suspect
fenoterol	H	OH	H	OH	C	C_3H_6phenyl	yes[$]
cimaterol	H	CN	NH_2	H	C	isopropyl	yes
"cimbuterol"	H	CN	NH_2	H	C	t-butyl	yes

* This list is far from exhaustive, names between quotation marks are fancy names given by the Benelux working group "Hormones and Anti-hormones" and the reference center "Anaref".

$ Also used in sports doping.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

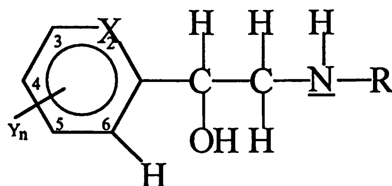


Figure 1: General structure of beta-agonists of the N-alkylphenylethanolamine type; n = 2, 3, 4 or 5.

A restricted controlled use of some specified anabolic agents (zeranol and some steroids) is legalized in e.g.: the USA and Canada, Australia and New Zealand and in some countries in South America, Asia and Africa. Beta-agonists up to the present are always banned for growth promoting purposes.
For the protection of consumers and for the benefit of international trade a total ban of anabolic agents for growth promoting purposes in slaughter animals is effective in The Netherlands since 1961, in Belgium since 1962-1969, in all Benelux Countries since 1973 and in the EU since 1988.
As a consequence up to 1988 the differences in approach and attitude towards the "hormone problem" in the various EU member states regularly resulted in conflicts between these states and also between the "hormone free" states and other countries outside the EU like the USA. The different opposing aspects are summarized below.

Legal use	versus	Illegal use
White market	versus	Black market
Well defined drugs	versus	Undefined drugs
Solid implants	versus	Liquid cocktails
Residue tolerance levels	versus	Zero tolerance level
Residues in edible tissues	versus	Residues in excreta
Control at slaughter	versus	Control at the farm
Ministry of Agriculture	versus	Ministryof Public Health
Producers Lobby	versus	Consumer Lobby
USA	versus	EU
Harmonized methods	versus	Harmonized performance criteria
Single Laboratories	versus	Hierarchy of Laboratories
QA via GLP	versus	QA via GLP + EN

In general, the last three items apply to all veterinary drugs.

Residue control

As a "mini EU" the three Benelux countries started a joined laboratory control programme in 1972 within the framework of the Benelux Economic Union. To coordinate and support this control for anabolic residues within the Benelux countries as a part of the Benelux Working Group "Hormones and Anti-Hormones" in 1979 the Anabolic Reference Center BNL ("Anaref") was established. This Center, located in

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

The Netherlands at RIVM can be considered as the direct precursor of and as a model for the corresponding European Communities Reference Laboratories designated in 1991 by the EU Council.

Analytical strategies

Residue analyses related to e.g. public health, international trade or environmental problems have to be performed fast and reliable, based on an integrated analytical chemical approach and professional consensus within a limited budget. This requires good professional behaviour (GPB) of all involved officials as well as quality assurance such as good laboratory practices (GLP) (21-23).

Triggered by a continuous series of residue scandals with illegal "anabolic hormones" in cattle, the European Commission (EC), instead of methods, developed analytical strategies for residue analysis of veterinary drugs and contaminants in food of animal origin. Council directive 86/469/EEC of 16 September 1986 concerning the examination of animals and fresh meat for the presence of residues was implemented to enforce uniform application throughout the EU of measures to ensure that meat was free from undesirable residues (24). For that purpose within the past 10 years a system was developed based on 4 fundamental corner stones and controlled by a series of hierarchically linked European Union and National reference laboratories. As shown in Figure 2 the 4 corner stones are a programme of reference materials (DG XII, BCR "Standards, Measurements and Testing Programme" (SMT)) (25, 26), a set of regularly updated mandatory minimum quality criteria for analytical techniques (DG VI) (27-31), a series of Reference Manuals (DG VI & XII) (32, 33) and a continuous series of laboratory workshops (DG VI) (12) plus a future Peer Review Group (DG III, V, VI and XII). Routine or field laboratories (RFLs) involved in the annual residue monitoring programmes (ANPs) are coordinated and controlled per EC member state by at least one National Reference Laboratory (NRL) designated by the National Government (28, 31). The NRLs are supported, advised and controlled by 4 Community Reference Laboratories (CRLs) (DG VI) designated in 1991 (34).

The 4 CRLs in the Netherlands, Germany, France and Italy respectively, are hierarchically equal, however, each of them is designated for a dedicated set of compounds:

- RIVM (Bilthoven, NL) stilbenes, thyreostats, steroids, zeranol;
- BGVV (Berlin, D) chloramphenicol, sulphonamides, beta-agonists;
- CNEVA-LMV (Fougères, F) antibiotics, quinolones, nitrofurans, nitro-imidazoles;
- ISS (Rome, I) Cd, Pb, Hg and As, PCB's, organochlorines, pesticides.

Powers and mandates of CRLs

The powers and conditions of operation of the CRLs concerning the examination of animals and fresh meat for the presence of residues are defined by the EU Council Decision of 6 March 1989 (35). In summary their tasks and duties are:

•to coordinate the application of GLP within the NRLs. Here GLP is defined as GLP according to OECD (21, 22). Within the revision process going on (CEC, (1993) COM (93) 441 def., unpublished) it is under discussion that accreditation according to the EN 45000 series (23) probably is a more appropriate base for QA of analytical chemical routine laboratories;

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

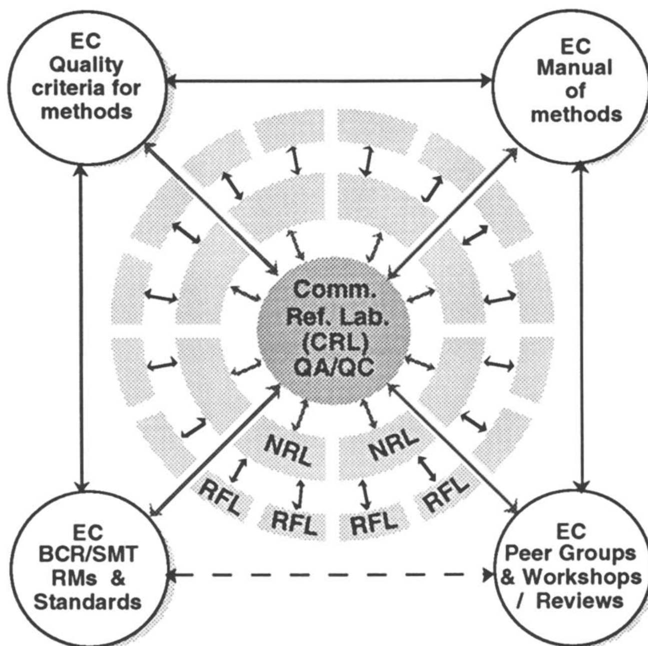


Figure 2:Analytical system for the determination / identification of residues and contaminants according to Council Directive 86/469/EC.

•to provide the NRLs within the EU and "third countries" with methods of analysis and technical advice. A series of Manuals (32, 33) is available and electronic data bases like CB\METHODS with information on methods validation are continuously updated. A whole series of reference materials and deuterated standards has been prepared or is in preparation (26, 36, 37);

•to promote and coordinate research into new methods (20, 38). In close cooperation with other EC programmes like BCR/SMT, HCM, AIR and VALUE inter EU Member State research programmes are running, to be started or under negotiations;

•to organize comparative ring tests between NRLs. A few ring tests have been completed, e.g. for chloramphenicol (39), diethylstilbestrol (40) and clenbuterol (41), others are running e.g. estradiol in bovine blood (42), or will be started in the near future;

•to conduct training courses for analysts of NRLs and RFLs. A continuous series of laboratory workshops have been organized, e.g. on immuno assays, HPLC and GC-MS. A workshop held in 1991 at RIVM was focussed on beta-agonists (12,13). The workshop in March 1994 at RIVM was focussed on Quality Assurance (QA) (53) ;

•to provide the EC and BCR with technical and scientific assistance. Support has been given in the evaluation of the result of the ANPs. New project proposals have been (co-)evaluated, e.g. for Agro-Industrial Research;

•last but not least to perform the "final analysis" in case of dispute between EU Member States. So far this challenge has not been met, however, discussions about the degree of reliability and the cost efficiency of such a "final analysis" are still going on.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

In the past few years the CRL-NRL system has been expanded and is still expanding for other topics, e.g. marine biotoxins (CRL in Vigo, Spain), Salmonellas (CRLs in Bilthoven, NL and Berlin, D), aquaculture (CRL in Aarhus, Danmark) and milk (CRL in Paris, F).

A chronological overview of the development of this EC system is reviewed in detail recently (43).

Cost effectiveness

To achieve a Regulatory Accepted Cost Effectiveness Balanced Approach (RACEBA) residue analysts in charge of setting up programmes to implement such RACEBAs worldwide more and more use at least two step programmes.

Such multi step programmes are based on Professionally Optimized Feasibility for an Agreed Purpose and Price (POFAPP). If the population of samples to be analyzed for residues consists mostly of samples that fulfil all regulatory requirements ("real negative samples") and only a relatively small fraction of samples that do not fulfil all regulatory requirements ("real positive samples") than it is worthwhile to apply in a two step programme an analytical screening followed, if appropriate, by an independent confirmation of the screening result.

To reduce false results and false conclusions the methods used in a screening step have to be suitable for large scale repetitive routine application, have to be relatively fast, simple and cheap and have to create no or only little false negative results. Such screening methods must be validated in a proper statistical design. Methods to be used in the confirmation of non-negative results from the screening step as real or true positive results, in contrast, are generally not simple, not fast and not cheap but highly reliable to prevent false positive results (44).

Various attempts and proposals were made in the past seven years to develop quantitative arguments on characteristics to support the implementation of RACEBA based on POFAPP. Especially a "Dutch School" of residue chemists involved and responsible for the implementation of POFAPP in The Netherlands was, and still is, active in this field. This sometimes happens in a quite unorthodox way, but that at least triggers fruitful discussions between politicians, regulators, administrators, managers and lawyers of all kind and professional analytical chemists.

So concepts and ideas are postulated about acceptable and feasible error probabilities for forensic or regulatory results of residue analysis (44-46), about experimental chemometrics based also on professional experience rather than only statistics (47), about minimum quality performance criteria for residue analyses (27-31, 48, 49), about uncertainty factors for analytical techniques (50) and about the balance of false negative and false positive analytical results in inspection procedures based on a multi step system (51, 52).

All these items are attempts to contribute to a new kind of approach for matching the present and future demands for chemical residue analyses and the available human resources, laboratory facilities and budgets which in general shown no increase parallel to the increase in demands.

As our experience and jurisprudence in Court of Law shows, residue analysts can defend themselves or their results only if they follow an approach which is based on a parallel series of quality assuring and controlling factors like the EC model.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

Closing remarks

Regulatory residue analysts should be able to prove their daily level of performance and competence not only by occasional participation into ring tests but preferably by daily operating along Good Laboratory Practice (GLP) guidelines and/or being accredited or certified by an appropriate independent organization (23). Last but not least exceptional non-typical excellent results should not be presented as daily "common practice". This will not only backfire sooner or later on the pretender, but also damages the image of residue analysts as a professional group. To improve not only this image but, more important, to improve the quality and reliability of residue analyses, analysts, chemometrists, lawyers, financiers, etc. are all invited to actively contribute to the attempts started in the last years to come to cost effective analytical residue strategies "suited for the purpose".

For additional background information and details the reader is referred to the a series of review papers (32, 33, 36, 37, 43, 44, 48).

Acknowledgement

Most of the RIVM work concerning residue analysis of veterinary drugs, anabolic agents and criteria for elements was performed on behalf of the Dutch Veterinary Chief Inspectorate of Public Health, often in cooperation with colleagues in Benelux, EC, ISO, IUPAC, WHO/FAO (Codex) working parties, in sports doping laboratories and Eurachem. Thanks are due to many of these colleagues for stimulating and sometimes challenging discussions and to the referees for very useful remarks.

Note: Any opinions, findings, conclusions and recommendations expressed in this paper are those of the authors and do not necessarily reflect the official views of the National Institute of Public Health and Environmental Protection or any of the International Bodies referred to.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

References

(O.J.E.C.: Official Journal of the European Communities, Luxembourg, Lux. CEC: Commission of the European Communities. Copies of referred documents, whole books excluded, outside of the regular public domain can be requested from the authors.)

1. *Domestication, conservation and use of animal resources*, Berende, P.L.M.; Ruitenberg E.J., Elsevier Publishers: Amsterdam, NL, **1983**; Chapter 10. ISBN 0-444-42068-1, pp 191-233.

2. *Proceedings International Symposium Steroids in animal production*, Jasiorowski, H., Ed.; Warsaw Agricultural University, Poland, **1981**; ISBN 83-00-01686-4, 261 pp.

3. *Environmental quality and safety*; Lu, F.C.; Rendel, J.; Coulston, F.; Korte, F., Eds.; Georg Thieme Publishers: Stuttgart, BRD, **1976**; Supplement Vol. V, ISBN 3-13-536101-2, 277 pp.

4. *Anabolics in animal production*; Meissonnier, E.; Mitchell-Vigneron, J., Eds.; Paris, Office International des Epizooties, France, **1983**; ISBN 92-9044-118-6, 572 pp.

5. *World Health Organization. Health aspects of residues of anabolics in meat*, **1982**, EURO Reports and Studies 59. ISBN 92-890-1225-0, 38 pp.
6. Ginkel, L.A. van; Stephany, R.W.; Zoontjes, P.W.; Rossum, H.J. van; Blitterswijk, H. van; Zuijdendorp, J. Tijdschrift Diergeneesk. **1989**, 114, pg. 311-314.
7. Meyer, H.H.D.; Hartman, F.-X.; Rapp, M. J. Chrom. **1989**, 489, pg. 173-180.
8. Rapp, M.; Meyer, H.H.D. J. Chrom. **1989**, 489, pg. 181-189.
9. Meyer, H.H.D.; Falckenberg, D.; Janowski, T.; Rapp, M.; Rösel, E.F.; Look, L. van; Karg, H. Acta Endocrinologica **1992**, 126, pg. 369-373.
10. Brabander, H.F. de; Hende, J. van; Batjoens, P.; Hendriks, L.; Raus, J.; Smets, F.; Pottie, G.; Ginkel, L.A. van; Stephany, R.W. Analyst **1994**, 119, pg. 2581-2585.
11. Vanoosthuyze, K.; Daeseleire, E.; Overbeke, A. van; Peteghem, C. van; Ermens, A. Analyst **1994**, 119, pg. 2655-2658.
12. *The use of immunoaffinity chromatography in multi-residue and confirmation analysis of beta-agonists in biological samples*. Ginkel, L.A. van; Rossum, H.J. van; Stephany, R.W., Eds.; Proceedings of the EC-workshop organized at RIVM, Bilthoven, The Netherlands, April **1991**, ISBN 90-6960-016-1, 136 pp.
13. Ginkel, L.A. van; Stephany, R.W.; Rossum, H.J. van J. AOAC International **1992**, 75, pg. 554-560.
14. *Beta-agonists and their effects on animal growth and carcass quality;* Hanrahan, J.P., Ed.; Elsevier Publishers: London, UK, **1987**; ISBN 1-85166-176-X and EUR 11167 EN, 201 pp.
15. *Proceedings International Symposium on the Analysis of Anabolizing and Doping Agents in Biosamples,* Ghent, B, May 16-19, 1988; Deyl, Z.; Leenheer, A. de, Eds.; J. Chrom. **1989**, 489, 253 pp.
16. *Proceedings EuroResidue I Conference on Residues of Veterinary Drugs in Food,* Noordwijkerhout, NL, May 21-23, **1990**; Haagsma, N.; Ruiter, A.; Czedik-Eysenberg, P.B., Eds.; University of Utrecht, NL, 1990 ISBN 90-6159-011-6, 431 pp.
17. *Proceedings EuroResidue II, Conference on Residues of Veterinary Drugs in Food,* Veldhoven, NL, May 3-5, **1993**; Haagsma, N.; Ruiter, A.; Czedik-Eysenberg, P.B., Eds.; University of Utrecht, NL, **1993**, ISBN 90-6159-016-7, 721 pp.
18. *Proceedings International Symposium on Hormone and Veterinary Drug Residue Analysis*, Ghent, B, May 19-22, 1992; Pardue, H.L.; Townshend, A.; Clerc, J.T.; Linden, W.E. van der; Worsfold, P.J., Eds.; Anal. Chim. Acta **1993**, 275, 362 pp.
19. *Proceedings 2nd International Symposium on the Analysis of Anabolizing and Doping Agents in Biosamples*, Ghent, B, May 16-18, 1990; Deyl, Z., Ed.; J. Chrom. **1991**, 564, 215 pp.
20. Peteghem, C. van, *Proceedings 2nd International Symposium on Hormone and Veterinary Drug Residue Analysis*, Bruges, B, May 31 - June 3 1994;; et al., Eds.; Analyst. **1994**, 119, pg. 2519-2768.
21. Council of the European Communities, O.J.E.C. **1987**, L 15, pg. 29-30.
22. Council of the European Communities, O.J.E.C. **1988**, L 145, pg. 35-37.
23. Klooster, H.A. van 't; Deckers, H.A.; Baijense, C.J.; Meuwsen, I.J.B.; Salm, M.L. TrAC **1994**, 13, pg. 419-425.
24. Council of the European Communities, O.J.E.C. **1986**, L 275, pg. 36-45.
25. BERM-4, Fresenius J. Anal. Chem. **1990**, 338, pg. 359-580.
26. CEC,. *The BCR programme on applied metrology and chemical analysis*. Projects and results 1988-92. Report EUR 14800. ISBN 92-826-4968-7, **1992** 312 pp.
27. CEC, O.J.E.C. **1987**, L 223, pg. 18-36.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

28. CEC, O.J.E.C. **1989**, L 351, pg. 39-50.
29. CEC, O.J.E.C. **1990**, L 286, pg. 33-39.
30. CEC, O.J.E.C. **1993**, L 118, pg. 64-74.
31. CEC, O.J.E.C. **1993**, L 118, pg. 75-79.
32. CEC,. Veterinary drug residues. Residues in food producing animals and their products: Reference materials and methods. R.J. Heitzman (Ed). Luxembourg, Lux. Report EUR 14126. ISBN 92-826-4095-7, **1992** 345 pp.
33. CEC, Veterinary drug residues. Residues in food producing animal and their products: Reference materials and methods. R.J. Heitzman (Ed.). Blackwell Scientific Publications, Oxford, UK. Report EUR 15127-EN. ISBN 0-632-03786-5, **1994** 512 pp.
34. Council of the European Communities, O.J.E.C. **1991**, L 368, pg. 17-18.
35. Council of the European Communities, O.J.E.C. **1989**, L 66, pg. 37-38.
36. Stephany, R.W.; Ginkel, L.A. van Fresenius J. Anal. Chem. **1990**, 338, pg. 370-377.
37. Stephany R.W., **1993**, See ref. 17, pg. 89-98.
38. Maxwell, R.J.; Stolker, A.A.M.; Lightfield, A.R. J. High Resol Chromatogr. **1995**, 18, pg. 231-234.
39. Preiss, A.; Balizs, G.; Benesch-Girke, L; Gude, Th.; Boenke, A.; Kroker, R., See ref. 17, **1993**, pg. 558-562.
40. Boenke, A. Anal. Chim. Acta. **1993**, 275, pg. 3-8.
41. Ginkel, L.A. van; Stephany, R.W.; Rossum, H.J. van; Bos, M., See ref. 17, **1993**, pg. 308-312.
42. Ginkel, L.A. van; Stephany, R.W.; Spaan, A.; Sterk, S.S. Anal. Chim. Acta. **1993**, 275, pg. 75-80.
43. Stephany, R.W.; Ginkel, L.A. van; Schothorst, R.C. Analyst **1994**, 119, pg. 2707-2711.
44. Stephany, R.W. J. Chrom. **1989**, 489, pg. 3-9.
45. Schuller, P.L.; Stephany, R.W., *Proceedings International Symposium Food Policy Trends in Europe*, Deelstra, H.; Fondu, M.; Ooghe, W.; Havere, R. van (Eds.); Ellis Horwood Ltd, West Sussex, UK. ISBN 0-7476-0075-9, **1991** pg. 140-148.
46. Stephany, R.W.; Jansen, E.H.J.M.; Freudenthal, J. Tijdschrift Diergeneesk. **1985**, 110, pg. 654-661.
47. Ginkel, L.A. van; Stephany R.W., See ref. 17, **1993**, pg. 303-307.
48. Ruig, W.G. de; Stephany, R.W.; Dijkstra, G. J. Assoc. Off. Anal. Chem. **1989**, 72, pg. 487-490.
49. Ruig, W.G. de; Weseman, J.M. J. Chemometrics **1990**, 4, pg. 61-77.
50. Ruig, W.G. de; Dijkstra. G.; Stephany, R.W. Anal. Chim. Acta. **1989**, 223, pg. 277-282.
51. Ruig, W.G.; Huf, F.A.; Jansen, A.A.M. Analyst **1992**, 117, pg. 425-430.
52. Stephany R.W.; 1990, Benelux Economic Union, Brussels, B. Document SP/LAB/h (90)
15.
53. Schothorst, R.C.; Ginkel, L.A. van; Sterk, S.S.; Stephany, R.W.; Eds. *Quality Assurance and Quality Control for National Reference Laboratories for Detecting Residues in Biological Samples*. Proceedings of the EU-workshop organized at RIVM, Bilthoven, The Netherlands, March 21-25 **1994**, ISBN 90-6960-053-6, 263 pp.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch004

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

Chapter 5

Evaluation of Analytical Methods Within a Context of Use

John J. O'Rangers[1] and David B. Berkowitz[2]

[1]Center for Veterinary Medicine, U.S. Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855
[2]Center for Devices and Radiological Health, U.S. Food and Drug Administration, 9200 Corporate Boulevard, Rockville, MD 20850

There is no such thing as a "good method" or a"bad method" because methods must be evaluated for a particular purpose. A method which is ideal for one purpose may be totally inadequate for another. Thus, to ask if a method is good is to ask only half of a question; one must ask if it is good *for a particular purpose*. Methods must be evaluated within a context of use. Good methods frequently evolve in a context of use, and are developed for a particular purpose. The USDA STOP TEST was designed for the rapid determination of antibiotics in meat. A sterile cotton swab is exposed to tissue fluids and placed on a petri plate seeded with bacteria sensitive to several antibiotics. If the animal fluids contain antibiotics, a zone of inhibition is seen around the cotton swab. The test is based on well-known principles, and is simple, inexpensive, portable, and sensitive. As a screening test it is excellent, but as a confirmatory test legal action it would be totally unsuitable, because the cause of the inhibition is not identified; it could be from an antibiotic residue or from an elevated serum component of the animal. The suitability of the test must rest on the context of its use.

Since 1985, there has been a growth of interest in the private and government sectors, in the application of screening tests for the detection of animal drug residues. Although screening tests can be based on any aspect of analytical technology, most of the screening tests being commercially produced for animal drug residues are based on immunoassay or biological receptor technologies. The commercial products are manufactured in a "test kit" format which are intended to be a self contained, complete analytical test for animal drug residues similar to test kits that are marketed for human diagnostic purposes.

The main point is that methods can not be evaluated in isolation. There is no such thing as a "good method" or a "bad method" because methods must be evaluated

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

for a particular purpose. A method which is ideal for one purpose may be totally inadequate for another. Thus, to ask if a method is good is to ask only half of a question; one must ask if it is good *for a particular purpose*. Methods must be evaluated within a context of use.

Screening tests provide several exciting capabilities to animal drug residue detection. For example, because the tests do not require complicated instrumentation, they are usually very rapid in performance with analytical results being achieved in minutes. Not only can more tests be performed in a given time period, but many screening tests can be used outside the laboratory. This capability is an advantage in residue control and public health protection, since an initial analysis is a real possibility at the level of drug use. The use of screening tests in residue detection can be exemplified by the tests that are commercially available for drug testing in raw, bulk tank milk.

The acceptance of animal drug screening tests for regulatory or public health uses should be based on adequate fulfillment of the following three considerations:

1. The screening test must be available for use. The use or recommendation of a screening test by public health or regulatory agencies must be practical. Often tests and methods are described in the scientific literature that would appear to offer good potential for screening test application. However, the general accessibility of these methods is usually very limited unless the reagents or tests can be purchased commercially or will be provided by some other means, such as government contract or drug sponsor. It is important to keep is mind that research is only the first step in the development of useful tests.

A mature, fully characterized and practical screening test that is suitable for routine regulatory or public health use requires additional work to ready the test for real world application

2.The performance of the test must be adequately characterized. The test must be demonstrated to perform with acceptable figures of merit for accuracy, specificity and reproducibility. Time will not allow a detailed discussion of all the technical aspects of screening test performance evaluation, however there are no esoteric performance requirement that are peculiar to screening tests. The performance elements for screening tests are similar to the performance elements that are assessed for other analytical methods.

3. All screening tests should have confirmatory methods. While screening tests provide presumptive information on the existence of a drug residue in a test sample, they do not provide definitive information on the identity of specific drug residues. Often, screening tests are designed to test for multiple residues of various drugs. An example of such a test is the Charm II receptor assay for beta-lactams in milk. This test can give an initial alert that a beta-lactam residue may be present in a milk sample. However, the determination of the specific beta-lactam drug that may be present in the sample requires verification by more specific analytical techniques.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

Furthermore, many screening test for drugs in milk that are currently on the market give a "yes" or "no" answer. i.e. they are designed to provide an indication of the presence of a drug residue in milk above a defined quantitative level.
These tests do not give a quantitative assessment of the residue that may be present. In many cases, it is important to know how much of a given residue may be present in order to take appropriate action. The required confirmatory or quantitative information can be provided by a suitable method.

Good methods frequently evolve in a context of use, and are developed for a particular purpose. The USDA STOP TEST was designed for the rapid determination of antibiotics in meat. (1). A sterile cotton swab is exposed to tissue fluids and placed on a petri plate seeded with bacteria sensitive to several antibiotics. If the animal fluids contain antibiotics, a zone of inhibition is seen around the cotton swab. The test is based on well-known principles, and is simple, inexpensive, portable, and sensitive. As a screening test it is excellent, but as a confirmatory test leading to legal action it would be totally unsuitable, because the cause of the inhibition is not identified; it could be from an antibiotic residue or from an elevated serum component of the animal. The suitability of the test must rest on the context of its use.

A screening test can be simply defined as a test the gives a reliable indication that the analyte(s) of interest are not present in the sample at hazardous or violative levels.(2) This requires that the screening test be developed with a limit of detection (LOD) at a level that will give a high degree of confidence that the tolerance or violative level will be detected. In designing a screening test, this usually means that the LOD of the test will be optimized below the hazardous or violative level so that the higher hazardous level will have a high probability of causing a positive result, or in the jargon of test kit users,"lighting up the test".

This technical specification also means that the screening test can give positive results below the tolerance or violative level. The frequency of positive results will be somewhat smaller than at the tolerance level, but can, and probably will occur.

Without further quantitative analysis or comparison to standards, it is difficult to be sure whether a positive screening test result is actually at the tolerance or violative level. This is a technical cost that is incurred in developing screening tests to monitor hazardous or violative levels or animal drugs and a major technical objective in the development of these tests is minimize the band of uncertainty for the screening test.

The great value of screening tests is the degree to which they reliably indicate samples for which there is no regulatory or public health concern so that the commodity can enter the food supply. A positive result indicates that there is reason to withhold the food commodity and that follow-up action is needed.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

It is apparent that the suitability of a method for a particular objective may be determined by factors external to the test itself. If the samples containing the analyte are well separated from the background or cross-reacting substances, the number of false positives will be minimized.
However, if the background level of the analyte or of some cross-reacting material is sufficiently close to the region of the analytical response given by the analyte, there would be a considerable amount of overlap in the analytical response regions of both the analyte and interfering materials and the percentage of false positive determinations may be unacceptable. For example, the use of the diazotization reaction for the determination of aromatic amines to determine sulfamethazine (3,4), may be perfectly suitable for drug samples, but in animal tissues the background level of aromatic amines is very high, so one must first separate the sulfamethazine from the cross-reacting material. The test is useful when the background is low, but not useful when the background is high.

Furthermore, screening tests can be based on any type of technology and may involve considerable technical complexity. Multi-residue chromatographic methods designed to separate and quantitate multiple drugs or pesticides require experienced laboratories and analysts to be used effectively.

These types of screening tests are intended to provide efficient economies of scale for the expert laboratory so that analytical coverage can be maximized. These types of screening methods will not be discussed.

Rather the types of screening tests that are of interest have the following characteristics:

1. They usually do not depend on complicated analytical instrumentation. Analytical signals are usually generated colorimetrically and use simple detector systems.

2. Although the tests do not depend on complicated instrumentation, they do generally depend on reagents that have complex scientific principles of operation. The quality of these reagents is usually dependent on the processes use to produce the reagents.

3. They generally don't require lengthy, multi-step sample extraction procedures although some minimal sample preparation may be required.

4. They usually determine multiple analytes although they can be designed to detect one analyte with relatively high specificity.

Screening tests having all or some of the above characteristics are usually ligand assays such as immunoassay or receptor assays and are typically provided in a test kit format. The manufacture of reagents into a test kit involves the configuring of reagents to achieve the desired performance characteristics for the test. For example, the sensitivity (limit of detection) of immunoassays partially depends on

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

the concentration of the antibody or other receptor used in the assay. Also the composition and components of the associated reagents such as buffers and substrates determine the performance of the test.

The important point is that the performance characteristics of any screening test kit depends on the unique interaction between the specific components of the test kit. Any changes in the individual test kit components usually can result in altered performance of the test kit.

When selecting a test kit for use or evaluation, the user may consult with the test kit manufacturer for the following information:

1. The test kit should have specific performance specifications set for the over-all test kit. This is the responsibility of the test kit developer or manufacturer. The test kit performance should be confirmed by the manufacturer by the use of alternative methods or definitive confirmatory techniques

2. If individual reagents are separately purchased to be used in a screening test procedure developed by the user,each individual reagent should have performance specifications established by the manufacturer so that the user can continue to select reagents that meet consistent performance specifications.

3. Evaluation of test kits should be done on the test kit as a whole for the claimed usage. This should have been performed by the manufacturer and should be verified by the user in an independent validation study. Alteration of any test kit component as part of the evaluation procedure may invalidate the test as an artefact of testing. Test kit evaluation protocols should observe the response of the test kit to appropriate physical and chemical tests. The evaluation protocol should not significantly change the characteristics of the test kit components as provided by the manufacturer.

4. The test samples used in the evaluation of the test kits should emulate as closely as possible the types of samples that are likely to be encountered by users of the test kit. This means that in addition to using fortified test samples, test samples that contain naturally incurred analyte(s) should also be used when possible.

If incurred samples are not routinely available, well designed field tests could be used to fulfill this recommendation. In the case of field tests, the results of the test kit should be compared to the results achieved by the accepted method of analysis for the tested analyte.

Using the information provided by the test kit manufacturer, the test kit user should develop a validation plan for all test kits that are being considered for use. The purpose of the validation plan is to assure that the test kit performs acceptably in the user's application and the data developed by the screening test is useful to the user to support the intended regulatory or public health action.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

All methods, including screening tests, have innate characteristics, the two most frequently discussed are sensitivity and specificity.
These characteristics are very helpful in judging the usefulness of an assay. However, sensitivity and specificity also vary with the intended use. Some examples are discussed below.

Sensitivity There are some differences in the way sensitivity is described. For example, sensitivity and the limit of detection are used in different ways. A method is frequently referred to as sensitive if it can detect a low level of analyte. Chemists refer to the lowest level of detection as the limit of detection, and method sensitivity as the increment in response relative to the increment in concentration. An example will illustrate the different usages. Assume that we have two methods, method A and method C. Furthermore assume that method C has a lower limit of detection than method A, but the response curve for method A has a greater slope than method C.

The typical conclusion is that method A is more sensitive because the analytical response is greater for each increment in concentration. However, limit of detection and sensitivity are not entirely independent. For example, method C has a lower limit of detection because it has a lower background. If the two methods had the same background, method A would have the lower limit of detection. This is because the point at which the sample response becomes significantly different from the background, i.e., the concentration at which the background and sample signals become significantly different, is lower in A than in C. To calculate this concentration, we take the simplest case and assume the standard deviations are equal at zero concentration and at the limit of detection. The variance of the difference is twice the variance, so the estimated standard deviation of the difference is $S = (2)^{1/2} S_0$ where S is the standard deviation of the difference, and S_0 is the standard deviation of the blank or sample, because they are assumed equal. At the 95% confidence level, the signal becomes significant when it is two standard deviations greater than the blank signal, and $2S = 2(2)^{1/2} S_0$. The limit of detection for method A is lower because the slope is greater; i.e., the signal becomes significantly different from the blank signal at a lower concentration. Thus, the improved sensitivity of method A reduces the limit of detection.

Many screening tests are dichotomous "yes" or "no" tests. If this is the case, and the test is designed to indicate the presence or absence of an analyte, the limit of detection must be known so that the lower concentration limit of what will be detected is known. If the purpose of the assay is to establish that the analyte does not exceed some established level, the limit of detection is far less important, and the reliability of the test must be known across the level of interest. This topic is discussed in more detail below.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

For immunoassays, obtaining this kind of information is somewhat more difficult because the antibody titration curve is sigmoidal rather than linear. Nonetheless useful information about the characteristics of a screening tests can be derived from the sigmoidal curve which is also known as "characteristic operating curve"
In this technique, a panel of test samples is produced by fortifying control matrix with the drug of interest.

The test is run on each sample at its respective concentration level. For good statistical confidence in the measurements, it is recommended that 15 to 20 replicates be run at each concentration level. For rapid screening tests this should not represent an undue analytical burden.

The results are plotted as the percent of samples that are positive at each concentration level. The data from this curve will indicate the following:

a. The concentrations of drug residue that can be detected and the confidence associated with each level.

b. The false positive samples that will be expected.

c. The ability of the test to discriminate between sample residue concentrations.

d. If the test is run over a given time period, the stability of the test can also be evaluated.

The operating characteristic approach is not new. It is an example of an adaptation of the Probit concept elaborated by a number of research workers notably Gaddum (5), Trevan (6), Bliss (7), and Finney (8) In addition, the four parameter logistic algorithm developed by Rodbard, et. al. (9), could also be adapted to analytically describe the operating characteristic curve of screening tests.

A note of caution is needed at this point. The degree of confidence that can be assigned to a result at any concentration level, is partly a function of the number of replicates assayed at each concentration level. While the use of 15 to 20 replicates will give a good assessment of the best confidence that is achievable with a given test, the actual confidence that can be assigned to each assay will depend on the number of replicates actually run in practice. This is a critical point to keep in mind when deciding how a screening test is to be used. If the test is to be used for quantitative purposes, a high degree of confidence in the analytical results is usually required. This requirement could require sample replications that are impractical and uneconomic for routine screening test application for quantitative purposes.

These difficulties are circumvented by use of the logit transformation which linearizes the curve.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

In general, an estimate of the precision at any concentrations level can be estimated from the empirical relationship derived by Horowitz (10). The equation for relative standard deviation is: RSD(%) = $2^{1-0.5\log C}$, where C is the concentration of interest.
For example, when the analyte is pure, C is 1, and the RSD should not exceed 2%. If the analyte is at 1 ppm, $C=10^{-6}$, and the RSD may be as high as 16%. This is a useful empirical equation, with some theoretical rationale

Specificity and Interferences The determination of assay specificity involves the evaluation of the extent to which the assay reacts only with the compound of interest. These studies are also known as cross-reactivity studies. They are performed by assessing the reactivity of the test with structural variants of the test molecule or related chemical substances that may also be present in the sample.

Specificity can be exquisite in immunoassays, and at the same time, it can be exquisitely vexing. For example, an immunoassay for the penicilloyl group is very sensitive and is able to detect the penicilloyl group at very low levels . However, when the assay was used to monitor the pharmacokinetics of penicillin elimination from the serum of treated animals, the situation became complicated. Although the antibacterial activity was all eliminated from bovine serum within 24 hours after injection, the serum levels measured by immunoassay remained high for at least several weeks. This was because the immunoassay measured not only the free drug, but also the penicilloyl groups bound to protein in the serum. The half-life of penicilloyl groups covalently bound to serum proteins are roughly equal to the half-life of the proteins in the circulation. Again, the intended use determines the suitability of the assay.

Advances in immunochemistry have provided research investigators with greater control over the required specificity of antibodies. The variability and unpredictability in specificity that is commonly experienced in polyclonal antibody development, can be greatly controlled by the investigator by using monoclonal antibodies. Producing and harvesting antibodies from a whole animal yields polyclonal antibodies. The use of polyclonal antibodies typically involves the use of antisera produced in an animal responsive the immunogen. The antisera produced actually contains and array of antibodies having different epitopic affinity and specificity characteristics. The characteristics of such an antisera reflects the total effect of the sum of all individual antibodies present in the antisera.

The selection of antibodies with the desired specificity characteristics can be done by use of monoclonal procedures. Monoclonal procedures operate at the level of the cell clones that produce antibodies in the whole animal. The antibody producing cells from an animal responsive to the immunogen are biochemically altered to enhance the growth characteristics of the cells. The hybrid cell clones that are producing the specific antibodies having the desired characteristics can be selected and maintained for production purposes. The availability of cell clones

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

capable of consistently producing antibodies of defined characteristics has had a major impact on making immunoassays much more predictable and practical in performance.

The definitions of sensitivity and specificity in the clinical literature are particularly useful, because they describe how a test performs in particular situations. In the clinical literature, sensitivity means the percentage of true positives detected as positive. Specificity is defined as the percentage of "negatives" reported as negative. Thus, the ideal test is both 100% sensitive and 100% specific. In some ways, an even more revealing measure is called the predictive value of a test. The predictive value of a positive test is plotted against the pretest probability of a sample being positive. Let's assume that we get 10% false positives and 10% false negatives, an that the true percentage of positives in the population is 1%.

If 1000 samples are measured , the pool should contain 10 positives (i.e. 1%), but only 9 are detected because one is a false negative. On the other hand, we have 990 true negatives, but of these, 10% or 99 are false positives.

We report 100 positives when, in fact, we have only 10. So the predictive value of a positive test is only 10%. But we have 990 true negatives and report 990 - 99 = 891, so the predictive value for negatives is about 90%. These estimates depend highly on the true frequencies in the population. Using the same false positive and false negative rates of 10%, but increasing the number of true positives to 10% makes the test more useful. In the population of 1000 we now have 100 true positives. We report 90. Thus, the predictive value of a positive test is 90%, a far better performance than was the case when the true frequency was lower.

Our certainty has been increased by a factor of 9 as a result of the situation in the population, i.e., a circumstance outside of the characteristics of the test itself. This is a dramatic demonstration of the influence of the intended use on evaluating test suitability.

Interference can be practically assessed by using the test under field conditions, using samples containing known quantities of test analyte. The test should provide accurate and reproducible results in the real world environment. Any difference in the performance of the test between the laboratory evaluation and the field test should be resolved before the test in incorporated into a testing program.

Assessment of method performance using actual test samples: The following sets of test samples should be used in the evaluation of the test:

Set 1: Background or blank samples should come from animals or other test articles that are known not to have been exposed to the test analyte. These samples will establish the negative control. The data from these samples are used to determine the false positive ratio which is used to establish the "diagnostic" specificity of the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

test. Efforts should be made to collect materials from control animals on different feeds or from different geographic locations. This will assist in selecting controls that reflect normal animal growth practices.

Set 2: Samples that are known to contain a definite quantity of the test analyte. Typically, these samples will be generated by "fortifying" or "spiking" a suitable matrix with the test analyte. The matrix could be a negative control or may be a sample containing potential cross-reacting or interfering substances.The data from these samples are used to determine the false negative ratio and to establish the "diagnostic" sensitivity of the test.

Set 3: Samples that contain residues of the test analyte that have been naturally incurred in samples from animals or other test article that have been exposed to the test analyte. Different cohorts could be examined in this experiment. For example, normal animals containing the test analyte would be tested followed by examination of cohorts of animals with pathologies or containing substances that are likely to occur and be associated with use of the test analyte. It should be important to understand the response characteristics of the screening test to these cohorts.

Ideally, these test samples should be maintained in a test panel that can be used to evaluate different lots of test kits or reagents. If a test panel is maintained, the stability of the analyte is the test matrix must first be determined.

Confirmatory analysis: There is no single correct procedure or strategy for confirming animal drug residues in meat, milk or eggs. The procedures or the extent of analysis that is needed for confirmation depends entirely on the intended use of the analytical results.

For example, a high degree of certainty is required in establishing the identity and quantity of a drug residue in meat, milk or eggs if the intent is to assess penalties against individuals or organizations for violation of laws or regulations. The very best scientific procedures should be used in these cases not only out of a sense of fairness and official responsibility, but ensure the capability to pursue future regulatory cases.

However, in public health monitoring, definitive identification of specific drug entities is desirable but not strictly necessary in order to take effective action. It is necessary that the analytical results indicate that there is a high probability that a food safety problem may exist in the sample and that further action is warranted to determine the disposition of the food commodity.

In some cases, confirmation may not be needed at all. For example, in establishing quality assurance programs for the acceptance of raw materials for manufacturing, specifications can be established that require the raw material to pass a specific test

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

or battery of tests. The only requirement in this case is that the tests used be well characterized and validated for the proposed use.

There are several approaches that can be used to address confirmation of results:

a. Use a definitive reference method to confirm the initial analysis. In regulatory analysis the method of choice is mass spectrometry. Mass spectrometry gives specific information on the identity and structure of the compound of interest. Coupled with techniques such as gas chromatography, this becomes a very powerful confirmatory tool for both quantitative and qualitative assessment of chemical residues in food. Heat labile chemicals can also be confirmed by interfacing HPLC with mass spectrometry, such as HPLC-Thermospray mass spectrometry.

b. Confirm with several methods or tests. Ideally, the tests used should assess different chemical characteristics of the analyte. The chromatographic behavior of the analyte under different conditions can be effectively used. Normal phase, reversed phase, size exclusion and ion exchange are all examples of chromatographic conditions that operate on different physico-chemical principles and when use with appropriate standards, can give a more definitive insight into the identity of a test analyte.

Different detectors can also be used to exploit the different chemical features that may be characteristic of a given analyte. Photodiode array, ultraviolet, fluorescence, and electrochemical detection are all commonly used in residue analysis and can be used in an on-line mode with chromatographic systems. Gentian violet is an example of a compound that can be determined using ultraviolet or electrochemical detection. (11)

The coupling of chromatographic procedures with immunochemical techniques can provide a very sensitive and specific method for either determinative or confirmatory analysis. Immunoaffinity chromatography has been used extensively in protein chemistry research and is finding increasing application in animal drug residue analysis. Immunoaffinity chromatography involves the coupling of antibodies to a chromatographic support thereby producing a relatively specific chromatographic media for the drug(s) of interest.

The utility of immunoaffinity chromatography in animal drug residue analysis is persuasively shown in the Proceedings of the EC-workshop on The Use of Immunoaffinity Chromatography in Multi-residue and Conformation Analysis of Beta-agonists in biological samples (12).

Immunoaffinity chromatography has been used successfully for the removal and concentration of aflatoxin B1, B2, G1 and G2 allowing the detection of as little as 0.5 ng. of aflatoxin (13)

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

Immunochemical reagents can also be used as an off-line chromatographic detector where fractions of the chromatographic eluate can be assessed be either RIA or ELISA for the analyte of interest. If the antibody used is very specific for the analyte of interest and the antibody reactivity is known to be sensitive to small variations in the structure of the analyte tested, positive reactions with the method are strongly indicative that and analyte of defined structural characteristics is likely present in the sample. Full rigorous confirmation, of course, would depend on further analysis by mass spectrometry. I have personally used this approach in the analysis of diethylstilbestrol in bovine liver. (14)

If the antibody used as a detector is not specific for the analyte of interest, the chromatographic conditions can be adjusted to optimally separate the analyte from other interfering or cross-reacting components that may be in the sample. Thus the power and flexibility of the linkage of chromatography with immunochemistry is readily apparent.

Useful screening technology using solid-phase techniques is not limited to immunoaffinity procedures. A new variation of solid-phase extraction has been developed which permits the very rapid extraction of drugs and other chemicals from complex biological matrices. This procedure is known as Matrix Solid Phase Dispersion (MSPD) and has been developed with the support of the U.S. Food and Drug Administration (15). This technique has been used for the separation of a variety of commonly used antibiotics from animal tissues. The use of this technique holds great promise for a simple clean-up procedure for immunoassay screening methods.

c. Define an existing method as a standard or reference and compare the new or propose test to the standard. This is a practical strategy which makes a great deal of sense when the standard method has been well characterized and demonstrated to be reliable.

It is quite likely that a reliable body of data on the analyte of interest already has been developed through use of the standard method.
This data will be a valuable source of historical control values for use in the evaluation of the new method.

A key point to keep in mind, is that the comparison of the new method with the standard method must be done on a standardized procedure for evaluating the data that each method generates. For example, any correction factors that are used, such as recovery corrections, must be normalized.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

Literature Cited

1. Performing the STOP test: USDA(FSQS) Training Manual U.S. Gov. Printing Office, FSQS-38. 1979.

2. O'Rangers, J. in Immunochemical Methods for Environmental Analysis pp 27-37 VanEmon J. and Mumma R. (eds) 1990

3. Bratton. A.C. and Marshall, E.K.: J. Biol. Chem. 128, 739, 1939.

4. Tischler, F., et. al.: J. Agric. Food Chem., 16, 50, 1968.

5. Gaddum, J. H.: Med. Res. Council Spec. Rept., 183, 1933

6. Trevan, J.W.: Proc. Roy. Soc. (London), 101, 483, 1927

7. Bliss, C.I : Ann Appl. Biol., 22, 307, 1935

8. Finney, D.J.: Probit Analysis (2nd edition) p65, Cambridge, London, 1952

9. Rodbard D. and Hutt, D.M.: Statistical Analytsis of Radioimmunoassay and Immunoradiometric Assays in Proc. Symp. on Radioimmunoassay and related Procedures in Medicine, IAEA, p62, Vienna, Austria, Unipub, N.Y. 1974

10. Howitz, W.,et al, JAOAC 63, Nr 6, 1344, 1980

11. Munns, R.K., et. al.: JAOAC 73, Nr5, 705, 1990

12. Van Ginkel, L.A., van Rossum, H.J. and Stephany, R.W. (eds), Commission of the European Communities, RIVM, Bilthoven, The Netherlands, 1991.

13. Candlish, A.A.G., Intl. J. Food Sci. Tech., 23,479, 1988.

14. O'Rangers, J.J. : (in press)

15. Barker, S.A., Long, A.R., J. Liq. Chromatog, 15,2071, 1992.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch005

Chapter 6

Dilemmas Associated with Antibiotic Residue Testing in Milk

Choices, Problems, and Issues

James S. Cullor

Dairy Food Safety Laboratory, School of Veterinary Medicine, University of California, Davis, CA 95616–8739

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Since mid-1994 there have been antibiotic residue assays that are Center of Veterinary Medicine/Food and Drug Administration (CVM/FDA) "accepted", Association of Official Analytical Chemists (AOAC) International "performance tested", and National Conference On Interstate Milk Shipments (NCIMS) "recommended" that: a) are used for tanker milk and have never been scientifically field tested on tanker loads of milk, b) are used for trace back on bulk tank milk and have never been field tested on bulk tank milk, and c) are routinely used on individual animal milk samples and have not gone through a validation protocol following scientific, epidemiological principles that take into account individual animal variation in milk constituents. The philosophies employed to permit the use of antibiotic residue assays in uncontrolled settings and the consequences of current assay performance will be discussed in the following pages.

Consumers are insisting upon production of safe and wholesome products, and processing plant personnel want the on-farm residue status of milk and dairy beef managed more closely. The veterinary profession has a long history of participating in the development and implementation of medical practices designed to ensure that food safety begins on the dairy, and that this premise is a major focus of dairy production. Hence, Preharvest Food Safety becomes the new agreement between the consumer and producer. Each party involved in the production of milk or meat must be equipped with the appropriate tools and information necessary to protect the food chain. One set of necessary tools, reliable antibiotic residue tests for individual animals, are not available in most cases, and this points out a serious need in future product development.

0097–6156/96/0636–0044$15.00/0
© 1996 American Chemical Society

Choices for the End-Users (Producers and Veterinarians)

Both the producer and veterinarian are committed to on-farm food safety. Therefore, they are interested in acquiring knowledge regarding what guidelines are provided for ensuring that high quality raw milk leaves the farm for the processing plant.

The Pasteurized Milk Ordinance Sets the Tone for Milk Safety.

The Grade A Pasteurized Milk Ordinance (PMO) is used as the sanitary minimum guideline for milk and milk products provided by interstate carriers, and is recognized by public health agencies, the milk industry, and others as the national standard for milk sanitation. Most importantly, the PMO is referenced in Federal specifications for procurement of milk and milk products by governmental agencies, etc. The PMO and its Appendixes is recommended by the United States Public Health Service/Food and Drug Administration for adoption by States, counties, and municipalities for improved uniformity in milk sanitation practices in the United States. Through its enforcement at the state and local level, the PMO facilitates the shipment and acceptance of milk and milk products in interstate and intrastate commerce. Specifically, the PMO defines milk, milk products, prohibits the sale of adulterated and misbranded milk/milk products, requires permits for the sale of milk/milk products, regulates the construction and inspection of dairy farms and milk processing plants, provides regulations regarding the examination, labeling, pasteurization, processing/packaging, distribution and sale of milk and milk products. The National Conference on Interstate Milk Shipments (NCIMS), in accordance with the Memorandum of Understanding with the FDA, convenes biennial conferences that may recommend changes and modifications to the PMO.

The Center for Veterinary Medicine in the Food and Drug Administration (CVM/FDA) Sets the Guidelines for the Protocols Used to Evaluate the Residue Assays. There is an interpretive memorandum (M-a-85) issued by the FDA (Milk Branch) that summarizes the in vitro evaluation of ß-lactam antibiotic residue screening tests. Various portions of this evaluation were carried out by test sponsors, independent laboratories and CVM/FDA. The evaluation protocol did not measure the performance of these tests in pasteurized milk, mammary gland secretions from individual cows, field samples of bulk tank milk, etc. CVM/FDA can only evaluate assays relative to their label claims. Yet, the PMO states that the tests must perform at the FDA established safe or tolerance levels ... not "at or below".

Appendix N of the PMO is the Section that Deals with Drug Residue Monitoring and Farm Surveillance. It was "established to reference safe levels and/or establish tolerances and to assure that milk supplies are in compliance with these safe levels or established tolerances for drug residues in milk." This appendix to the PMO clearly states that "drug residue detection methods shall be evaluated at the safe level or tolerance." It appears that it was never the intent, either

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

expressed or implied, that residue testing methods should be "accepted" when they are assay positive at several times below the established safe or tolerance level or safe concentration. Unless the producers or veterinarians are able to submit a problem at the biennial NCIMS conference and get it approved, they have little or no input on this important regulatory document.

The producer and veterinarian have little or no official choices in this matter of residue test kit approval under the current system. The PMO dictates that drug residue testing shall be done on raw, commingled milk at the safe or tolerance levels. CVM/FDA has been given the directive to initiate this evaluation and determine assay "acceptance." They have done so, and designed a protocol that permits the "acceptance" of tests that detect antibiotics in spiked milk samples several fold below the safe or tolerance levels. Next, the NCIMS Board of Directors gives their approval based upon the recommendation of their laboratory committee, which got their information from CVM, which got the majority of their information from kit manufacturers, independent laboratories, or AOAC International.

The result is that there are antibiotic residue assays that are CVM/FDA "accepted", Association of Official Analytical Chemists (AOAC) International "performance tested", and NCIMS "recommended" that: a) are used for tanker milk and have never been scientifically field tested on tanker loads of milk, b) are used for trace back on bulk tank milk and have never been field tested on bulk tank milk, and c) are used on individual animal milk samples and have not been subjected to standard epidemiological protocols for this purpose.

Problems: Antibiotic Residue Test Performance In "Real World" Scientific Studies.

We must be able to accurately assess the residue status of tanker trucks, bulk tanks and lactating cows on the farm. There is documentation that problems with antibiotic residue assays exist(*1-14*). Every processing plant and state regulatory agency has documented cases of unexplained residue positive loads or loads that could not be confirmed as containing antibiotic residues when using another assay. Some problems with correctly identifying the antibiotic status of bulk tank milk has been reported in Europe and the United States. False positive and presumed false negative residue assay outcomes on individual animal milk have been reported in scientific literature from Europe, Asia, and North America since 1984.

VanEenennaam et al. (*10*) performed antibiotic residue assays mammary gland secretions from 172 commercial dairy cows and heifers with cases of mild to moderate clinical mastitis. False positive assay results were recorded on pretreatment samples, non-treated animals, and samples obtained 21 days after the first treatments had been administered. The percentage of false positive results was 43.6% (n=839) for the ß-lactam CITE Probe®, 37.7% (n=839) for the Delvotest® P, 81.7% (n=387) for the Charm Farm™ assay, 2.6% (n=836) for the LacTek ß-lactam test, and 18.8% (n=819) for the disc assay (BsDA).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

The study also documented apparent problems with false negative outcomes for some of the test kits. One example of mention is at milking quarter sample 4, the CITE Probe® ß-lactam had a false negative rate of 15.3%.

Carlsson and Bjorck (*11*) examined bulk tank milk samples that were suspected of containing violative residues of inhibitory substances. All samples analyzed yielded assay positive results in the determination of tetracyclines and macrolides by the Charm II™ microbial receptor tests. Two agar free assays were evaluated in this investigation. The Arla™ microtest (SMR, Malmo, Sweden) employs a freeze-dried culture of *Bacillus subtilis* and the Valio™ T101 assay (Valio, Helsinki, Finland) uses growth inhibition of *Streptococcus thermophilous* as the indicator system. After incubation with the test sample, growth of the organism is indicated by a color change of a pH or redox indicator. The Delvotest® SP is based upon the agar diffusion principle and uses growth inhibition of *Bacillus stearothermophilus* as the indicator system. The investigators initially employed a liquid chromatography technique as a confirmatory test for the presence of tetracyclines in the test samples. In the course of the study, it became apparent that the inhibitory substance being detected by many of the assays was not a tetracycline antibiotic. The samples were found to be false positive for the presence of tetracyclines and macrolides by the Charm II™ assay. The study found that as little as 2-5% by volume of serum in the negative control milk resulted in immediate count reductions in the Charm II™ assays. Thus, indicating the "antibiotic receptors" were binding "antibiotic" when indeed none was present.

Their investigation demonstrated that lipolysis of milk fat can give rise to false positive antibiotic residue indications in the Arla microtest and the Valio T101 assay. Free Fatty Acids can also interfere with the Charm II™ determination of tetracyclines and macrolides by the microbial receptor tests. The authors determined that their earlier speculations of increased tetracycline usage by dairy producers that were based upon results indicated with these antibiotic residue tests, now appears to be less probable because of these false positive results. The lipolysis that occurred in the herd milk could have been stimulated by spontaneous action during transportation or stimulated by physical treatment, e.g., agitation and foaming. The outcomes of the assays were not due to the examination of "bad milk."

In this journal article (*14*), the author addresses the issue of residue test kits performance from a practitioner's point of view. Since none of the antibiotic residue assays have been validated for use on individual animals, practitioners are finding it necessary to evaluate them under field conditions. This presentation supplies an on-farm protocol that will aid in providing data to assess the accuracy of a residue assay on individual animal mammary gland secretions. A small field trial employing this protocol is presented and the results are discussed. In summary, the Charm Farm™, Delvotest® P, CITE Probe® and Penzyme® yielded too many false positive assay outcomes to meet the 90% specificity guideline set forth by CVM/FDA.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Issues that Must Be Addressed in the Interest of Scientific Merit

The consequences of false positive antibiotic residue test kit results may be summarized as follows: a) they lead to unwarranted waste of milk and economic loss; b) the socioeconomic impact can harm the dairy industry if antibiotic tests with inadequate biomedical specificity, the ability to correctly identify an untreated cow, are indiscriminately used to test individual cow samples. The false positive outcomes create a mistrust among the consumer and the producer, veterinarian, and regulatory personnel, because the tests are interpreted to mean that the safety of the milk is not being adequately monitored at the level of the bulk tank; c) false positive residue test results can lead to the inaccurate conclusion that a significant proportion of "normal" dairy cows are delivering residues each day into our milk supply; d) in the face of sincere efforts made by the dairy and medical industries to produce a safe and wholesome dairy product, widely publicized negative reports of residues in milk that are based upon inappropriately validated and applied technologies, will be the reports that the milk-consuming public remember and base their actions upon; e) the welfare of the individual dairy cow is at risk because too many positive assay outcomes after recommended withdrawal times have been followed will result in her being sent to the slaughterhouse. In this case, the false positive assay outcomes result in the untimely death of the dairy cow; f) eventually, this problem will have a negative impact on international trade because of the misconception that too many antibiotics are being administered to individual animals and are not being detected by the bulk tank monitoring system or at the meat processing plant.

The Recent CVM/FDA Test Kit Evaluation Process: Two salient points should be remembered in this discussion. First, the 1993 Grade A Pasteurized Milk Ordinance (PMO) guidelines (Section 6: p. 45): Laboratory techniques (first paragraph of #4) states: "In addition, methods which have been independently evaluated or evaluated by FDA and have been found acceptable by FDA for detecting drug residues at current safe or tolerance levels shall be used for each drug of concern." Second, the recent Center for Veterinary Medicine branch of the Food and Drug Administration (CVM/FDA) test kit acceptance protocol specifically applies to raw, commingled milk (tanker milk) ... not bulk tank or individual animal milk. The third important point is that neither CVM/FDA, A.D. Little (a private testing company), AOAC International, nor the kit manufacturers evaluated assay performance under the field conditions required to examine commonly tested milk samples (e.g., tanker truck milk, pasteurized milk, bulk tank milk, individual animal milk, or milk from single mammary gland quarters). However, it has been indicated by CVM/FDA that some antibiotic residue test kit manufacturers have label claims for such use.

The data released by FDA indicates that they gave "acceptance" to many test kits that detect antibiotics in spiked milk samples significantly below FDA established safe or tolerance levels or safe concentration of a compound that successfully went through CVM/FDA new drug approval. This will provide for tests to be "assay positive" when no regulatory actionable residue exists.

The intent of the monitoring system, as viewed by CVM/FDA, is to have no

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

false negative assay outcomes. When implementing this system, there will automatically be false positive assay outcomes. This is acceptable as long as provisions are made to identify the false positive results and correctly classify the true positive samples. However, at the time this chapter was written, no such provisions in the monitoring system have been provided to correctly classify the false positive samples. In addition, no interlaboratory collaborative study was required to be performed on the antibiotic residue assays. This provides a significant void in the ability to account for the inevitable laboratory to laboratory variation in performing the assays. The protocol does not contain the necessary, appropriate information on important controlled variables in sample taking that all biomedical tests must function under field conditions. In the context of milk residue status, the new test kit evaluation process did not account for the following: a) Bulk tank and tanker truck samples stratified by Somatic Cell Count (e.g., 0-250,000; 251,000-500,000; 501,000-750,000; 751,000-1,000,000), b) Herd size influence, e.g., constituents from small herd bulk tank samples can vary from large herd samples(*15*), c) feeding, housing(*16*), and milking practices that vary in each region of the country, and d) the time of year can also influence milk constituents in the bulk tank or tanker truck.

The producer must be considered innocent until proven guilty of a residue violation, and this validation protocol does not provide the appropriate tools to assess the situation. The current residue test kit evaluation protocol does not supply appropriately validated assays to employ as either regulatory or screening tests. This approach is not "erring on the safe side" as some would have you believe. The true outcome of false positive tests is that producers and veterinarians must defend themselves against false accusations that they are not following regulatory guidelines, and the loss of confidence in the entire milk monitoring system is not far behind.

Pharmaceutical companies have based their antibiotic withdrawal times upon FDA-established safe, tolerance levels and safe concentrations. Now that the tests are assay positive on spiked samples significantly below these established levels, the withdrawal times become essentially void because no one can tell if it is a violative residue or not in the milk sample. The laboratory reports a "positive" test, and this results in veterinarians and pharmaceutical companies being accused of false advertising, misbranding of their product, and adulterating the milk supply. In addition, the credibility of the corporate scientific data to get the antibiotic approved will be questioned, and the credibility of FDA will be called into doubt as well.

Processing plants will find it increasingly difficult to sell their product to end users. The end user will most often employ the qualitative assay system with the advertised lowest detection limit, and will falsely believe that a violative residue is present in the milk purchased from the processing plant. Thus, instead of the residue status of the nation's milk supply being regulated by the Pasteurized Milk Ordinance, we will now have an antibiotic residue monitoring program based upon the assay system advertised with the lowest detection limit, that has not been field tested for accuracy or reliability to perform this function. This will result in a defacto policy where the processing plant will adopt the same assay system, then will divert the product away from the fluid milk product line, while

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

going back to the producer and initiating actions against them.

An additional problem has already arisen when processing plants within a state or in other states chose to use different screening assays accepted for use to test under PMO guidelines. This has created the problem of the tanker truck load of milk being "assay negative" in processing plant A using test A and then being antibiotic residue "assay positive" at receiving plant B using test B. Now the dilemma becomes, which test is correct and what must be done with the milk? The decision is a difficult one to make and the producer rarely comes out the winner.

The veterinary practitioner must ask relevant questions regarding antibiotic residue test kits. Veterinarians as well as everyone else involved in this food safety issue must understand the basic definitions of sensitivity, specificity, and predictive value, etc., of antibiotic residue assays (Table I).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Table I. Definitions for validation of antibiotic residue test kits (e.g., individual animal)

- Specificity (Biomedical/Epidemiological): The probability of correctly identifying true-negative (nontreated) animals/milk.
- Laboratory Specificity: The ability of the assay to differentiate between antibiotic classes, i.e., penicillin vs. tetracycline.
- Sensitivity (Biomedical/Epidemiological): The probability of correctly identifying true-positive (antibiotic treated) animals/milk.
- Laboratory Sensitivity: The detection limit of the assay, i.e., ppm, ppb, etc.
- Predictive value (+) test: The probability that a test-positive animal truly has antibiotic in its milk.
- Predictive value (-) test: The probability that a test-negative animal does not have antibiotic in its milk.
- Prevalence: The proportion of the population (group) actually having antibiotic in the milk.
- Exclusionary testing: The process of correctly identifying the nonantibiotic status of milk being submitted for sale, i.e., tanker truck load, bulk tank, individual animal. It is the use of diagnostic testing for the rule-out process for milk suspected of containing antibiotics.
- Confirmatory test: The second assay to be employed in the series of tests with the expressed purpose of obtaining a correct determination of the milk's antibiotic residue status. Confirmatory tests are those assays with a high Predictive Value (+) capability, i.e., >90%. A screening test cannot be designated as a confirmatory test.

The laboratory definitions used during the initial development of the assays can not appropriately be applied in the field setting for individual animal or cowside tests, or any other biological sample (e.g., bulk tank milk, tanker truck milk). The underlying difference in the two sets of definitions is that the laboratory definition is a static measure of specificity in a medium that may or may not reflect the residue status existing in the population in which the test will be performed. The epidemiological definitions are required by established medical doctrine for this food safety issue, because they reflect the arena in which the test must assess the residue status of the milk. This set of appropriate scientific definitions account for the biological variation that occurs among animals and within animals over time in the absence of treatment. We have demonstrated that for some antibiotic residue tests, their epidemiological specificity will change over the course of a treated or untreated mastitic event. The fact is, the laboratory definition employed by CVM/FDA, AOAC, test kit companies, etc., is a subset of the broader, more appropriate epidemiological definition. The problem with the definitions employed in laboratory assay development is that they fail to represent the heterogeneity in subgroups in the population to be evaluated.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Relevant Questions Veterinary Practitioners Must Ask

The following are the significant questions that processing plants, producers, and veterinarians must assess each day. After all, the goal is to produce a safe and wholesome product for the consumer, as well as document the performance of animal agriculture to provide them this service.

Performance of the Assays on "Unknown" Milk Samples: What would be the outcome of the antibiotic residue assay(s) if someone prepared milk samples and provided the manufacturers with the following questions:
a. Is there antibiotic in this sample?
b. If so, which class of antibiotic?
c. If the answer is "a ß-lactam antibiotic", please tell me which one.
d. How much antibiotic is really in the sample, and/or does this sample contain a violative level of antibiotic residues?

What Constitutes Misbranding of a Test Kit? When antibiotic residue assays are advertised to provide important information regarding on-farm food safety, what are the appropriate answers to the following questions when viewed from the perspective of medicine and science?
a. If the term "Quantitative" is a part of the name, in the label claims, or in the expressed or implied advertising, how well does the kit perform under the "unknown sample" criteria mentioned above?
b. What is the intent of a tanker truck or bulk tank test that has the term "cowside" or "individual animal" in its name?
c. If the kit has "cowside" or "individual animal" either in its name or in the label claims, has it gone through the appropriate validation protocol as suggested by the National Mastitis Council Research Committee?

d. If the test can pick up an antibiotic in a spiked milk sample, does that mean it can perform in a real world setting?
e. If the test is positive before the cow is treated and is assay positive after she is treated and FDA approved labeled withdrawal times have been honored, is this really an accurate or reliable test for determining the residue status of the cow?
f. Can the assay really tell the difference between 1 ppm or 5 ppm of ß-lactam antibiotic in the sample?

Let's do Some Numbers: What's the True Status of Antibiotic Residues in Milk? Suppose that the assay is given the task of being 90% specific. In simple terms, this means that it can correctly identify milk not contaminated with antibiotics 9 out of 10 samples. Thus it is predicted to yield false positive results on 1 of every 10 samples. This is considered not so bad until one discovers that their state produces 500,000 tanker loads of milk each year. Simple math, without including other variables, indicates that there is a potential for 50,000 false positive outcomes. This is quite worrisome, so when one asks the regulatory personnel about the number of tankers determined to have residues on these same tanker samples, the response is usually "0.02% to 0.04%. Even if one of every ten of these is false positive, that's not too bad to error on the safe side." This philosophy should make producers and veterinarians paying fines feel much better.

In contrast, let's suppose the bottom line is that the test must be 90% sensitive, meaning that it can correctly identify milk contaminated with antibiotics at this rate of accuracy. Then, without factoring in any other variables, this test will have a false negative rate of 1 for each 10 samples. This means that if a state produces 500,000 tanker loads of milk each year, there is a potential of 50,000 false negative tanker loads going into the food chain.

This scenario becomes problematic because regulatory officials clearly state that their intent is to have no false negative readings by the test system. However, a system that is allowed to be 90% sensitive with a 95% confidence interval cannot provide 0% false negative assay outcomes. Therefore, if the first section in this discussion is correct and we have few or no false positives, it must mean that there are many false negative tanker loads of milk going into the food chain of this imaginary state. Which explanation is the correct answer to our inquiry into the true antibiotic status of the milk supply? Perhaps neither, because the answer to this last question is not known. In reality, none of the assays have been evaluated according to appropriate field trials designed to determine the correct epidemiological sensitivity, specificity, or (+/-) predictive values. Tables II and III introduce information appropriate for assessing the predicted false positive or false negative outcomes a various levels of assay performance.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Table II. Predicted False Positive Outcomes

Desired Assay Specificity	False Positive Outcomes
90 %	1/10
95 %	1/20
99 %	1/100
99.9 %	1/1,000

It is interesting to note that when the prevalence of the antibiotic contamination of tanker truck loads of milk is low as presented by regulatory agencies, i.e., 0.05%, some problems arise with the testing systems. Specifically, the predictive value of a positive test outcome is very low. In this case, with a prevalence of tanker truck contamination at 0.05%, an assay with a 90% laboratory sensitivity rate as required by CVM/FDA, and testing 500,000 tanker trucks, the following numbers become realized. Of the 500,000 tanker truck loads, 44, 978 would be expected to have false-positive outcomes, and 25 expected false-negative assay results would occur in this scenario. The predictive values for a negative test outcome would be virtually 100%, while the predictive value of a positive test outcome would be 5%. Thus, under these conditions, a positive test outcome on a tanker truck load of milk has a 5% chance of being the correct answer.

Table III. Predicted False-Negative Outcomes with 500,000 Samples Tested

Desired Assay Sensitivity	False-Negative Outcomes
90 %	50,000
95 %	25,000
99 %	5,000
99.9 %	500

Let's suppose that a residue test system yields a 2% false positive rate on normal, antibiotic-free milk from individual cows. That doesn't seem too bad, right? Well let's put it to some numbers and see what the dairy producer may think. At a 2% false positive rate, a 1500 cow milking dairy would have 30 cows a day test positive. One must remember that this false positive rate is never determined under regulatory test kit protocols; therefore, this information is not known by the regulatory agency or the test kit manufacturer, plus it is not required to be on the label of the product. Now a consumer group, investigative reporter, etc., could interpret this as not 30 false positive outcomes, but rather that 30 cows are putting violative residues into the milk supply each day and the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

tanker truck/bulk tank monitoring system cannot detect this problem. How do you explain this to the consumer?

The correct identification of milk as contaminated with antibiotics requires that both a screening assay and a confirmatory assay be employed. This two-tiered system is common in the medical world with respect to public health issues. The public health ramifications of potential antibiotic contamination of the milk supply would therefore also require that this same medical principle be implemented. Thus, it is important to note that a screening assay cannot be used to confirm itself, neither can a designated screening assay be used as a confirmatory test. Employing screening assays with a that possess a high Predictive Value (-) and using a Confirmatory assay with a high Predictive value (+) on all screening assay positive samples is the scientifically sound approach to be applied to this public health issue. Unfortunately, this approach is not being implemented under the current regulatory guidelines.

One approach that may lend itself to address everyone's concerns regarding the safety of the milk supply could include a few compromises from absolute scientific methodology. First, one may allow the current screening assays to stay in place for Appendix N testing. However, a consistent protocol for milk sample chain of custody from the farm to the processing plant must be put in place to assure sample identification and retention for further testing if necessary. Next, a second assay system that is capable of "quantitating" antibiotic residues must be established for verifying screening assay results.

Now the process is in place to address both possible outcomes of a screening assay result. If the tanker load of milk is determined to be screening assay positive at the processing plant, the tanker load of milk is dumped and does not enter the food chain. However, the sample must be subjected to quantitative assay analysis. If the sample is determined to contain a violative level of antibiotics, the producer is subjected to the regulatory penalties; thus, the consumer is protected and the offender is punished. On the other hand, if the quantitative assay does not verify an illegal residue: 1) the producer is paid for the milk that was dumped, 2) no penalties are assessed, and 3) the processor pays for the confirmatory assay procedure. Under these conditions, the producer is not falsely accused of adulterating the milk supply, and the processor learns more about the accuracy of the screening assay. If the processor pays for enough loads of milk, they will change screening assays. If the producer if found to be tainting the milk supply, established regulatory actions will be undertaken by the authorities.

Discussion

The medical truth seems to be that there is no problem with adverse reactions due to antibiotics in our milk supply. A review of medical journals dating back to the 1950's reveals a paucity of documented case reports of adverse consequences of drinking milk found to be contaminated with antibiotics. An interesting exercise regarding medical concerns of antibiotics in milk would be to ask your local pediatrician, internist, or specialist in mastitis in women the following question: How many days do you recommend to discontinue breast

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

feeding an infant when you recommend antibiotics to the lactating mother?

It is clear from reviewing the literature that false positive assay outcomes are most common on individual animal samples, occurs in bulk tank samples somewhat less frequently when compared to individual animal assay performance, and probably have occurred in tanker milk. The strategy of "erring on the safe side", while admirable, is an incomplete philosophy and has already resulted in milk being discarded for an inappropriate reason. This approach has already become an animal welfare issue because thousands of dairy cows will meet untimely deaths as a result of the false positive assay outcomes and/or producers and veterinarians have ceased using antibiotics to treat patients because of fears of residue violations. Outside observers view the assays as infallible, thus the results are not considered "false positive", "false violative", or "nonactionable", but "positive" and "laboratory proof" that a residue violation has occurred. Therefore, it is imperative that the appropriate biomedical control, the pretreatment assay performance, is available in individual animal test kit evaluation protocols. It is now evident that some of the assays cannot differentiate between normal host defense and antibiotics in the milk (*1-14*). How is it possible to make sound medical judgments on the residue status of a patient when the assay for antibiotic residues is positive both before the patient is given medication and after the approved regulatory withdrawal time? We must be able to accurately assess the residue status of lactating cows on the farm.

There are many issues pertaining to appropriate test kit validation that must be resolved in order to make more factual interpretations of these assay outcomes. Caution must be exercised when interpreting the laboratory sensitivity figures quoted by the residue test kit manufacturer and regulatory agencies for the current antibiotic residue assay kits. These laboratory sensitivity figures, more accurately described as the detection limit of the assay, are based upon milk samples that are spiked (fortified) with the parent compound of a known antibiotic, then the assay is performed. If the assay outcome is positive, the test result is reported as such, and interpreted to the consumer as a good test. Spiking bulk tank or pasteurized milk with parent compound of an antibiotic has very little, if any, biological relevance to treating an active case of mastitis, then assessing the residue status of the patient.

A principal goal of individual animal or cowside testing for various types of residues is to aid the production of milk free of violative levels of antibiotics or other categories of residues. Milk samples have considerable compositional diversity, especially the variation in bacteriologic contamination, somatic cell count, and natural antimicrobial substance activity. Many test kit formats or regulatory assays cannot differentiate between an antibiotic-free, convalescing animal producing saleable milk, and a patient that is undergoing therapeutic intervention. This may also be true for bulk tank samples where the dilution factor of normal milk is notably different between a small, 25 cow milking string and a much larger 1,200 cow dairy.

The reliability of an antibiotic residue assay positive result is important to the dairy industry in assessing appropriate management decisions to assure a safe product is being delivered to the processing plant. Diagnostic and screening tests are employed as tools in medical practice to reduce uncertainty in the diagnosis.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Unless or until the antibiotic residue assays undergo the National Mastitis Council Research Committee protocol for individual animal evaluation, implementing an individual field evaluation is the only tool available to the practitioner, processing plant, or producer to assess the reliability of these assays. Validation protocols based upon spiked (fortified) milk samples have little or no biological relevance. There's never a right way to apply either incomplete or wrong science. Therefore, appropriate scientific evaluation of these assays with respect to their reliability for assessing the antibiotic residue status of tanker truck and bulk tank milk must also be conducted as soon as possible. This issue is of extreme importance to the veterinary profession, especially in the context of food safety and adulteration of the food supply.

Acknowledgments

The University of California-Davis School of Veterinary Medicine is a charter member of the Food Animal Production Medicine Consortium. I would like to thank the University of California-Davis Livestock Disease Research Laboratory and the California Dairy Food Research Center for their support of the Dairy Food Safety Laboratory.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Literature Cited

1. Egan, J.; Meaney, W. J. *Ir. J Food Sci Technol*, **1984,** 8:115-120.
2. Okada, Y. *Jap Vet Med Assoc*, **1986,** 39:97-101.
3. Tyler, J. W.; Cullor, J. S.; Erskine, R. J.; Smith, W. L.; Dellinger, J. D.; McClure, K. *J Am Vet Med Assoc*, **1992,** 201(9):1378-1384.
4. Carlsson, A.; Bjorck, L.; Persson, K. *J Dairy Sci*, **1989,** 72(12):3166-75.
5. Seymour, E. H.; Jones, G. M.; McGilliard, M. L. *J Dairy Sci*, **1988,** 71:539-544.
6. MaCauley, D. M.; Packard, V. S. *J Food Protection*, **1981,** 44(9):696-698.
7. Van Der Leek, M. L.; Donovan, G.A.; Saltman, R. L.; Moja, R. J. *J Dairy Sci*, **1991,** 74:433-435.
8. Cullor J. S.; VanEenennaam, A. L.; Dellinger, J. D.; Perani, L.; Smith, W. L.; Jensen, L. *Vet Med*, **1992,** (May) 477-494.
9. Sischo, W. M.; Burns, C. M. *JAVMA*, **1993,** 202(8):1249-54.
10. VanEenennaam, A. L.; Cullor, J. S.; Perani, L.; Gardner, I. A.; Smith, W. L.; Dellinger, J. D.; Guterbock, W. *J Dairy Sci*, **1993,** 76:3041-3053.
11. Carlsson, A.; Bjorck, L. *J Food Protection*, **1992,** 55(5):374-378.
12. Cullor, J. S.; VanEenennaam, A. L.; Gardner, I. A.; Smith, W. L.; Perani, L.; Dellinger, J. D.; Jensen, L.; Guterbock, W. *Proceedings 32nd Annual National Mastitis Council*, **1993,** Feb. 15-17, 133-143.
13. Cullor, J. S.; VanEenennaam, A. L.; Gardner, I. A.; Perani, L.; Dellinger, J. D.; Smith, W. L.; Thompson, T.; Payne, M. A.; Jensen, L.; Guterbock, W. *J AOAC International*, **1994,** 77(4):2238-2247.
14. Cullor, J. S. *Vet Med*, **1994,** (May) 462-472.

15. Corlett, N. J. Turcinov, J. V. *Proceedings 33th Annual National Mastitis Council,* 31 Jan-2 Feb., **1993,** 345-346.
16. Lehenbauer, T. L.; Jones, T.; Collar, L. *Proceedings 33th Annual National Mastitis Council,* **1993,** 31 Jan-2 Feb., pp 250-261.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch006

Chapter 7

Potential for Oxytetracycline Administration by Four Routes To Cause Drug Residues in Milk of Lactating Cattle

K. L. Anderson,[1] W. A. Moats,[2] J. E. Rushing,[3] and J. O'Carroll[1]

[1]Department of Food Animal and Equine Medicine, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606
[2]Meat Science Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705
[3]Department of Food Science, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC 27606

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch007

The potential for oxytetracycline (OTC) to cause milk residues above the FDA safe level was studied by administering OTC by 4 routes to lactating cows and measuring milk OTC concentrations over time using 2 analytical methods. Milk concentrations of OTC were determined from 18 lactating cows (6 cows per route) following administration of OTC by the intravenous (16.5 mg/kg once), intramuscular (11 mg/kg once), and intrauterine (2 grams once) routes. Milk was collected prior to and at twice daily milkings for 156 hours after OTC administration. Milk OTC was determined by an HPLC method and the Charm II test for tetracyclines. The intravenous and intramuscular routes were associated with considerable potential for violative milk residues. When OTC was administered orally at 5X the label dose for 3 days, milk OTC concentrations above the FDA safe level were not detected. Bulk milk samples from 5 dairies feeding oral chlortetracycline did not contain milk residues above the FDA safe level.

Residues of tetracyclines have been reported in milk (*1–2*). This is a concern for the milk industry and regulatory officials. Other than for low-level administration by the oral route, tetracyclines are not approved for use in lactating cattle. However, tetracyclines are used by veterinarians in therapy of some serious diseases of lactating cattle under FDA extra-label use guidelines. Oxytetracycline (OTC) is one drug which is available and administered parenterally in such cases. Routes of administration of tetracyclines which may potentially lead to milk residues include intravenous (IV), intramuscular (IM), intrauterine (IU), oral, and others.

Based upon reports of tetracycline residues above the FDA safe level of 30 ng/ml in milk, four routes of OTC administration were investigated. Data on the potential for residues following IV, IM, and IU administration have been published (*3*) and will

0097–6156/96/0636–0058$15.00/0
© 1996 American Chemical Society

only be summarized here. This communication will focus on investigation of the oral route of administration.

Analytical Methods

HPLC Analysis of Milk. Milk OTC and chlortetracycline (CTC) concentrations were determined by the HPLC procedure of White et al (*4*) and Moats and Harik-Khan (*5*). The limit of OTC quantitation was approximately 2 ng/ml (*5*).

HPLC Analysis of Mineral Supplements. One gram of mineral concentrate was accurately weighed and transferred to a volumetric flask with a water rinse. Then 10 ml of 1N HCl and 10 ml of 0.2M tetramethyl ammonium chloride were added to the flask. After evolution of gas ceased, the contents of the flask were diluted to 100 ml, mixed thoroughly and allowed to stand 10 minutes. An aliquot of the contents of the flask was filtered through a plug of glass wool prior to analysis.

The analytical procedure used a 4.6 × 150 mm, 5 um particle-size, 100 angstrom pore diameter column (Polymer Laboratories PLRP-S). The HPLC mobile phase consisted of 0.02M H_3PO_4, 0.01M sodium decanesulfonate-acetonitrile (68+32). The flow rate was 1 ml/min and detection was at 380 nm UV.

HPLC standards for CTC and OTC (Sigma Chemical Co., St. Louis, MO) were prepared as stock solutions of 1 mg/ml with appropriate corrections for purity, when known, in 0.01N HCl. Working solutions of 100, 10 and 1 ug/ml were prepared in 0.01N HCl and were stored at refrigerator temperature.

Charm II Competitive Assay for Tetracyclines. The Charm II test (Charm Sciences Inc., Malden, MA) for tetracyclines was performed as previously described (*3*). Milk samples from individual cows were centrifuged and skimmed prior to analysis. Concentrations were determined from a standard curve. When dilution was required, final concentrations in samples were determined by multiplying the derived concentration times the dilution factor. The limit of detection of the method was 5 ng/ml.

Potential for Residues Following IV, IM and IU Administration

Results of the investigation of potential for milk residues following IV, IM and IU administration of OTC (Liquamycin 100, Pfizer, Inc., New York, NY) have been published previously (*3*) and will only be summarized here. Values reported represent concentrations determined in pooled weigh jar milk samples from machine milkings at approximately 12 hour intervals after treatment. The potential for OTC milk residues following IV, IM and IU administration is summarized in Table I.

Intravenous (IV). Lactating Holstein cows (n=6) administered OTC IV at 16.5 mg/kg exhibited peak milk concentrations of OTC of approximately 3,700 to 4,200 ng/ml at the first milking after administration (Table I). Mean milk OTC concentrations rapidly declined and reached the FDA safe level at ≤ 96 hours after administration. Assuming equal volumes of milk from all herd cows, a single cow at

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch007

peak concentrations could cause pooled herd milk to exceed the FDA safe level in herds of approximately 120–140 cows.

Table I. Potential for OTC Residues Following a Single Administration by the IV, IM and IU Routes (n=6 per route, summarized from reference 3)

Administration Route	*Dose*	*Maximum OTC Milk Concentration at First Milking After Treatment*	*Time for Mean OTC Milk Concentrations to Reach FDA Safe Level*
IV	16.5 mg/kg	3,700 to 4,200 ng/ml	≤ 96 hours
IM	11 mg/kg	2,200 to 2,600 ng/ml	≤ 132 hours
IU	2 gm/cow	186 to 192 ng/ml	≤ 84 hours

Intramuscular (IM). Lactating Holstein cows (n=6) administered OTC IM at 11 mg/kg exhibited peak milk concentrations of OTC of approximately 2,200 to 2,600 ng/ml at the first milking after administration (Table I). Mean milk OTC concentrations declined less rapidly than for the IV administration and reached the FDA safe level following IM administration at ≤ 132 hours. Assuming equal volumes of milk from all herd cows, a single cow at peak concentrations could cause pooled herd milk to exceed the FDA safe level in herds of approximately 70–85 cows.

Intrauterine (IU). Lactating Holstein cows (n=6) administered OTC IU at 2 grams/cows exhibited peak milk concentrations of OTC of approximately 186 to 192 ng/ml at the first milking after administration (Table I). Mean milk OTC values slowly declined and reached the FDA safe level at ≤ 84 hours after administration. Assuming equal volumes of milk from all herd cows, a single cow at peak concentrations could cause pooled herd milk to exceed the FDA safe level in herds of approximately ≤ 6 cows.

Potential for Residues Following Oral Administration

The potential for residues above the FDA safe levels (30 ng/ml for OTC and CTC) following oral administration was investigated by 2 methods. A controlled experimental approach was used as well as measurement of tetracycline residues from 5 herds feeding oral tetracyclines to lactating cows at approved levels.

Experimental Approach. Lactating Holstein cows (n=6), weighing 679 ± 65 kg (mean ± SD), were used in these studies. The cows averaged 4.1 ± 1.4 years of age (mean ± SD) and 8.1 ± 1.7 months (mean ± SD) in lactation. They had not received any antibiotics in the 30 days prior to the study. Cows were administered 375 mg OTC/head/day for 3 days by oral administration of feed-grade OTC (85 grams/head/day of Terramycin Crumbles, Pfizer, Inc., New York, NY) in gelatin capsules. This represents 5X the approved label dose of 75 mg OTC/head/day. Cows were machine milked at approximately 12 hour intervals, and samples of

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch007

pooled total milk from individual treated cows were collected for analysis of OTC in milk by Charm II and HPLC methods. Results of testing by method over time are given in Table II.

Table II. Milk OTC Concentrations (ng/ml) by Charm II and HPLC Test Methods after Oral Administration of OTC at 375 mg/head/day × 3 days to 6 Lactating Cows

		Hours After First Treatment[+]													
Animal #		*0*	*12*	*24*	*36*	*48*	*60*	*72*	*84*	*96*	*108*	*120*	*132*	*144*	*156*
1	Ch II	<--------------------all <5 ng/ml---------------------------------->													
	HPLC	<--------------------all <2 ng/ml---------------------------------->													
2	Ch II	<--------------------all <5 ng/ml----------->										ND	ND	ND	<5
	HPLC	<2	<2	4.2	<------all <2 ng/ml----------->							ND	ND	ND	<2
3	Ch II	<--------------------all <5 ng/ml----------->										ND	ND	ND	<5
	HPLC	<--------------------all <2 ng/ml----------->										ND	ND	ND	<2
4	Ch II	6	<------------------all <5 ng/ml----------->									ND	ND	ND	<5
	HPLC	<--------------------all <2 ng/ml----------->										ND	ND	ND	<2
5	Ch II	<--------------------all <5 ng/ml----------->										ND	ND	ND	<5
	HPLC	<--------------------all <2 ng/ml----------->										ND	ND	ND	<2
6	Ch II	<--------------------all <5 ng/ml----------->										ND	ND	ND	<5
	HPLC	<--------------------all <2 ng/ml----------->										ND	ND	ND	<2

Ch II = Charm II. Charm II limit of detection = 5 ng/ml; HPLC limit of detection = 2 ng/ml.

ND = Not determined.

[+]Sample at 0 hours just prior to drug administration. OTC administered at 0, 24, 48 hours after first administration.

The results demonstrated that no milk sample for any cow exceeded the FDA safe level for OTC in milk. No sample was found to exceed 6 ng/ml OTC. Nearly all samples were below the limit of detection of the 2 analytical methods.

Milk Residues in Herds Feeding Oral Chlortetracycline. Bulk tank milk samples were collected on multiple days (n = 2 for 4 herds, n = 1 for the fifth herd) from 5 North Carolina dairy herds feeding oral chlortetracycline in lactating cow rations at approved levels. Herds were selected on the basis that no tetracyclines other than feed additives had been administered to any herd cow in the 30 days prior to sample collection. Bulk tank samples were collected, frozen and analyzed by Charm II technology within 2 weeks of collection. Duplicate samples were analyzed by

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch007

HPLC. Mineral supplements from the respective herds were also analyzed by HPLC as described previously to determine content. Results of milk analysis are given in Table III.

Table III. Bulk Tank HPLC and Charm II Analysis of Milk on Dairies Feeding Oral Chlortetracycline

Herd	*Milk Sample*	*HPLC* ng/ml CTC*	*Charm II Test ng/ml CTC*	*Charm II Control Point Analysis*[†]
1	1	<2	0+	Pass (Negative)
	2	<2	0+	Pass (Negative)
2	1	4.2	2+	Pass (Negative)
	2	6.6	4.5+	Pass (Negative)
3	1	9.4	3+	Pass (Negative)
	2	8.2	3.5+	Pass (Negative)
4	1	8.4	<15[‡]	Pass (Negative)
	2	6.3	<15[‡]	Pass (Negative)
5	1	5.6	<15[‡]	Pass (Negative)

*Mean of duplicate determinations

[+]Values from standard curve; actually < limit of detection (LOD).

[†]Detects presence or absence (pass) of CTC at 30 ng/ml.

[‡]Different lot of tetracycline tablets; more precise quantitation not pursued.

All bulk tank samples contained <15 ng/ml CTC and were negative (passed) by Charm II control point analysis (indicating <30 ng/ml CTC). Mineral samples were found to contain expected levels of CTC and would have provided doses of CTC within FDA guidelines.

Discussion

The purpose of this study was to investigate the potential for administration of tetracyclines by 4 routes to cause milk residues above the FDA safe level. Doses evaluated were within the upper range occasionally used by veterinarians to treat lactating cattle using FDA extra-label use guidelines.

Short-term oral administration of OTC at up to 5X the label dose and administration of CTC at label dose did not produce milk levels of tetracyclines above the FDA safe level. This does not preclude the possibility that excessive overdosing via the oral route may cause milk tetracyclines to exceed the FDA safe level. However, it appears that the likelihood for such residues is small when tetracyclines are used according to label directions. Administration of OTC by the IV and IM routes was found to be associated with a considerable potential for producing

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch007

violative OTC residues in milk. The results of these investigations suggest that efforts to reduce violative residues of tetracyclines in milk should be concentrated on extra-label use of the drug following IV or IM use. OTC is occasionally used by veterinarians for therapy of some diseases in lactating cows, using FDA extra-label use guidelines. When used in this manner, it is critical that appropriate withholding times be practiced for milk and meat to avoid violative tetracycline residues.

Acknowledgments. This work was supported by the North Carolina Dairy Foundation, the State of North Carolina, and the United States Department of Agriculture--Agricultural Research Service. The technical assistance of individuals within the cooperating institutions is gratefully acknowledged.

Literature Cited.

1. *Food Safety and Quality: FDA Surveys Not Adequate to Demonstrate Safety of the Milk Supply.* GAO/RCED-91-26, Washington, DC. United States General Accounting Office, November, 1990.
2. Ingersoll, B. *Wall Street Journal*, Dec. 29, **1989**, p. 1.
3. Anderson, K. L.; Moats, W. A.; Rushing, J. E.; Wesen, D. P.; Papich, M. G. *J. Am. Vet. Med. Assoc.* **1995**, *56*, pp. 70–77.
4. White, C. R.; Moats, W. A.; Kotula, K. L. *J. AOAC. Intl.* **1993**, 76, pp. 549–554.
5. Moats, W. A.; Harik-Khan, R. *J.Agr. Food Chem.* **1995**, 43, pp. 931–934.

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch007

Chapter 8

Evaluation and Testing of the *Bacillus stearothermophilus* Inhibition Test with Tissues and Fluids from Hogs Injected with Penicillin G Procaine

Gary O. Korsrud, Craig D. C. Salisbury, Joe O. Boison, Lily Keng, and James D. MacNeil

Health of Animals Laboratory, Agriculture and Agri-Food Canada, 116 Veterinary Road, Saskatoon, Saskatchewan, S7N 5E3, Canada

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch008

Penicillin G concentrations were determined using liquid chromatography in kidney, muscle, plasma and urine from 43 market hogs injected IM with procaine penicillin G. Results were compared with those obtained using the qualitative Charm Farm screening test for antimicrobial residues. Overall there was 94 % agreement observed between the Charm Farm Test results and the liquid chromatographic results. There were 3 (7 %) Charm Farm Test false positive muscle results, one (3 %) Charm Farm Test false positive plasma result and possibly 3 (8 %) Charm Farm Test urine false positive results and no Charm Farm Test kidney false positive results. There were 4 (9 %) Charm Farm Test false negative kidney results and those samples contained 12 - 21 μg penicillin G/kg. There were no false negative Charm Farm Test results for muscle, plasma or urine. The Charm Farm Tests were positive for all the kidney, plasma and urine samples from all the 2 and 3 day withdrawal hogs that contained penicillin G in their muscles.

The Swab Test On Premises (STOP) (*1*) and the Calf Antibiotic and Sulfa Test (CAST) (*2*) have been used for the past decade at Canadian packing plants to screen meat for antimicrobial residues (*3*). Alternative test kits now being marketed need to be evaluated and tested. The Charm Farm Test is a qualitative microbial inhibition test available from Charm Sciences Inc. (Malden, MA, USA) for testing for antimicrobial residues in meat, serum and urine. The test is based on the inhibition of the growth

0097-6156/96/0636-0064$15.00/0
© 1996 American Chemical Society

of *Bacillus stearothermophilus*. Acid production in the presence of microbial activity is visualized with a pH indicator or measured with a pH meter. Positive samples remain blue and negative samples turn green during a 3 hr incubation.

In another study the Charm Farm Test was run in conjunction with Agriculture and Agri-Food Canada's routine confirmation analyses for suspect samples (Korsrud, G.O.; Salisbury, C.D.C.; Fesser, A.C.E.; MacNeil, J.D. *J. Food Prot.*, in press). For the current report the Charm Farm Test was applied to kidney, muscle, plasma and urine samples from hogs that had been injected with procaine penicillin G. The results were compared to those obtained with a liquid chromatographic method (*4*) for penicillin G.

Materials and Methods

Experimental Animals and Drug Administration. Market hogs weighing an average of 100 kg were injected IM daily in the neck with Ethacilin (procaine penicillin G, 300,000 IU/mL, Rogar/STB, Pointe Claire-Dorval, Quebec, Canada) at the label dosage rate of 15,000 IU/kg body weight for three consecutive days. The label withdrawal time is 5 days.

Experimental Design. Groups of 6 hogs (3 male and 3 female) were slaughtered at a local commercial abattoir at each of days 1, 2, 3, 4 and 8, respectively, after the last injection. One group of 13 hogs was slaughtered 5 days after the last injection. Two hogs served as untreated controls.

Samples of muscle (front leg), kidney, heparinized blood and urine were collected from each hog. The blood was centrifuged in the laboratory at 4,500 X g for 10 minutes to collect plasma. Samples were stored at the laboratory at - 76° C prior to analysis.

Analysis of Residues. The Charm Farm Test (Charm Sciences Inc., Malden, MA, USA) was used according to the manufacturer's protocols. Tissue was cut into small cubes. Two grams of muscle were added to 6 mL of extraction buffer or one gram of kidney was added to 7 mL of extraction buffer in a small plastic bag and then crushed with a rubber mallet. After centrifugation, 0.5 mL of tissue extract was added to 0.5 mL of deionized water and one Buffer M tablet was added to each test tube. For urine, 0.1 mL of the sample was pipetted into a test tube and diluted with 5.0 mL of deionized water. One mL of the diluted urine sample was added to a test tube and one Buffer M tablet was added. For plasma, 30 μL of the sample was added to one mL deionized water in a test tube and one Buffer M tablet was added. After a 6 minute incubation one microbial tablet was added to each test tube and the tubes were then placed in a programmed incubation block to provide heat activation of the spores, after which a tablet containing growth medium was added and the tubes were

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch008

incubated for the indicated time period. The reaction was inactivated by heat at the end of the incubation period. The color of the sample was observed immediately in fluorescent light and compared to the color references. Results were based on duplicate determinations and were compared with liquid chromatographic analyses for penicillin G, using previously described methodology (*4, 5*) which utilized derivatization with 1,2,4-triazole-mercuric chloride and ultraviolet detection at 325 nm.

Results and Discussion

The minimum detectable levels (MDLs), according to Charm Sciences Inc., for the Charm Farm Test were 5, 10, 25 and 50 μg/kg, respectively, for muscle, kidney, serum and urine. The limits of quantitation for penicillin G for the liquid chromatographic method were 5 μg/kg, and 15 and 100 μg/L, respectively, for tissue, plasma and urine.

Individual penicillin G levels as determined by liquid chromatography and Charm Farm Test results are presented in Table I. The results are summarized in Table II.

Table I. Penicillin G concentrations determined by liquid chromatography (LC) (μg/kg or μg/L) and Charm Farm Test (+ = positive, - = negative) results for tissues and fluids from individual 100 kg market hogs injected intramuscularly with 15,000 I.U. procaine penicillin G/kg body weight once daily for 3 consecutive days.

Withdrawal (days)	Kidney		Muscle		Plasma		Urine	
	LC[a]	CFT[a]	LC	CFT	LC	CFT	LC	CFT
1	270	+	10	+	97	+	na[b]	na[b]
	2,600	+	52	+	370	+	na	na
	570	+	18	+	230	+	na	na
	350	+	17	+	120	+	na	na
	1,800	+	29	+	230	+	na	na
	1,900	+	54	+	380	+	na	na
2	380	+	11	+	47	+	26,000	+
	<5	-	<5	-	<15	-	<100	-
	66	+	<5	-	<15	+	6,100	+
	240	+	16	+	82	+	38,000	+
	<5	-	<5	-	<15	-	<100	+
	5	+	<5	-	<15	-	1,800	+

[a]LC = liquid chromatography, CFT = Charm Farm Test.
[b]na = samples not analyzed.

(continued)

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch008

Table I. (continued)

Withdrawal (days)	Kidney		Muscle		Plasma		Urine	
	LC[a]	CFT[a]	LC	CFT	LC	CFT	LC	CFT
3	31	+	<5	-	<15	-	1,100	+
	<5	-	<5	-	<15	-	<100	-
	48	+	<5	-	<15	-	210	+
	<5	-	<5	-	<15	-	<100	-
	240	+	<5	-	32	+	16,000	+
	1,100	+	20	+	65	+	320	+
4	<5	-	<5	-	<15	-	<100	-
	<5	-	<5	-	<15	-	<100	+
	21	-	<5	-	<15	-	<100	-
	13	-	<5	-	<15	-	580	+
	5	-	<5	-	<15	-	200	+
	<5	-	<5	-	<15	-	<100	-
5	<5	-	<5	-	<15	-	<100	-
	<5	-	<5	-	<15	-	<100	-
	<5	-	<5	-	<15	-	<100	-
	<5	-	<5	+	<15	-	<100	-
	<5	-	<5	-	<15	-	750	+
	38	+	<5	-	<15	-	6,400	+
	<5	-	<5	-	<15	-	<100	-
	<5	-	<5	-	na[b]	-	<100	-
	<5	-	<5	+	na	-	<100	-
	<5	-	<5	-	na	-	<100	-
	<5	-	<5	-	na	-	<100	-
	<5	-	<5	-	na	-	<100	-
	7	-	<5	-	na	-	<100	+
8	<5	-	<5	+	na	-	<100	-
	<5	-	<5	-	na	-	<100	-
	12	-	<5	-	na	-	<100	-
	<5	-	<5	-	na	-	<100	-
	<5	-	<5	-	na	-	<100	-
	14	-	<5	-	na	-	<100	-
Control	<5	-	<5	-	<15	-	<100	-
	<5	-	<5	-	<15	-	<100	-

[a]LC = liquid chromatography, CFT = Charm Farm Test.
[b]na = samples not analyzed.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch008

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

Table II. Summary of the Comparison of Charm Farm Test Results with Liquid Chromatographic Penicillin G Results for Tissues and Fluids from Market Hogs Injected Intramuscularly with 15,C00 I.U. Procaine Penicillin G/kg Body Weight Once Daily for 3 Consecutive Days.

Results		Kidney	Muscle	Plasma	Urine
Agree	Positive	15	9	10	12
	Negative	24	31	20	22
False	Positive	0	3	1	3?
	Negative	4[a]	0	0	0

[a]Penicillin G concentrations: 12 - 21 μg/kg.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch008

Overall there was 94 % agreement between the liquid chromatographic results and the Charm Farm Test results for kidney, muscle, plasma and urine samples. There were 3 false positive Charm Farm Test results for muscle, one for plasma, possibly three for urine and none for kidney. The three urine samples that were positive by the Charm Farm Test could have contained penicillin G concentrations between the 50 μg/kg detection limit claimed for the Charm Farm Test for urine and the 100 μg/L limit of quantitation observed for the liquid chromatographic method. There were 4 false negative Charm Farm Test results for kidney (those samples contained 12 - 21 μg penicillin G/kg). Two samples contained penicillin G concentrations below the 10 μg/kg detection limit claimed by Charm Sciences Inc. No false negatives were found for muscle, plasma or urine.

For a screening test, false negatives are undesirable but a low false positive rate is acceptable because these samples will be eliminated by the confirmatory analyses. In this study there were no Charm Farm Test false positive kidney results but there were 3 (10 %) Charm Farm Test false positive muscle results. The concentration of penicillin G in the 4 kidneys that were Charm Farm Test negative were all below the Maximum Residue Limit (MRL) of 50 μg/kg established for bovine tissues in Canada but above the MDL of 10 μg/kg. An MRL has not been established in Canada for swine tissues so the detection of penicillin G residues at any concentration in the muscle will result in the condemnation of the carcass. The Codex Alimentarius Commission has recommended an MRL of 50 μg/kg for penicillin G in kidney and muscle of swine (*6*).

Currently in Canada kidney samples are screened at the packing plants with the STOP or the CAST and when the results are positive then both muscle and kidney samples are sent for confirmatory laboratory testing. Penicillin G was not detected in any of the corresponding muscle samples from the 4 hogs with Charm Farm Test false negative kidneys. The Charm Farm Tests were positive for all the kidney, plasma and urine samples from all of the 2 and 3 day withdrawal hogs that were found by LC analysis to contain penicillin G in the corresponding muscle tissue.

The Charm Farm Test is relatively easy to perform and does not require significant technical expertise to obtain valid results. Expensive equipment is not required. The incubation time is shorter than for the STOP and CAST, allowing same day results, and the costs are similar. The microorganisms and media for the Charm Farm Test are provided in tablet form and are claimed to be stable at room temperature for one year.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch008

Acknowledgments

We acknowledge the technical assistance of Wieslaw Wolinski, Greg Sliva and Keiko Tanioka-Man.

Literature Cited

1. Johnston, R. W.; Reamer, R. H.; Harris, E. W.; Fugate, H. G.; Schwab, B. A. *J. Food Prot.* **1981**, *44*, 828-831.
2. United States Department of Agriculture. *Performing the calf antibiotic and sulfa test. A self-instructional guide.* First revision September **1984**. Food Safety and Inspection Service, Meat and Poultry Inspection Program, Technical Services, Program Training Division.
3. Korsrud, G.O.; MacNeil, J.D. *J. Food Prot.* **1988**, *51*, 43-46.
4. Boison, J. O.; Salisbury, C. D. C.; Chan, W.; MacNeil, J. D. *J. Assoc. Offic. Anal. Chem.* **1991**, *74*, 497-501.
5. Boison, J.O.; Korsrud, G.O.; MacNeil, J.D.; Keng, L.; Papich, M. G. *J. Chromat.* **1992**, *576*, 315-320.
6. Joint FAO/WHO Food Standards Programme Codex Alimentarius Commission, *Residues of veterinary drugs in food;* Codex Alimentarius (Second Edition), **1993**, *Vol. 1*, pp. 4.

Chapter 9

Ceftiofur Sodium: Absorption, Distribution, Metabolism, and Excretion in Target Animals and Its Determination by High-Performance Liquid Chromatography

Maria G. Beconi-Barker, Ryan D. Roof, Tom J. Vidmar, Rex E. Hornish, Evan B. Smith, Cathy L. Gatchell and Terry J. Gilbertson

Pharmacia and Upjohn, Inc., 301 Henrietta Street, Kalamazoo, MI 49007

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

After intramuscular injections of [^{14}C]-ceftiofur sodium to beef, dairy cattle, swine and sheep, the radiolabeled cephalosporin was absorbed rapidly into the blood and eliminated mostly in the urine (>65% for all species). In all species, the tissue where highest residue concentrations were observed at 12 h after the last dose was the kidney. The major metabolite of ceftiofur was desfuroylceftiofur (DFC), which is microbiologically active, and was found conjugated to cysteine, glutathione and plasma and tissue proteins. An HPLC method was developed and validated for the determination and quantitation of ceftiofur-related metabolites that have the potential to be microbiologically active in bovine and swine muscle, kidney, liver and fat and sheep muscle and kidney. This method is based on the cleavage of the disulfide and/or thioester bonds between the metabolites and their conjugate sulfur containing moiety using dithioerythritol to yield DFC, which is then stabilized by derivatizing to desfuroylceftiofur acetamide.

Ceftiofur is a broad spectrum cephalosporin antibiotic used solely in veterinary medicine. It can be synthesized in many different salt forms due to the molecule's zwitterion characteristics. The sodium salt of ceftiofur, **NAXCEL/EXCENEL Sterile Powder**, (The Upjohn Company), has been approved by the FDA and other world wide regulatory agencies for the treatment of respiratory diseases (shipping fever, pneumonia) in beef and dairy cattle, swine and horses and infections in day old chickens (*1-3*).

The metabolism of ceftiofur following intramuscular treatment has been extensively studied in rats, beef and dairy cattle, swine and sheep (*4-7*). (Figure 1). From these animal species it is known that after intramuscular treatment, ceftiofur is rapidly metabolized to desfuroylceftiofur (DFC). Free DFC, which contains an intact β-lactam ring, is the microbiologically active metabolite of ceftiofur. DFC is seldom found as itself in plasma, urine or tissues. The sulfhydryl group of DFC conjugates rapidly to molecules like cysteine and glutathione to form the desfuroylceftiofur cysteine disulfide

0097–6156/96/0636–0070$15.00/0
© 1996 American Chemical Society

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

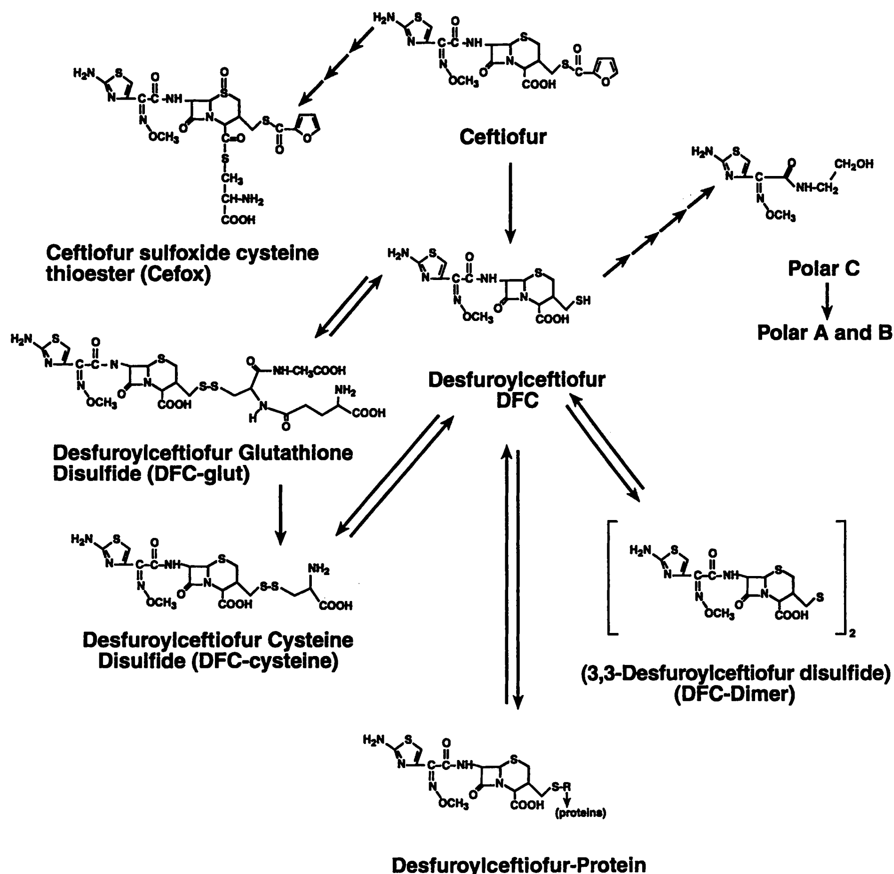


Figure 1. Proposed metabolism of ceftiofur in rats, cattle, swine and sheep.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

Table I. Total Residue Levels of ^{14}C-Ceftiofur Equivalents (CE) in Tissues of Different Species After Multiple Administrations of Ceftiofur Sodium (Values are expressed as µg CE/g tissue ± s.d)

Species[1]	kidney	lung	liver	fat	muscle
Swine	4.47±0.81	2.93±0.56	1.55±0.18	1.49±0.54	0.76±0.24
Sheep	9.02±1.15	0.63±0.12	0.62±0.15	0.12±0.03	0.13±0.02
Cattle	5.54±1.18	1.18±0.14	1.35±0.22	0.56±0.31	0.23±0.47

[1] Swine were slaughtered 12 h after the last of three intramuscular injections at 5.2 mg CFAE/kg bw at a 24 h interval (*6*), sheep were slaughtered 12 h after the last of five intramuscular injections at 2.2 mg CFAE/kg bw at a 24 h interval (*7*), and beef cattle were slaughtered at 8 h after the last of five intramuscular injections at 2.2 mg CFAE/kg bw at a 24 h interval (*The Upjohn Co., unpublished data*).

Table II. % Recovery of Total Dose (as [^{14}C]) in Urine and Feces From Swine, Cattle and Sheep

Species[1]	% feces ± s.d.	% urine ± s.d.
Swine	10.80 ± 5.10	61.8 ± 4.70
Sheep	6.5 ± 0.8	92.6 ± 3.7
Beef Cattle	29.1 ± 4.2	57.4.0 ± 4.5
Dairy Cattle	35.7 ± 9.6	62.8 ± 7.6

[1] Accountability of the dose in excreta was obtained as follows: swine, 12 h after the last of three intramuscular injections at 5.2 mg CFAE/kg bw at a 24 h interval (*6*), sheep, 12 h after the last of five intramuscular injections at 2.2 mg CFAE/kg bw at a 24 h interval (*7*), beef cattle, 8 h after the last of five intramuscular injections at 2.2 mg CFAE/kg bw at a 24 h interval (*The Upjohn Co., unpublished data*), and dairy cattle, 12 h after the last of five intramuscular injections at 2.2 mg CFAE/kg bw at a 24 h interval (*5*).

(DFC-cysteine) and glutathione (DFC-glutathione) conjugates. It binds to plasma and tissue proteins. The biotransformation of DFC to disulfides and conjugation to proteins is reversible (*4*). Thus, disulfides and protein conjugates probably act as reservoirs of desfuroylceftiofur in plasma and tissues and, as a result, determine the duration of action of desfuroylceftiofur or half life. The position of equilibrium in a given animal species or system would probably be determined by the redox potential of the system, by the concentration of desfuroylceftiofur and the concentration of endogenous sulfhydryl and disulfide compounds present. In biological systems, disulfides can interact with thiols either by a thiol-disulfide interchange or by reduction catalyzed by a reductase requiring NADPH or NADH (*8*). The most abundant thiol compounds are cysteine and glutathione which are maintained intracellularly predominantly in their reduced form. Therefore, desfuroylceftiofur bound to proteins of plasma and tissues of animals may be reduced and thus provide a steady supply of desfuroylceftiofur over a longer period for biological activity.

Since the binding of desfuroylceftiofur to biological molecules is reversible, all of the ceftiofur related metabolites that contain the DFC residue have the potential to be microbiologically active. Thus, to measure the concentration of DFC-related metabolites that have the potential of being microbiologically active, an analytical method was developed to determine the total concentration of both free and bound DFC.

Overview of [^{14}C]-Ceftiofur Sodium Tissue Residue Concentration, Excretion and Metabolite Profiles in Target Animals

[^{14}C]-Distribution and Excretion. Tissue residue concentrations and/or dose accountability in excreta were determined in dairy and beef cattle and sheep (*4,5,7*) after intramuscular administration of [^{14}C]-ceftiofur sodium at 2.2 mg ceftiofur equivalents (CE)/kg body weigth (bw) for five consecutive days at a 24 hour interval, and in swine (*6*) after intramuscular administration of [^{14}C]-ceftiofur sodium at 5.2 CE/kg bw for three consecutive days at a 24 h interval. In these studies swine and sheep were slaughtered at 12 h after the last dose, while cattle were slaughtered at 8 h after the last dose. The pattern of disposition of [^{14}C]-ceftiofur-related residues in tissues was similar between the species studied (Table I). For all species, kidney was the tissue with the highest [^{14}C]-ceftiofur-related residue concentrations, followed by lung which is the clinical target tissue as ceftiofur is used for the treatment/control of bacterial respiratory diseases. Next in concentration were the liver, fat and muscle.

Concentration levels of [^{14}C]-ceftiofur-related residues in kidneys of sheep administered five consecutive doses of [^{14}C]-ceftiofur sodium at 2.2 mg CE/kg bw were higher on the average than those observed in kidneys of swine administered three consecutive doses at 5.2 mg CE/kg bw (9.02 ± 1.15 and 4.47 ± 0.81, respectively), indicating that sheep accumulated a larger percentage of the dose in the kidneys than swine. Concentration levels of [^{14}C]-ceftiofur-related metabolites in muscle, the meat most consumed by the public was 0.76 ± 0.24, 0.23 ± 0.47 and 0.13 ± 0.02 mg CE/g tissue for swine, cattle and sheep, respectively.

[^{14}C] recovery in urine accounted for approximately 60% of the dose in swine, beef and dairy cattle (Table II). In sheep, however, recovery of [^{14}C] in urine accounted for 92.6 ± 3.7 % of the dose, indicating that, during the period of time evaluated, sheep clear more of the drug by renal excretion than cattle and swine. [^{14}C] recovery in feces accounted for more than 10% of the dose for all species except sheep which cleared only 6.5 ± 0.8% of the dose via the feces (Table II).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

Metabolite Profile.

Plasma. Incubation of sheep and cattle plasma samples from [^{14}C]-ceftiofur treated animals with the reducing agent dithioerythritol indicated that DFC is the only metabolite of ceftiofur found in plasma (*7,9*). This metabolite, DFC, is rarely found free. In all species 50 to 100% of DFC was found conjugated to plasma proteins. The fraction that remained not conjugated to proteins was usually found in the form of the DFC-cysteine conjugate (*6,7*).

Urine. [^{14}C]-ceftiofur-related metabolites in urine samples from beef and dairy cattle, swine and sheep (*The Upjohn Co, unpublished data, 5-7*) can be grouped into polar metabolites, devoid of the DFC moiety/β-lactam ring and, thus devoid of microbiological activity; and into DFC containing metabolites which have the potential to be microbiologically active upon liberation of the DFC moiety. Polar metabolites comprised less than 10% of the total [^{14}C]-activity found in the urine of beef and dairy cattle and in swine. In sheep however, polar metabolites accounted for approximately 40% of the [^{14}C]-activity found in urine, indicating once more that sheep metabolize ceftiofur faster than either swine or cattle. In swine, the most abundant [^{14}C]-ceftiofur-related residue was DFC-cysteine (21.11% of the total urinary [^{14}C]-activity). Swine urine also contained DFC-dimer (23.66%). In cattle and sheep, however, DFC-dimer was the most abundant [^{14}C]-ceftiofur related metabolite (55.0 and 47.58% of the total urinary [^{14}C]-activity, respectively), and DFC-cysteine was also present in sheep urine (8.73 %). Minor components of urine of cattle and swine included DFC and parent ceftiofur. Neither of the later metabolites were found in sheep urine.

Tissues. A large percentage of the [^{14}C]-activity found in kidney of animals treated with [^{14}C]-ceftiofur was associated with macromolecules in the tissue matrix at 12 h after the last dose (62.6 and 95.7% for swine and sheep, respectively) (*6-7*). After incubating the tissue samples with the reducing agent dithioerythritol, over 70% of the tissue [^{14}C]-activity was recovered and the only [^{14}C] metabolite found was DFC, indicating that most of the [^{14}C]-activity in kidney corresponded to DFC associated with tissue macromolecules. In swine, the kidney residues not associated with macromolecules consisted of DFC-cysteine and polar metabolites (12.3 and 23.2% of the total [^{14}C]-activity). In sheep, however, all of the [^{14}C]-activity not associated with tissue macromolecules corresponded to polar metabolites (4.3% of the total [^{14}C]-activity). In swine liver (*6*), DFC-glutathione was identified as one of the ceftiofur metabolites. In sheep liver the total [^{14}C]-activity not associated with macromolecules (9.5%) corresponded to polar metabolites (*7*).

Determination of Ceftiofur and its Related Metabolites by HPLC.

Method Principle. An HPLC method, hereafter referred as HPLC-DCA, developed for the determination of ceftiofur and desfuroylceftiofur-related metabolites that have the potential to be microbiologically active (contain an intact β-lactam ring) in swine kidney, muscle, liver and fat (Beconi-Barker et al. *J. Chrom.*, in press) was applied to bovine muscle and kidney, liver and fat and to sheep muscle and kidney. This method is based on the cleavage of the disulfide and/or thioester bonds between the metabolites and their conjugate sulfur containing moiety using dithioerythritol (DTE) to yield desfuroylceftiofur (Figure 2). After incubation with DTE, DFC is converted to the stable, desfuroylceftiofur acetamide (DCA). DCA is concentrated using a C-18 solid-

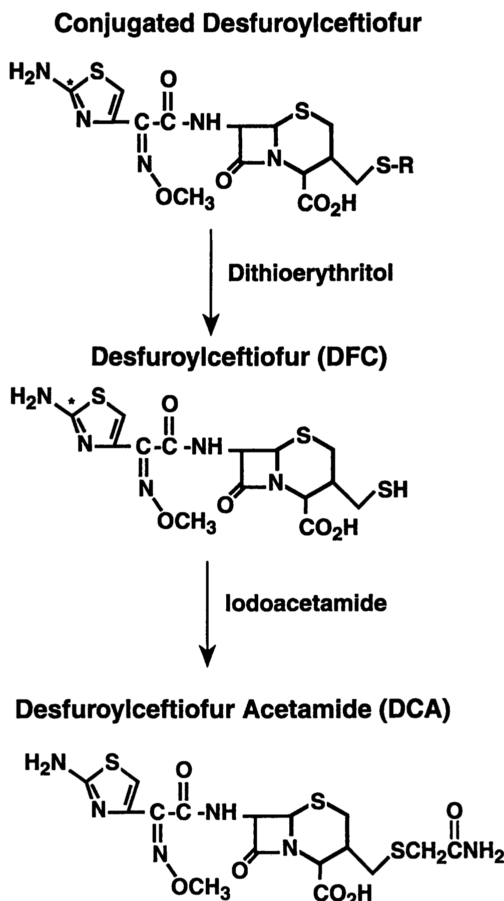


Figure 2. Cleavage of desfuroylceftiofur (DFC) from the conjugated metabolites and stabilization to desfuroylceftiofur acetamide (DCA).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

phase extraction (SPE) cartridge and further purified by anion and cation exchange SPE cartridges. DCA is separated using gradient HPLC and detected by UV absorption at 266 nm. DCA concentrations are calculated using the slope and intercept of the calibration line (weighing factor = $1/\text{concentration}^2$) of standards prepared in buffer. Extraction and chromatographic conditions were adapted specifically for each tissue.

For evaluating this method, ceftiofur itself was used for fortification since the cleavage of its thioester bond by DTE parallels the reduction of the bonds between DFC and its conjugate, thus generating DFC *in situ*. All steps in this method are critical. Successful results can only be obtained by following closely all points considered in the methods section. Twelve samples can be prepared, in duplicate, by an experienced analyst in approximately 6-8 hours, depending on the tissue. The assay makes use of HPLC equipment, columns and reagents that are commercially available to laboratories engaged in residue determination worldwide.

Analytical Reference Standard. Ceftiofur hydrochloride (ceftiofur syn-oxime, U-64,279A), lot no. Upjohn Control Reference Standard, Issue E, 893 µg/mg potency as ceftiofur free acid equivalents (CFAE), was supplied by the Upjohn Company.

Method Procedures. The HPLC-DCA method developed and validated for swine muscle, kidney, fat and liver (Beconi-Barker et al. *J. Chrom.*, in press), was evaluated for bovine muscle, kidney, fat and liver tissues and sheep muscle and kidney tissues as follows: Bovine, kidney, muscle and liver and sheep kidney and muscle were extracted, processed and analyzed as previously described for swine kidney and muscle (Beconi-Barker et al. *J. Chrom.*, in press). (Note that due to the background conditions of the bovine liver, the HPLC-DCA method for bovine liver was evaluated using the conditions determined for swine muscle and kidney). Bovine fat was extracted, processed and analyzed as previously described for swine fat (Beconi-Barker et al. *J. Chrom.*, in press). Briefly, quadruplicate control bovine kidney samples were fortified with ceftiofur at concentrations of 0, 0.107, 2.14 and 5.35 µg ceftiofur free acid equivalents (CFAE)/g tissue, the rest of the tissue samples were fortified in triplicate with ceftiofur at concentrations of 0, 0.1, 1 and 10 µg CFAE/g tissue.

Calculation of concentrations. A standard curve was generated from the DCA peak area vs. the ceftiofur concentration (µg CFAE/g tissue) of the standards. The accuracy of the regression (observed concentration/backcalculated concentration) was checked and recorded. Weighted regression (weighing factor = 1/concentration^2) proved to be the best fit. Sample DCA concentrations were calculated as follows:

$$\textit{Concentration } (\mu g \textit{ CFAE } / g \textit{ tissue}) = \frac{\textit{DCA area} - \textit{intercept}}{\textit{Slope} \times 1g \textit{ (equivalent sample wt)}}$$

The calibration curves were linear for the entire calibration range of 0.05 to 10.0 µg CFAE/g tissue. Calibration standard concentrations were back-calculated on each day of analysis using the corresponding regression line. Back-calculated values were in all cases within 10% of the theoretical value.

Method Accuracy (% Recovery). The accuracy (% recovery) was determined by comparing measured concentrations of fortified tissue extracts with their theoretical concentrations as presented in Table III. Average recovery values in bovine tissues ranged from 87.98-89.16%, 88.44-96.82%, 87.5-93.51% and 89.37-92.23% for kidney, muscle, liver and fat, respectively, across concentrations. Average recoveries in sheep tissues ranged from 79.98-91.83% and 83.46-84.75% for kidney and muscle, respectively, across concentrations. These recovery values were similar to those previously reported for swine tissues of 79.57-88.0%, 71.58-85.01%, 88.27-94.90%, 89.37-92.23% for kidney, muscle, liver and fat, respectively, across concentrations. Representative chromatograms obtained from different control and fortified matrices are shown in Figure 3.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

Table III. Accuracy (% Recovery) of Ceftiofur and Related Metabolites Containing an Intact β-lactam Ring from Fortified Bovine and Sheep Tissues.

Tissue	Fort. Level (μg/g)	AVERAGE RECOVERY (%)[A] Day 1	2	3	4	5	Total Av.(CV%)
Bovine Kidney	0.107	89.8	89.8	98.7	89.3	72.3	87.98 (10.92)
	2.14	84.7	85.9	86.9	91.0	88.4	87.38 (2.79)
	5.30	83.8	85.0	85.0	96.8	95.2	89.16 (7.05)
Bovine Muscle	0.1	92.7	96.3	96.6	102.8	98.8	88.48 (10.02)
	1.0	94.4	92.5	93.1	89.5	73.0	0.89 (9.88)
	10.0	93.0	87.4	91.9	90.5	90.0	90.57 (2.36)
Bovine Liver	0.1	91.25	90.08	94.02	93.26	90.02	91.97 (1.99)
	1.0	88.34	92.22	90.29	84.48	82.17	87.50 (4.72)
	10.0	92.44	93.35	88.21	93.33	101.4	93.51 (5.24)
Bovine Fat	0.1	93.7	84.7	93.6	92.9	96.3	92.23 (4.79)
	1.0	88.4	78.6	89.4	95.5	94.9	89.37 (7.61)
	10.0	70.1	76.7	94.7	96.0	96.0	90.68 (9.04)
Sheep Kidney	0.1	79.6	83.5	82.4	80.9	73.5	80.0 (4.89)
	1.0	86.06	104.5	96.2	88.0	83.6	91.83 (9.27)
	10.0	83.7	84.0	107.4	79.2	87.6	88.36 (12.50)
Sheep Muscle	0.1	68.5	87.8	79.2	88.8	93.0	83.46 (11.66)
	1.0	72.5	87.1	88.7	88.1	87.4	84.75 (8.11)
	10.0	73.2	79.4	93.9	86.0	88.9	84.26 (9.64)

[A]Average of quadruplicate samples per day for bovine kidney and triplicate samples per day for the remaining tissues.

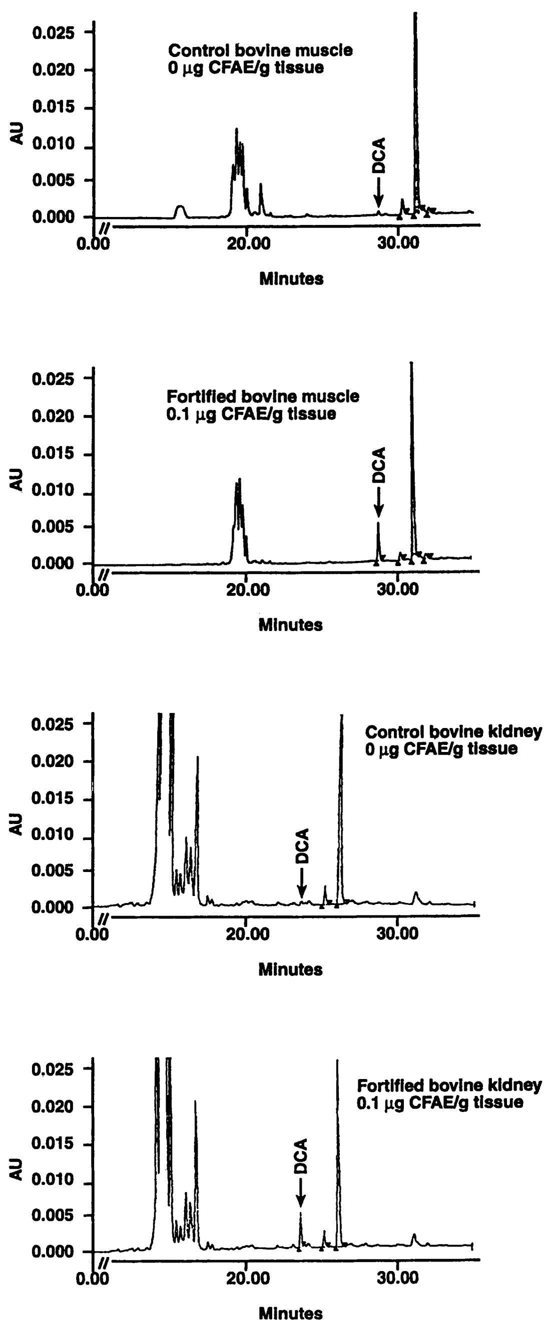


Figure 3. Representative chromatograms obtained from various control and fortified tissue matrices processed by the HPLC-DCA method.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

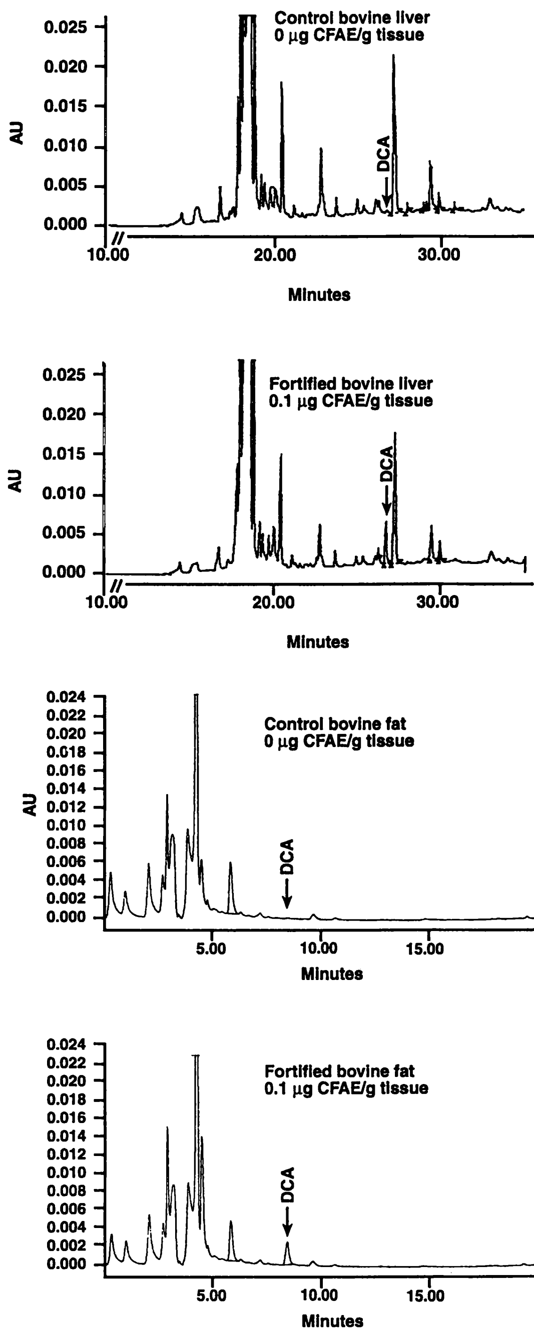


Figure 3. Continued

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

Method Precision. The within-day precision was assessed using the coefficient of variation (relative standard deviation) calculated from the replicates measured for the same concentrations for that day. The total (ignoring day) precision was assessed using the coefficient of variation (relative standard deviation) calculated from all replicates obtained for the same concentrations during the study. The accuracy was calculated as the percent difference between the measured and the theoretical concentration. For all tissues and fortification levels analyzed the within and total CV were less than 15%. (Table IV).

Limit of detection (LOD), limit of quantitation (LOQ) and limit of determination/decision (LODe). The LOD is defined as the lowest concentration of that residue in the sample which can be detected, but not necessarily quantified, under the prescribed experimental conditions. It is normally calculated as the mean value of the matrix blank readings ($n \geq 20$) plus 3 standard deviations of the mean, expressed in analyte concentration (*10*). If the variances across concentrations are not homogeneous, it is recommended that the LOD be calculated from the linear regression, as a function of the slope and the standard deviation of the y-intercept (*11*). The latter one, assumes that the variance of the calibration standards at a specific concentration is similar to the variance of the fortified matrix at that same concentration. These two criteria could not be used in this study: there was no background response from the control samples, the variances increased with increasing concentrations and the variation of the calibration standards was different from the variation of the fortified matrices. Thus, the LOD was calculated as 3 times the square root of the mean square error ($MSE^{½}$) of the lowest fortified sample. This is a conservative approach since the estimate of variability from the matrix blank readings should be much less than the estimate of variability from the lowest fortification level used. Since extrapolation beyond the standard curve is not acceptable, for this study the LOD was the lowest calibration standard used.

The LOQ is defined as the lowest concentration of the solute in the sample that may be determined with acceptable accuracy and precision (*10*). The LOQ for this study was the lowest fortified sample for which precision and accuracy were determined and found acceptable.

The Limit of Determination/Limit of Decision (LODe) is a measure of the lowest predictable value of concentration for which the corresponding instrument response can be differentiated from the intercept with a certain statistical confidence (*11*). It is usually defined as the mean value of the matrix blank readings ($n \geq 20$) plus 6 standard deviations of the mean, expressed in analyte concentration (*10*), or can be calculated from the linear regression as a function of the slope and the standard deviation of the y-intercept, when the variances across concentrations are not homogeneous (*11*). Using the same argument described for the LOD, the LODe was calculated as 10 times the $MSE^{½}$ of the lowest fortified standard (Table V).

Method Specificity. The specificity of the HPLC-DCA assay was evaluated against the following commercially available cephalosporins and other antibiotics: Cephapirin (Sodium salt, SIGMA Chemical Co), Dihydrostreptomycin Sulfate (U.S. Reference Standard), Neomycin Sulfate (Upjohn Control Reference Standard), Penicillin G-sodium (Upjohn Control Reference Standard), Spectinomycin Sulfate Tetrahydrate

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

Table IV. Precision of the HPLC-DCA assay

Tissue	Fort. Level (μg/g)	Calculated Concentration (μg/g) Average[A] (CV%) Day					
		1	2	3	4	5	Total Av.(CV%)
Bovine Kidney	0.107	0.096 (1.68)	0.096 (2.42)	0.106 (7.48)	0.078 (7.79)	0.096 (5.45)	0.09 (11.70)
	2.14	1.81 (1.08)	1.84 (2.68)	1.86 (1.22)	1.95 (1.16)	1.89 (9.37)	1.87 (4.74)
	5.30	4.49 (1.37)	4.55 (2.16)	4.55 (4.05)	5.18 (3.27)	5.09 (13.46)	4.77 (8.93)
Bovine Muscle	0.1	0.094 (5.35)	0.096 (2.94)	0.097 (1.23)	0.103 (4.03)	0.099 (7.41)	0.10 (5.31)
	1.0	0.94 (1.84)	0.92 (3.60)	0.93 (3.79)	0.90 (5.71)	0.73 94.38)	0.89 (9.88)
	10.0	9.30 (2.66)	8.74 (9.49)	9.19 (3.76)	9.05 (4.02)	9.00 (0.71)	9.06 (4.72)
Bovine Liver	0.1	0.092 (2.76)	0.090 (3.72)	0.094 (5.62)	0.093 (6.01)	0.090 (1.34)	0.09 (4.08)
	1.0	0.88 (0.20)	0.92 (5.12)	0.90 (6.73)	0.84 (5.80)	0.82 (3.78)	0.88 (6.04)
	10.0	9.19 (2.55)	9.33 (1.27)	8.82 (5.44)	9.33 (2.86)	10.14 (3.99)	9.35 (5.68)
Bovine Fat	0.1	0.094 (3.60)	0.085 (1.61)	0.094 (4.28)	0.093 (3.91)	0.096 (5.02)	0.09 (5.54)
	1.0	0.88 (3.55)	0.79 (5.41)	0.89 (3.83)	0.96 (5.36)	0.95 (3.71)	0.89 (7.97)
	10.0	9.01 (1.43)	7.67 (1.29)	9.47 (2.25)	9.60 (4.76)	9.60 (3.96)	9.07 (8.80)
Sheep Kidney	0.1	0.080 (1.90)	0.084 (1.51)	0.082 (2.43)	0.081 (1.34)	0.074 (1.98)	0.08 (4.86)
	1.0	0.86 (3.32)	1.05 (2.36)	0.96 (1.23)	0.89 (1.90)	0.84 (0.69)	0.92 (8.76)
	10.0	8.37 (1.46)	8.40 (11.41)	10.74 (6.44)	7.92 (5.59)	8.76 (7.38)	8.84 (13.08)
Sheep Muscle	0.1	0.069 (2.89)	0.088 (7.19)	0.079 90.54)	0.089 (3.96)	0.093 (2.55)	0.08 (11.37)
	1.0	0.73 (0.61)	0.87 (0.84)	0.89 (1.16)	0.88 (3.64)	0.87 (2.06)	0.85 (7.71)
	10.0	7.32 (4.38)	7.94 (1.34)	9.39 (4.70)	8.60 (3.02)	8.89 (2.44)	8.42 (9.39)

[A]Average of quadruplicate samples per day for bovine kidney and triplicate samples per day for the remaining tissues.

Table V. Limit of Detection (LOD), Limit of Quantitation (LOQ) and Limit of Determination (LODe) of the HPLC-DCA method in Different Tissue Matrices

Tissue	Study		Calculated		Recovery Adjusted	
	LOD (μg/g)	LOQ (μg/g)	LOD (μg/g)	LODe (μg/g)	LOD (μg/g)	LODe (μg/g)
Bovine Kidney	0.05	0.11	0.02	0.04	0.02	0.04
Bovine Muscle	0.05	0.10	0.012	0.04	0.01	0.04
Bovine Liver	0.05	0.10	0.015	0.05	0.02	0.05
Bovine Fat	0.05	0.10	0.016	0.05	0.02	0.06
Sheep Kidney	0.05	0.10	0.004	0.01	0.01	0.013
Sheep Muscle	0.05	0.10	0.01	0.04	0.01	0.05

(Upjohn Control Reference Standard), Tetracycline Hydrochloride (Upjohn Control Reference Standard), Cefquinome Sulfate (Hoechst AG, Germany), Cefoperazone Sodium (Cefobid, Roerig, Pfizer), and Cephacetril Sodium (Ciga-Geigy) following procedures previously described (Beconi-Barker et al. *J. Chrom.*, in press) using approximate concentrations of 10 μg antibiotic/g tissue. No interference was observed with the non-cephalosporin antibiotics dihydrostreptomycin, neomycin and spectinomycin when they were chromatograph without derivatization or following processing by the HPLC-DCA method. The β-lactam antibiotic, penicillin G, absorbed weakly at 266 nm or had impurities which showed up in the chromatogram without derivatization. Tetracycline also absorbed at 266 nm when not derivatized, but at a considerably longer retention time than DCA. Once derivatized and processed by the HPLC-DCA method, both penicillin G and tetracycline no longer appeared in the chromatograms.

All of the cephalosporins, cephapirin, cefquinome, cefoperazone and cephacetril, when not derivatized, absorbed at 266 nm and were observed in the chromatograms. Only cefquinome had a retention time similar to DCA. When these cephalosporins were subjected to the derivatization and purification process of the HPLC-DCA method cephacetril and cefoperazone were completely removed and did not appear in the chromatograms. Cephapirin, however, appeared in the chromatograms after derivatization and purification by the HPLC-DCA method as a small peak that eluted with approximately a 1 min difference in retention time from DCA. Cephapirin should not interfere in the HPLC-DCA assay for ceftiofur metabolites because of its different retention time with DCA. In case of doubt co-chromatography is recommended. When cefquinome was processed in swine liver, a background peak appeared at the retention time of DCA. Thus, at an approximate concentration of 10 μg cefquinome/g tissue in swine liver, cefquinome can potentially interfere with the HPLC-DCA assay. In all other tissues, cefquinome was completely removed and did not appeared in the chromatograms after processing by the HPLC-DCA method.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

Conclusions

The absorption, distribution, metabolism, and excretion of ceftiofur sodium in beef and dairy cattle, swine and sheep is similar (*4-7*). In these species the injected [^{14}C]-ceftiofur was absorbed rapidly into the blood and was primarily eliminated in the urine (>65% for all species). Sheep eliminated more ceftiofur metabolites via the urine than the other species (~92.55%). In all species, the tissue where highest residue concentrations were observed at 12 h after the last dose was the kidney.After intramuscular administration ceftiofur was rapidly metabolized to DFC which was mostly found conjugated to cysteine, glutathione and proteins. The microbiologically active metabolite of ceftiofur is DFC. Since the binding of DFC is reversible (4), all of the DFC-conjugates have the potential of exhibiting microbiological activity. In addition, polar metabolites devoid of the intact β-lactam ring and lacking microbiological activity, have been identified in tissues and urine of all species.

An HPLC method developed to detect and quantify ceftiofur-related metabolites that contain the DFC in swine and bovine kidney, muscle, liver and fat (Beconi-Barker et al. *J. Chrom.*, in press) was evaluated in bovine kidney, muscle, liver and fat and in sheep kidney and muscle. This HPLC method (HPLC-DCA) is based on the cleavage of the disulfide and/or thioester bonds between the metabolites and their conjugate sulfur containing moiety using dithioerythritol (DTE) to yield desfuroylceftiofur, derivatization of DFC to the more stable derivative DCA, and concentration and purification by SPE columns. The HPLC method allows for determination and quantitation of ceftiofur-related metabolites containing an intact β-lactam ring in tissues at concentrations of 0.05 to 10 μg CFAE/g tissue with precision and accuracy. The resulting calibration curves are linear with correlation coefficients > 0.99 over the range evaluated. The HPLC-DCA method makes use of HPLC equipment, columns and reagents that are commercially available to laboratories engaged in residue determination worldwide and has been successfully implemented in laboratories in France (Dr. L. Millerioux, Cephac Research Centre, France) and Japan (Mr. Kazunari Uchida, Research Institute for Animal Science in Biochemistry and Toxicology, Japan). It can be used to predict total ceftiofur related residues (residues containing the DFC moiety plus polar ones) in swine tissues under certain conditions (Beconi-Barker et al. *J. Chrom.*, in press). It is specific for ceftiofur and related metabolites when evaluated against several commercially available antibiotics for swine, cattle and sheep.

Literature Cited.

1. Food and Drug Administration; Animal Drugs, Feeds and Related Products - Ceftiofur Sterile Powder; Fed. Regist. 1988, 53, 5369-5370.
2. Food and Drug Administration; Implantation Injectable Dosage Form; Animal Drugs not Subject to Certification - Ceftiofur Sterile Powder; Fed. Regist. 1991, 56, 12-119.
3. Food and Drug Administration; Implantation Injectable Dosage Form; New Animal Drugs - Ceftiofur Sterile Powder for Injection; Fed. Regist. 1992, 57, 41862.
4. Jaglan, P.S., Kubicek, M.F., Arnold, T.S., Cox, B.L., Robbin, R.H., Johnson, D.B., and Gilbertson, T.G. "Metabolism of Ceftiofur. The Nature of Urinary and Plasma Metabolites in Rats and Cattle." *J. Agri. Food Chem.* 1989, 37, 1112-1118.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

5. Jaglan, P.S., Yein, F.S., Hornish, R.E., Cox, B.L., Arnold, T.S., Roof, R.D., and Gilbertson, T.J. "Depletion of Intramuscularly Injected Ceftiofur From the Milk of Dairy Cattle." *J. Dairy Sci.* 1992, 75, 1870-1876.
6. Gilbertson, T.J., Roof, R.D., Nappier, J.L., Zaya, M.J., Robins, R.H., Stuart, D.J., Krzeminski, L.F. and, Jaglan, P.S. "Disposition of Ceftiofur Sodium in Swine Following Intramuscular Treatment." *J. Agri. Food Chem.* 1995, 43, 229-234.
7. Beconi-Barker, M.G., Davison, K.L., Hornish, R.E., Arnold, T.S., Craigmill, A.L., Gilbertson, T.J., Smith, E.B., Vidmar, T.J., Hoffman, G.A., and Gatchell, C.L. "[14]Ceftiofur Absorption, Distribution, Metabolism, and Excretion in Sheep following Intramuscular Injections." *J. Agric. Food Chem.* 1995, 43, 1589-1597.
8. Jocelyn, P.C. Biochemistry of SH Group, Academic Press, London. (1972), p. 121.
9. Jaglan, P.S., Cox, B.L., Arnold, T.S., Kubicek, M.F., Stuart, D.J., and Gilbertson, T.J. "Liquid Chromatography Determination of Desfuroylceftiofur Metabolite of Ceftiofur as Residue in Cattle Plasma." *J. Assoc. Off. Anal. Chem.*, 73, (1990) 26-30.
10. AOAC® Peer-Verified Methods Program. Manual on Policies and Procedures. (1994), pp 8 - 9.
11. Oppenheimer, L., Capizzi, T.P., Weppelman, R.M., andMehta, H. "Determining the Lowest Limit of Reliable Assay Measurement." *Anal. Chem.*, 55, (1983) 638-643.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch009

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

Chapter 10

Rapid Determination of Tetracycline Antibiotics in Milk and Tissues Using Ion-Pairing High-Performance Liquid Chromatography

William A. Moats and Raida Harik-Khan[1]

Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705–2350

Most analytical procedures for tetracycline antibiotics include a clean-up step using solid-phase extraction. By use of ion-pairing with alkyl sulfonates, tetracyclines could be separated from interferences in sample extracts. Samples were extracted/ deproteinized with HCl-acetonitrile. Acetonitrile was removed by evaporation or by adding hexane and methylene chloride and collecting the water layer. Residues could be concentrated on-line by injecting the water layer into the LC system and eluting with a gradient. They could also be concentrated by evaporation and analyzed by isocratic LC. For analysis, a Polymer Laboratories PLRP-S column was used. The LC mobile phases were H_3PO_4 or H_3PO_4-KH_2PO_4, mixtures with sodium decane sulfonate or sodium dodecylsulfate as ion-pairs, and acetonitrile. Recoveries were 70-100% depending on the compound and substrate. Limits of quantitation were about 20-50 ppb in tissues and 4-8 ppb in milk.

The tetracycline group of antibiotics is widely used in treatment of farm animals, both therapeutically and as feed additives to promote growth. These uses have the potential to produce residues in tissues and milk from the animals. It is therefore important to have reliable methods to detect such residues in milk and tissues, both to detect positive samples and to clear samples which are free of residues.

Several years ago, a new and more sensitive screening test for tetracyclines, the Charm II procedure, was introduced for testing milk. Investigators using this procedure reported widespread contamination of the commercial milk supply with tetracycline antibiotics (*1-2*). At the time, no liquid

[1]Current address: Gerontology Research Center (BLSA), NIA, 4940 Eastern Avenue, Baltimore, MD 21224

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

chromatographic (LC) or other confirmatory tests of comparable sensitivity were available to establish the reliability of these test results. These reports nevertheless were and continue to be of major concern to regulatory agencies and the daily industry, (*3*).

To be useful, LC determinative procedures should meet the requirements of regulatory agencies for sensitivity and also equal or exceed the sensitivity of screening tests. Otherwise, a perception may be created that residues are present which are not being picked up by the determinative procedure.

Considerable progress has been reported in recent years in the development of LC methods for determination of tetracycline antibiotics in both milk and tissues. Several sensitive LC procedures capable of determining tetracycline antibiotics in milk at levels of 20 ppb or less have been reported in recent years (*4-9*). These are well within the official levels of concern of 80, 30, and 30 ng/ml for tetracycline (TET), oxytetracycline (OTC) and chlortetracycline (CTC), respectively, in the USA (*10*) and 100 ng/ml recommended by the World Health Organization (*11*).

A number of LC methods have been described for determination of residues of tetracycline antibiotics in tissues (*12-36*). Many of these use some type of solid-phase extraction cartridge for cleanup (*20-22, 24, 25, 27-29, 31, 32*). Farrington, et al. (*12*) used chelating sepharose for cleanup. Others have used partitioning cleanup with the aid of complexing agents (*14, 16, 19, 30, 34*).

The use of ion-pairing as a technique for changing the retention time of analytes is well known. Ion-pairs of the same charge as the analyte may decrease retentions while ion-pairing agents of opposite charge increase retention on reversed-phase columns, presumably by forming a complex which is less polar than the original analyte. Retentions can be further increased by adding ionic compounds with hydrocarbon tails. Retention is then related to the length of the hydrocarbon tail. Carignan, et al. (*36*) added alkyl sulfonates as ion-pairs to separate oxytetracycline from interferences in extracts of fish tissue, thus eliminating the need for further sample cleanup. White, et al. (*6*) found that addition of sodium decanesulfonate was effective in separating tetracyclines from interferences in milk extracts. Milk was extracted/deproteinized with 1N HCl-acetonitrile (Table I). The resulting filtrate was mixed with hexane and methylene chloride and the water layer which formed was collected. This required further concentration to achieve adequate sensitivity using HPLC analysis with UV detection. The approach used by White, et al., (*6*) was to use on-line concentration in the HPLC system followed by elution with an acetonitrile gradient. White, et al., (*37*) noted the advantages of using the Polymer Laboratories PLRP-S column for determination of tetracyclines. The results with this procedure are shown in Figure 1. Separation of tetracyclines from one another and from interferences was good. The peak shape was equally good with early and late eluting compounds. Recoveries (Table II) were also good but quantitation at 10 ng/ml was more difficult as shown by the high standard deviations. The gradient elution procedure gave a slightly uneven base-line which was a problem at low levels. It was rather lengthy and appeared to stress the polymeric HPLC column used. We therefore explored the use of isocratic analysis procedures (*9*). However, for isocratic analysis, some

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

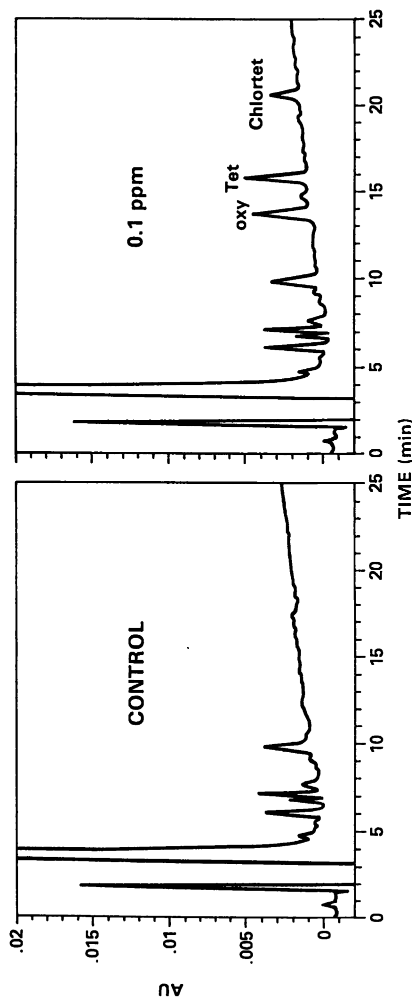


Figure 1. Chromatogram of milk sample with 0.1 ppm of three tetracyclines. Gradient elution: 0.05M pH2 oxalate buffer, 0.005 M sodium decanesulfonate-acetonitrile 80:20 (0-3 min), 62:38 (25 min). Adapted from Ref. 6.

concentration of the sample extract was required to achieve adequate sensitivity. The filtrates could be concentrated to 1-2 ml by evaporation under reduced pressure using the Buchler Vortex evaporator. However, evaporation to dryness resulted in degradation of the tetracyclines. Two evaporation procedures were evaluated:

1. Evaporation of the water layer formed after addition of hexane and methylene chloride.
2. Direct evaporation of the acetonitrile filtrate.

These worked equally well. The recoveries are summarized in Table II. Figure 2 shows isocratic analysis of three tetracyclines at the 0.01 ppm level. Separation of tetracycline and oxytetracycline from interferences was improved at a lower (72 + 28) acetonitrile concentration. However, the retention time of chlortetracycline became impracticably long.

These approaches have also been used for determination of tetracyclines in animal tissues. In earlier studies, tissues were blended in 1N HCl and then acetonitrile. The tetracyclines were recovered in the water layer formed by addition of hexane and methylene chloride. Oxytetracycline was determined by on-line concentration and gradient elution (*38*). However, tetracycline was markedly unstable in extracts prepared in this manner. After some investigation, we found that better results were obtained by first homogenizing tissue in water and then treating the homogenate in a manner similar to that described for milk. The tetracyclines were stable in the resulting filtrate. They could be analyzed using one of the three approaches described for milk. However, the HPLC mobile-phase required some modification from that used for milk, especially for some liver and kidney extracts. The HPLC mobile-phases used are summarized in Table III. Modifications included switching from decane- to dodecylsulfate and adding some KH_2PO_4 to modify the pH of the buffer. Figures 3 and 4 show oxytetracycline at 0.1 ppm in beef muscle and kidney, respectively. Suspect samples could be reanalyzed using a different HPLC mobile phase to provide further evidence that the analyte is indeed a tetracycline.

Recoveries from beef muscle and beef kidney are summarized in Tables IV and V. They were somewhat lower from muscle and decidedly lower from kidney. Recoveries from liver (not shown) were generally in the 70-78% range.

The limits of detection will depend somewhat on the equipment used and were markedly lower on a diode array as compared with a conventional UV detector. With our equipment detection limits in milk were about 0.002 ppm for oxytetracycline and tetracycline and 0.004 ppm for chlortetracycline, well within U.S. levels of regulatory concern. The limits of quantitation in tissues were about 0.020 ppm for oxytetracycline and tetracycline and 0.050 ppm for chlortetracycline, again, within the U.S. tolerances of 0.1 ppm.

The use of fluorescence detection for tetracyclines has also been described. Blanchflower, et al., (*31*) converted chlortetracycline to a fluorescent derivative in pH12 glycine buffer. Croubels, et al, (*8*) used post-column mixing with zirconium cation at alkaline pH to produce a fluorescent compound while Haagsma and Scherpenisse (*34*) used mixing with magnesium ion at alkaline pH

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

to produce fluorescence. Post-column mixing is required in both cases since the tetracyclines are not stable under these conditions. Duggan (*39*) reported a method using luminescence detection which requires a special detector. All these procedures require more elaborate equipment and special detectors as compared with UV detection. They may, however, offer better sensitivity and specificity.

The technique described using ion-pairing with alkyl sufonates and UV detection is simple and does not require special equipment. Sensitivity is adequate to determine residues in meat and milk at or below U.S. tolerances.

Table I. Extraction/Deproteinization of Milk and Tissue

Milk	To 5 ml of milk, add 1 ml 1N HCl and 15 ml acetonitrile, mix, final volume = 20 ml. Decant through a plug of glass wool, collect 12 ml filtrate.
Tissue	Blend with 3 V/W of water. To 4 ml homogenate, add 16.5 ml acetonitrile, mix, and add 0.5 ml 1N HCl, mix, final volume = 20 ml. Decant through a plug of glass wool, collect 15 ml filtrate.

Table II - Recoveries of Tetracyclines from Milk by Various Procedures

		% Recovery		
Amount Added (ppm)		*On-line (6) concentration*	*Sep Funnel (9) Evaporation*	*Direct (9) Evaporation*
OTC	1	98 ± 3	101 ± 1	104 ± 7
	0.1	97 ± 5	97 ± 8	103 ± 8
	0.01	95 ± 16	99 ± 5	106 ± 8
TET	1	97 ± 3	93 ± 3	101 ± 8
	0.1	93 ± 6	81 ± 2	92 ± 7
	0.01	91 ± 6	83 ± 4	87
CTC	1	92 ± 3	93 ± 6	85 ± 3
	0.1	87 ± 7	103 ± 2	94 ± 6
	0.01	99 ± 48	100 ± 19	110 ± 3

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

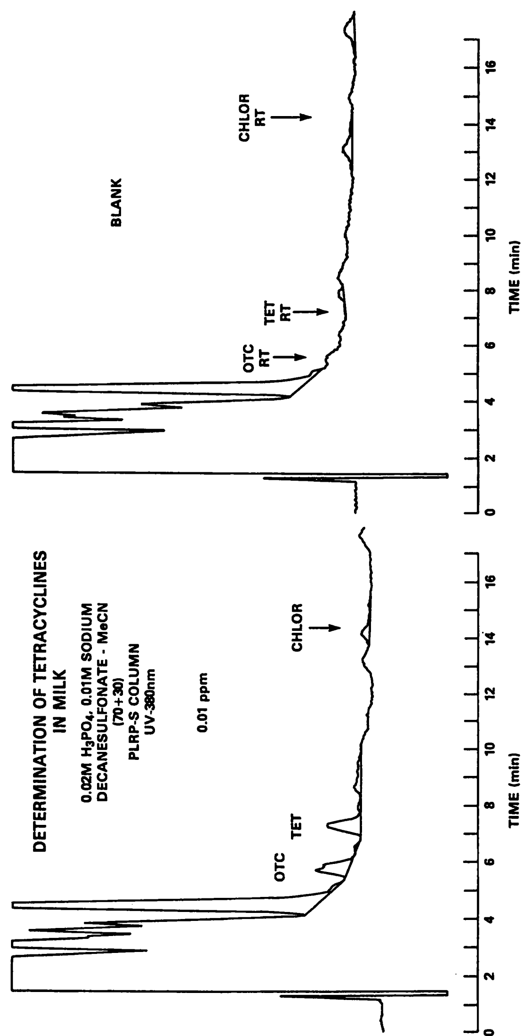


Figure 2. Chromatogram of milk sample with 0.01 ppm of three tetracyclines. Isocratic analysis. Adapted from Ref. 9.

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

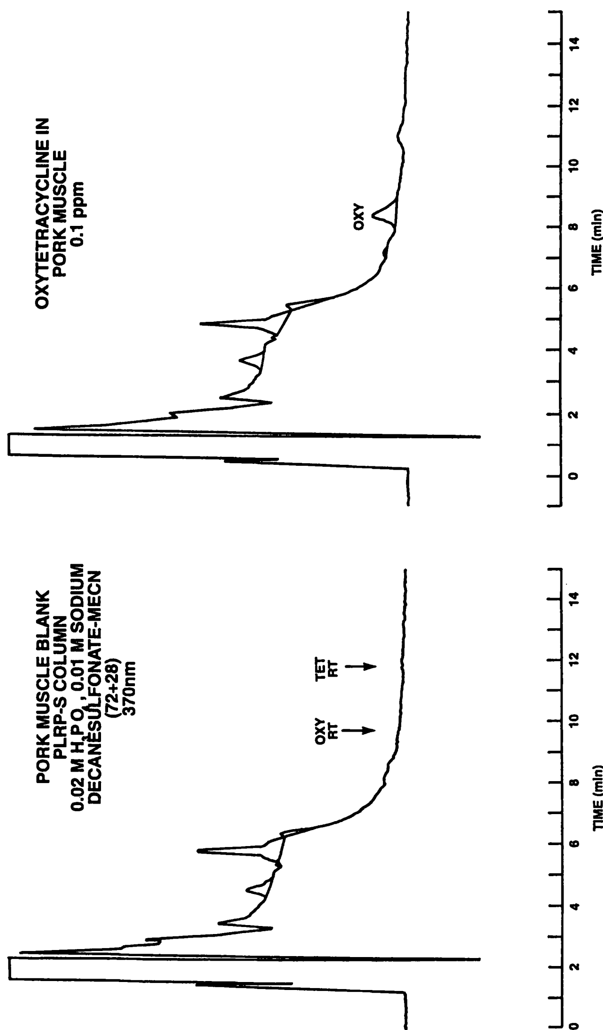


Figure 3. Chromatogram of pork muscle with 0.1 ppm oxytetracycline.

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

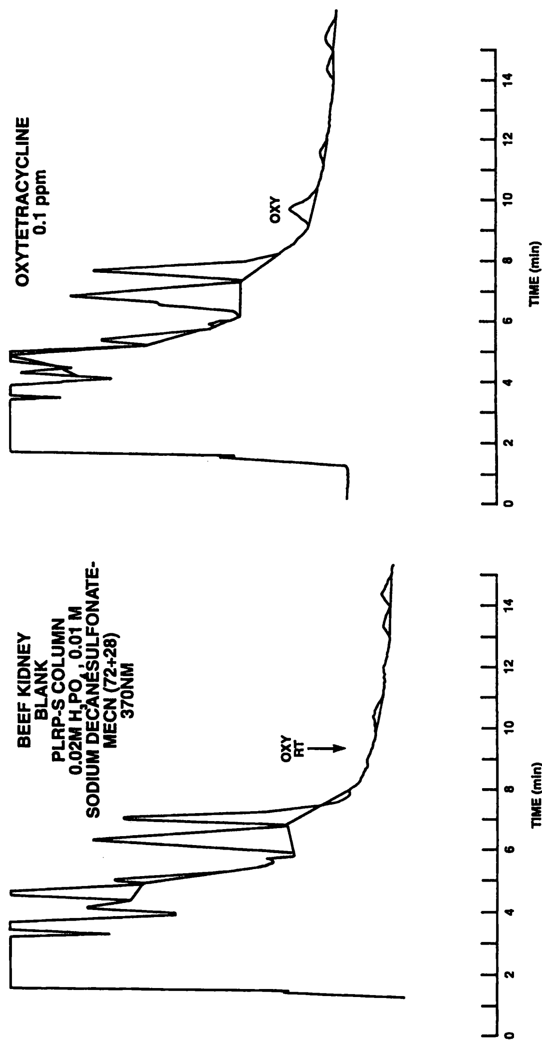


Figure 4. Chromatogram of beef kidney with 0.1 ppm oxytetracycline.

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

Table III. HPLC Mobile-phases

Matrix	*Mobile-phases*	
Milk, muscle, beef kidney	0.02M H_3PO_4, 0.01M sodium decanesulfonate-acetonitrile	
	72 + 28	OTC, TET
	68 + 32	CTC
Beef, pork liver	0.015M H_3PO_4, 0.0075M sodium dodecylsulfate-acetonitrile	
	65 + 35	OTC, TET
	62 + 38	CTC
Pork kidney	0.01M H_3PO_4, 0.005M KH_2PO_4, 0.01M sodium dodecylsulfate	
	65 + 35	OTC, TET
	62 + 38	CTC

*Polymer Laboratories PLRP-S column.

Table IV - Recoveries of Tetracyclines from Beef Muscle

Amount Added (ppm)		*% Recovery*	
		Sep. Funnel Evaporation	*Direct Evaporation*
TET	1	81	85
	0.1	79	91
OTC	1	89	88
	0.1	80	75
CTC	1	89	88
	0.1	80	75

Table V. Recoveries of Tetracycline from Beef Liver

Amount Added (ppm)		*% Recovery*	
		Sep. Funnel Evaporation	*Direct Evaporation*
TET	1	76	75
	0.1	73	70
OTC	1	72	74
	0.1	91	72

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

References

1. Brady, M. S.; Katz, S. E. *J. Food Prot.* **1988**, *51*, 8.
2. Collins-Thompson, D. L.; Wood, D. S.; Thomson, I. A. *J. Food Prot.* **1988**, *51*, 632.
3. United States General Accounting Office: Food Safety and Quality: FDA Strategy needed to address animal drug residues in milk. GAO/RCED-92-209, Washington, D.C., August, 1992.
4. Thomas, M. H., *J. Assoc. Off. Anal. Chem.* **1989**, *72*:564
5. Fletouris, D. J.; Psomas, J. E.; Botsoglou, N. A. *J. Agric. Food Chem.* **1980**, *38*, 1913.
6. White, C. R.; Moats, W. A.; Kotula, K. L. *J. AOAC Int'l.* **1993**, *76*, 549.
7. Carson, M. C. *J. AOAC Int'l.* **1993**, *76*, 329.
8. Croubels, S.; Van Peteghem, C.; Baeyens, W. *Analyst* (Cambridge, U.K.) **1994**, *119*, 2713-16.
9. Moats, W. A.; Harik-Khan, R. *J. Agric. Food Chem.* **1995**, *43*, 931.
10. Anon, *Food Chemical News*, **Dec. 17, 1990**. p. 58.
11. Del Pozo, E. *Rev. Cubana Cienc. Vet.* **1985**, 16, 15.
12. Farrington, W. H. H.; Tarbin, J.; Bygrave J.; Shearer, G. *Food Addit. Contam.* **1991**, *8*, 55-64.
13. Long, A. R.; Hsieh, L. C.; Malbrough, M. S.; Short, C. R.; Barker, S. A. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 379.
14. Sharma, J. P.; Bevill, R. F. *J. Chromatogr.* **1978**, *166*, 213.
15. Bocker, R.; Estler, C. J. *Arzneim.-Forsch.* **1979**, *29*, 1690.
16. Sharma, J. P.; Perkins, E. G.; Bevill, R. F. *J. Chromatogr.* **1977**, *134*, 441.
17. Onji, Y.; Uno, M.; Tanigawa, K. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 1135.
18. Ashworth, R. B. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 1013.
19. Nelis, H. J. F. C.; De Leenheer, A. P. *Clin. Chim. Acta* **1980**, *103*, 209.

20. Oka, H.; Matsumota, H.; Uno, K.; Horada, K.-I.; Kadowaki, S.; Suzuki, M. *J. Chromatogr.* **1985**, *325*, 265.
21. Oka. H.; Ikai, Y.; Kawamura, N.; Uno, K.; Yamada, M.; Harada, K.-I.; Suzuki, M. *J. Chromatogr.* **1985**, *322*, 265.
22. Ikai, Y.; Oka, H.; Kawamura, N.; Yamoda, M.; Haroda, K.; Suzuki, M. *J. Chromtogr.* **1987**, *411*, 313.
23. Moats, W. A. *J. Chromatogr.* **1986**, *358*, 253.
24. Mulders, E. J.; Van De Lagemaat, D. *J. Pharm. Biomed. Anal.* **1988**, 7, 1829.
25. Nordlander, I.; Johnson, H.; Osterdahl, B. *Food Addit. Contam.* **1987**, *4*, 1987.
26. Kondo, F.; Morikawa, S.; Tetayama, S. *J. Food Prot.* **1989**, *52*, 41.
27. Reimer, G. J.; Young, L. M. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 813.
28. Rogstad, A.; Harmazabal, V.; Yndestad, M. *J. Liq. Chromatogr*, **1988**, *11*, 2337.
29. Murray, J.; McGill, S.; Hardy, R. *Food Addit. Contam.* **1988**, *5*, 77.
30. Botsoglou, N. A.; Vassilopoulis, V. N.; Kufidis, D. C. *Chim. Chron.* **1984**, *13*, 37.
31. Blanchflower, W. J.; McCracker, R. J.; Rice, D. A. *Analyst* **1989**, *114*, 421.
32. Walsh, J. R.; Walker, L. V.; Webber, J. J. *J. Chromatogr.* **1992**, *596*, 211.
33. Riond, J. L.; Hedler, M.; Tyczkowska, K.; Riviere, J. E. *J. Pharm. Sci.* **1989**, *78*, 44.
34. Haagsma, N.; Scherpernisse, P. "*Residues of Veterinary Drugs in Food*" Haagsma, N.; Ruiter, A., Czedik-Exsenberg, P. O., Eds. Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, **May 3-5, 1993**, pp. 342-346.
35. Moretti, V. M.; Maggi, G. L.; Albertini, A; Bellagamba, F; Luzzana, U. *Analyst* (Cambridge, U.K.) **1994**, *119*, 2749.
36. Carignan, G; Carrier, K.; Sved, S. *J. AOAC Int'l.* **1993**, 76, 325.
37. White, C. R.; Moats, W. A.; Kotula, K. L. *J. Liq. Chromatogr.* **1991**, 14, 2499.
38. Ibrahim, A.; Moats, W. A. *J. Agric. Food Chem.* **1994**, *42*, 2561.
39. Duggan, J. X. *J. Liq. Chromatogr.* **1991**, *14*, 2499.

September 14, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch010

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

Chapter 11

Interfacing High-Performance Liquid Chromatography with Rapid Screening Kits for Detection and Measurement of β-Lactam Residues

Raida Harik-Khan[1] and William A. Moats

Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agricultural, Beltsville, MD 20705–2350

Liquid chromatographic (HPLC) methods are important for the confirmation of ß-lactam antibiotic residues. A procedure for expediting these methods is described. This procedure integrates ß-lactam residue detection kits with the multiresidue automated HPLC cleanup method developed in our laboratory. Spiked milk was processed and subjected to reverse phase HPLC using a gradient program which concentrated the ß-lactams. Amoxicillin, ampicillin, cephapirin, ceftiofur, cloxacillin, and penicillin G, eluted into five fractions which were then tested for activity using three screening kits. Quantification of the ß-lactams in the positive fractions was done using analysis HPLC methods. The HPLC cleanup method separated ß-lactam antibiotics from each other and from interferences in the matrix, and also concentrated the antibiotics, thus increasing the sensitivity of the kits. The procedure, extended for the analysis of fractions from spiked kidney and muscle, is useful for identifying and measuring ß-lactam antibiotics present in incurred milk and meat samples.

The presence of antibiotic residues in milk and food is a topic of concern to the general public, government regulatory agencies, and industry. For this reason, screening of milk for ß-lactam antibiotic residues is now mandatory in the United States (*1*), where the Food and Drug Administration has set tolerance "safe levels" for the most commonly used ß-lactam antibiotics (*2*).

In addition, a residue surveillance and monitoring program, the 10 Point Milk and Dairy Beef Quality Program, has been in effect since 1992 (*3*). This

[1]Current address: Gerontology Research Center (BLSA), NIA, 4940 Eastern Avenue, Baltimore, MD 21224

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

program was developed jointly by the National Milk Producers Federation and the American Veterinary Medical Association.

To comply with these regulations and recommendations, commercial kits which can detect ß-lactams at concentrations equal to or lower than the tolerance levels, are used to screen milk for the presence of ß-lactams. However, these kits cannot identify or quantify the ß-Lactams, and false positives have also been reported to occur especially in mastitic milk. For these reasons it is important to have confirmatory methods in order to check the authenticity of the screening kit results.

Recently (*4*) our laboratory developed and evaluated a multiresidue liquid chromatography method for the detection of the six most commonly used ß-lactams: penicillin G, amoxicillin, cloxacillin, cephapirin, ampicillin, and ceftiofur. For confirmation, a replicate was run after treating the milk or the tissue homogenate with ß-lactamase. The method includes a single unified extraction and HPLC cleanup procedure suitable for all ß-lactam antibiotics in milk. The gradient HPLC cleanup procedure separated the six ß-lactams into five fractions (*Figure 1*). Penicillin G and ceftiofur eluted together, while the other four were well separated. Fractions that correspond to the ß-lactams of interest were collected and analyzed by isocratic HPLC using different combinations of columns and mobile phases (*4*). This multiresidue LC method was intended for use with milk samples found positive by one or more of the ß-lactam rapid screening kits. However, separate HPLC analysis of all fractions is rather tedious since different conditions are required for each fraction. Screening of the cleanup fractions with the rapid commercial kits would greatly expedite the process of identifying and measuring the suspect ß-lactams (*5*). Only those fractions that test positive are subjected to further analysis by HPLC.

In this study, we evaluated the use of commercial ß-lactam kits for the screening of HPLC cleanup fractions from milk (*5*), and meat samples. This approach is similar to that of the HPLC receptorgram method reported by Zomer et al. (*6*), where liquid chromatography was interfaced with a microbial receptor assay (*Charm II*), for the identification and quantification of several sulfonamide residues in milk.

Use of Screening Kits with Fractions from Spiked Milk. Six screening kits were tested with fractions of spiked milk and/or with ß-lactam standards dissolved in the cleanup buffer (0.01M KH_2PO_4). The Delvotest-P, LacTek ß-lactam, and LacTek Ceftiofur kits were selected for use in this study. The kits were used according to the manufacturers' instructions, except that cleanup buffer fractions were used instead of milk. Fractions from blank and penicillinase treated samples, were usually included in the assays.

Spiking of Milk Samples. Spiking of samples was done at three levels: 1) half the safe level, 2) the safe level, 3) double the safe level. The safe levels varied, from 5 ppb for penicillin G, 10 ppb for amoxicillin, ampicillin, and cloxacillin, 20 ppb for cephapirin, and 50 ppb for ceftiofur. In some instances milk samples were simultaneously spiked with two or more ß-lactams. Each set contained a blank, as well as a spiked milk sample treated with penicillinase.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

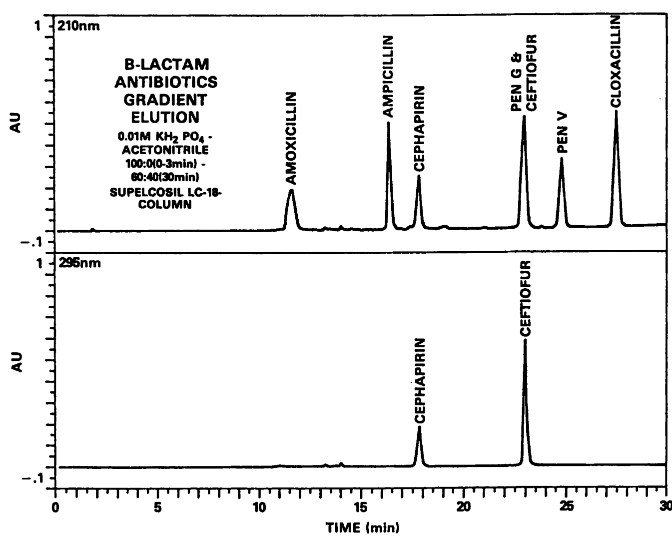


Figure 1. Gradient elution of six ß-lactam standards.

Extraction/Deproteinization and LC-Cleanup. Milk samples, 10 or 5 mL, were extracted and deproteinized with acetonitrile as described earlier (*4*). After filtration, and evaporation, the extract was subjected to a gradient HPLC cleanup procedure reported by our laboratory (*4*). The mobile phase used was 0.01 M KH_2PO_4 (A)-acetonitrile (B). The gradient program was as follows:

100 A : 0 B (0-3 min) - 40 A : 60 B (40 min) - 100 A : 0 B (41 min).

Single or multiple fractions corresponding to the ß-lactam(s) of interest were collected. For some samples, including unknowns, the five fractions that contained the six ß-lactams of interest were collected. The final volume of the collected fractions was adjusted to 1 mL. Residues in the cleanup fractions were concentrated 2 or 4 fold over what was originally present in the milk, depending on whether the extracted volume was 5 or 10 mL respectively. The fractions were divided into two equal parts, one was analyzed by isocratic HPLC as reported earlier (*4*), and the other was screened with the test kits. The concentration of the ß-lactams was calculated using peak areas.

Screening Kit Results. The following are the results obtained when the multi-residue LC cleanup method developed in our laboratory was used to process milk samples spiked with penicillin G, amoxicillin, ampicillin, cephapirin, ceftiofur, or cloxacillin. Each set contained two controls: (*a*) a non-spiked milk sample, and (*b*) a spiked milk sample treated with penicillinase. Fractions collected from these controls tested negative with the screening test kits, and by HPLC analysis. This confirmed the presence of ß-lactams in the fractions of spiked samples which tested positive, and showed that the peaks of interest in HPLC analysis chromatograms did not have interfering components.

Table I shows results obtained when penicillin G, ampicillin, amoxicillin, and cephapirin cleanup fractions of milk (10 mL in volume), spiked with the aforementioned ß-lactams, were tested with Delvotest-P, and LacTek ß-lactam screening kits. The penicillin G fractions from samples spiked with this ß-lactam tested positive with the two kits. These results demonstrate that, at the levels of spiking used here, the kits were useful in detecting penicillin G in the HPLC cleanup fractions in which the antibiotic eluted. When the extracted volume of milk was reduced from 10 to 5 mL, the concentration of penicillin G in the fractions was reduced correspondingly (results not shown). However, this did not affect the ability of the kits to detect penicillin G, since its concentration was still higher than the limits of detection of the two kits. Moreover, the presence of penicillin G in cleanup buffer rather than in milk did not affect the limits of detection of the kits to this antibiotic (results not shown).

Table I also shows results obtained when cleanup fractions of milk spiked with ampicillin, amoxicillin and cephapirin were tested with the two screening kits. As in the case of penicillin G, the Delvotest-P, and LacTek ß-Lactam were equally effective in detecting these ß-lactams at the levels of spiking used (1/2, 1, 2 X safe levels). Likewise, as in the case of penicillin G, the extraction and the HPLC cleanup procedures separated ampicillin, amoxicillin, and cephapirin,

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

Table I. ß-lactam Test Kit Results for LC-Cleanup Fractions of Milk Spiked with Pen-G, Ampicillin, Amoxicillin or Cephapirin

Spiking (ppb)	*N*	*Delvo-P Result*	*LacTek Result*
	Penicillin G		
0	4	-	-
0 + P-nase	2	-	-
2.5	8	+	+
5	8	+	+
10	8	+	+
10 + P-nase	2	-	-
	Ampicillin		
0	4	-	-
5	5	+	+
10	5	+	+
20	5	+	+
20 + P-nase	2	-	-
	Amoxicillin		
0	2	-	-
0 + P-nase	1	-	-
5	5	+	+
10	5	+	+
20	5	+	+
20 + P-nase	1	-	-
	Cephapirin		
0	2	-	-
10	6	+	+
20	6	+	+
40	5	+	+
40 + P-nase	2	-	-

Cleanup HPLC fractions were concentrated four fold (see test).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

from each other, from other ß-lactams, and from matrix interferences, thus enabling us to identify and measure them.

Results of testing cloxacillin and ceftiofur containing cleanup fractions, from cloxacillin or ceftiofur spiked milk, are represented in Table II. Cleanup fractions from milk, spiked with cloxacillin at safe levels (10 ppb) tested positive with Delvotest-P and LacTek ß-lactam. However, only LacTek ß-lactam was able to detect cloxacillin in all fractions tested. Delvotest-P and LacTek Ceftiofur were found to be equally effective in detecting ceftiofur when the volume of the extracted milk was 10 mL. However, only LacTek Ceftiofur was able to detect ceftiofur when 5 mL of milk was spiked at half the safe level (results not shown).

In Table III the concentration of cloxacillin in the HPLC cleanup fractions, as determined by analysis HPLC, was compared with results obtained using the Delvotest-P and LacTek ß-lactam test kits. The latter had a higher sensitivity for cloxacillin than the Delvotest-P. In contrast, the ability of the two kits to detect Penicillin G appears to be the same at all concentration levels (*Table IV*).

The multiresidue method was also evaluated with samples of raw milk, spiked with penicillin G, amoxicillin, cephapirin, or cloxacillin. Similar results were obtained when the fractions of interest were tested with the Delvotest-P and LacTek ß-lactam, then analyzed by HPLC.

In addition, this procedure was evaluated using twenty samples of milk provided by the Center for Veterinary Medicine, FDA, Beltsville, MD. The samples consisted of single, and double spikings with ß-lactams, as well as one incurred sample, and four blanks, all of which were unknown to us. The multiresidue procedure, in conjunction with the screening kits allowed us to correctly identify all of the samples.

Our procedure was also used to process samples, sent to our laboratory for confirmation by a State Health Department, and an FDA laboratory. The samples had tested positive when screened with commercially available ß-Lactam antibiotic residue kits. Using this procedure, we were able to identify and quantify the violative ß-lactams in 1-2 days.

Incurred milk samples from mastitic cows treated with cephapirin were also processed using the above procedure. HPLC cleanup fractions corresponding to cephapirin and desacetyl-cephapirin, a metabolite of cephapirin (Fig 2), were collected, tested with the Delvotest-P and LacTek ß-lactam screening kits, then analyzed by HPLC. Analysis of samples obtained from four cows show that cephapirin is metabolized to desacetyl cephapirin, and that the two kits are able to identify desacetyl cephapirin in HPLC cleanup fractions when it is present in milk at levels smaller than 5 ppb (*Table V*).

The elution time of other antibiotics with our HPLC cleanup program was determined. This is of special significance when processing unknown samples, which in addition to the ß-Lactams, might contain other antibiotics. It was important to determine the fate of some common antibiotics after undergoing the extraction and HPLC cleanup program; if they co-eluted with any of the ß-lactams of interest; if so, whether they interfered with the Delvotest-P and LacTek results. Table VI summarizes the results obtained. Of

Table II. ß-Lactam Test Kit Results for LC-Cleanup Fractions of Milk Spiked with Cloxacillin and Ceftiofur

Spiking (ppb)	*N*	*Delvo-P Result*	*LacTek Result*
	Cloxacillin		
0	2	-	-
5	4	+, ?	+
10	5	+	+
20	6	+	+
20 + P-nase	2	-	-
	Ceftiofur		
0	2	-	-
25	3	+	+
50	3	+	+
100	3	+	+
100 + P-nase	1	-	-

Cleanup HPLC fractions were concentrated four fold. ? denotes ambiguous.

Table III. Comparison of Test Kit Results with HPLC Analysis for Cloxacillin Present in HPLC Fractions

HPLC Conc. (ppb)	*Delvo-P (+) N*	*LacTek (+) N*
>20	100% (11/11)	100% (11/11)
10-20	50% (2/4)	100% (4/4)
5-10	0% (0/3)	100% (3/3)
2-5	0% (0/2)	0% (0/2)
0	0% (0/7)	0% (0/7)

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

Table IV. Comparison of Test Kit Results with HPLC Analysis for Penicillin G Present in Cleanup Fractions

HPLC Conc. (ppb)	*Delvo-P (+) N*	*LacTek (+) N*
>10	100% (18/18)	100% (18/18)
5-10	100% (8/8)	100% (8/8)
2.5-5	100% (5/5)	100% (5/5)
<2.5	0% (0/1)	0% (0/1)
0	0% (0/8)	0% (0/8)

Table V. Detection of Desacetylcephapirin in Cleanup Fractions of Incurred Milk from Cows Injected with Cephapirin

Conc. in Milk (ppb)	*Delvo-P (+)*	*LacTek β-Lactam (+)*
>20	100% (15/15)	100% (6/6)
10-20	100% (2/2)	-
5-10	100% (2/2)	-
1-5	100% (7/7)	67% (2/3)
0	5% (1/19)	8% (1/12)

Table VI. Antibiotic Elution Using the Cleanup Gradient Program

Antibiotic	*Retention Time (min)*	*Detection by Delvo-P & LacTek in Cleanup Fractions*
tetracyclines	1.5 - 5.5	
lincomycin	24.5 - 25.5	Delvo-P +
novobiocin	39-41	
chloramphenicol	29-30	Rida screen chloramphenicol +
tylosin	no peak	
sulfamethazine	24-25	Rida screen sulfamethazime +

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

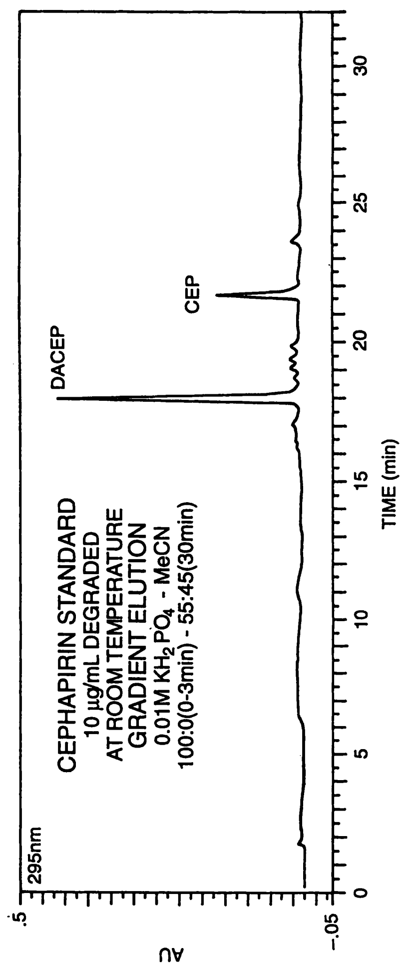


Figure 2. Gradient elution of an aged cephapirin standard showing degradation to desacetylcephapirin, the main metabolite.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

the antibiotics that eluted in the zone of interest (10-30 min), only lincomycin was detected by Delvotest-P.

Single spikings of milk with chloramphenicol and sulfamethazine showed that these antibiotics survived the extraction and cleanup HPLC program. They were detected by Ridascreen chloramphenicol, and Ridascreen sulfamethazine kits, (r-biopharm, Darmstadt, Germany) when the two antibiotics were present in cleanup buffer at 1, and 10 ppb respectively.

Use of Screening Kits with Fractions from Spiked Meat. Limits of ß-lactam antibiotic residue in edible tissue range from 10 ppb for amoxicillin, ampicillin and cloxacillin, 50 ppb for penicillin G, to 100 ppb for cephapirin (*7*). Unlike milk, where testing for these residues is mandatory, only carcasses suspected of containing violative amounts, are tested using rapid screening kits. Samples that test positive are subjected to further analysis for confirmation. As in the case of milk, integrating HPLC with the screening kits, would facilitate and expedite the confirmatory process.

Spiking, Extraction, and HPLC Cleanup/Analysis of Meat Samples. Kidney and muscle samples were spiked with ß-lactams at three levels: 10, 100, and 1000 ppb. In addition to the blank, penicillinase-treated spiked samples were included in the assays as controls. Following homogenization, aliquots were extracted and deproteinized using acetonitrile, as described for milk. The meat extracts were subjected to the same procedure as milk extracts, fractionated, tested with Delvotest-P and LacTek, then analyzed with HPLC. Results obtained when pork or beef muscle was spiked with amoxicillin, ampicillin, cephapirin, penicillin G, and cloxacillin at the above levels, showed that the kits detected the ß-lactams in all the fractions except for Delvotest-P, which detected cloxacillin only in fractions of meat samples spiked at levels equal or greater than 100 ppb. It is noteworthy that muscle tissue homogenates metabolized cephapirin to desacetylcephapirin.

Likewise, when pork and beef kidney were spiked with amoxicillin, ampicillin, and ceftiofur, then processed and tested as above, the test kits detected the ß-lactams in the fractions. Moreover, kidney homogenates, spiked with ceftiofur (at 10 ppm) converted the latter to several metabolites which appeared in different HPLC cleanup fractions. These metabolites were detected by both LacTek Ceftiofur and Delvotest-P screening kits.

Table VII summarizes the current status of the multiresidue analysis of ß-lactams in tissues. At present, HPLC analysis of cleanup fractions corresponding to amoxicillin and ampicillin is difficult due to the presence of interfering peaks from the matrix. It would be very desirable to use the rapid kits, particularly those that lend themselves to quantification, not just to screen the cleanup fractions, but also to measure the concentration of ß-lactams in meat fractions.

Our results show that the three kits, Delvotest-P, LacTek ß-Lactam, and LacTek Ceftiofur are useful for screening milk and meat cleanup fractions for the presence of ß-lactams. Fractions testing negative will not be further analyzed by HPLC, whereas positive and suspect fractions will be subjected to the

Table VII. Multiresidue Analysis of β-Lactams in Tissues

Compound	Comment
o Amoxicillin	Detected by screening kits. No HPLC analytical method.
o Ampicillin	Detected by screening kits. HPLC analytical method can detect 10 ppb. Needs improvement.
o Cephapirin	Degrades to desacetylcephapirin in tissue homogenates. Parent compound and metabolite detected by screening kits and can be determined by HPLC.
o Penicillin G	Detected by screening kits; can be determined by HPLC at 10 ppb.
o Ceftiofur	Degrades to metabolites which can be detected by screening kits. Analytical HPLC method under development for major metabolites.
o Cloxacillin	Detected by screening kits; can be determined by HPLC at 10 ppb.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

appropriate HPLC analysis methods for confirmation and measurement of the suspect ß-lactams. The integration of the rapid screening kits with the multiresidue method, therefore expedites the process of identifying and measuring the violative ß-lactams. A potential valuable use of these rapid kits would be to measure the ß-lactams of interest in the corresponding HPLC cleanup fractions.

The LacTek kits were found to be the most sensitive and rapid for screening the fractions for the presence of ß-lactams. A simpler and less expensive but slower test may be adequate if results are not required quickly. Cost per test, number of samples to be tested, ease of use, equipment needed, and time required for performing assays are important considerations in choosing kits for the testing of the HPLC cleanup fractions.

Acknowledgement

We wish to thank Max Paape and Mike Zurakowski from the Milk Secretion and Mastitis Laboratory, Beltsville, for providing raw milk samples and Pat Sgambati from MSRL for assistance in preparing this manuscript.

References

(1) United States Food and Drug Administration, Pasteurized Milk Ordinance, Appendix N, **1991**.
(2) *Food Chemical News* **1992**, **March 16**, p. *15*.
(3) Herrick, J.B. *JAVMA* **1991**, *199*, 1268.
(4) Moats, W.A. and Harik-Khan, R. *J. AOAC Intl.* **1995**, *78*, 49.
(5) Harik-Khan, R. and Moats, W.A. *J. AOAC Intl.* **1995**, (*in press*).
(6) Zomer, E., Saul, S., and Charm, S. E. *J.Assoc. Off. Anal. Chem.* **1992**, *75*, 987.
(7) United States Department of Agriculture, FSIS, Compound Evaluation and Residue Information, FSIS Chemistry Division, Washington, D.C., **1994**.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch011

Chapter 12

Toward a Regulatory Method: Comparison and Validation of Multiresidue Procedures for Determination of β-Lactam Antibiotics in Milk

Mary C. Carson, Pak-Sin Chu, and Jurgen von Bredow

Center for Veterinary Medicine, U.S. Food and Drug Administration, BARC-East, Building 328A, Beltsville, MD 20705

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

Intramammary infusion of β-lactam antibiotics is often used to treat mastitis in lactating dairy cattle. Failure to follow label guidelines for dosage or milk discard time may result in residues of these drugs (amoxicillin, ampicillin, cloxacillin, cephapirin and penicillin G) entering the raw milk bulk tank. Under the U.S. Pasteurized Milk Ordinance, industry must now monitor each milk tanker for the presence of β-lactams prior to accepting the milk for processing. Several screening tests are currently accepted by the Food and Drug Administration (FDA) for use in this program. Screening tests are designed to pass safe milk; a negative result indicates that the milk contains no unsafe residues of the drugs the test detects. By themselves, screening tests generally do not meet FDA criteria for a regulatory method. Use of a regulatory determinative procedure adds certainty that milk testing positive by a screening test contains violative drug residues. We conducted a laboratory evaluation of several determinative procedures developed by other laboratories for their suitability as part of a regulatory method. The criteria the procedures were evaluated against include: 1) number of residues detected at their tolerance/safe levels; 2) accuracy; 3) precision; 4) ruggedness; and 5) practicability. The procedures studied include: 1) a 2-dimensional liquid chromatographic (LC) assay developed by W.A. Moats; 2) a liquid-liquid extraction procedure employing diazomethane derivatization followed by capillary gas chromatography developed in the lab of M. Petz; 3) a "receptorgram" assay developed by Charm Sciences, Inc.; and 4) an LC procedure using pre-column derivatization with triazole/$HgCl_2$ developed by J. Boison. A comparison of these procedures and our validation results will be presented.

Analytical procedures should be evaluated within the context of their intended use. In a regulatory environment, there are three kinds of procedures: screening, determinative, and confirmatory. Screening procedures are typically fast, inexpensive, qualitative in nature,

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

and give only presumptive evidence that a residue may be present. There are currently many commercially available test kits for use in screening raw bulk tank milk for antibiotic (principally β-lactam) residues. Determinative procedures are quantitative and give specific identification of the residue present. For highest confidence in residue identification, an additional confirmatory procedure may be used. These are typically complex and expensive procedures which involve mass spectral analysis of the suspect violative sample.

One of the Center for Veterinary Medicine's (CVM's) highest method priorities is to find or develop a suitable determinative procedure, preferably multiresidue, for β-lactam antibiotic residues in milk. Such a procedure would initially be evaluated against the following CVM method performance guidelines for residue concentrations <0.1 ppm (*1*):

Precision	Intralaboratory coefficient of variation <20%
Accuracy	60 to 110% recovery for fortified samples
Specificity	<10% interference from endogenous milk peaks
Practicability	Be capable of being performed by a reasonably experienced analyst in a typically equipped analytical lab in a timely and safe manner

The procedure should meet these criteria at the residues' tolerances or concern levels. Once these initial criteria have been met, the procedure is further evaluated against the following criteria:

Specificity	1) No interferences from other approved drugs 2) No interferences observed in milk produced in different regions of the country
Incurred residues	Evaluation for accuracy (usually using "exhaustive extraction") and precision by analysis of milk from cows dosed with the drug

This paper will describe our initial evaluation results for β-lactam determinative procedures developed by other labs (*2-5*) using control and fortified milk samples.

General Experimental Design

All four procedures were evaluated using raw milk from cows that had not been treated with any β-lactam antibiotics during the month previous to the milking. In most instances, this milk ("control milk") was subdivided into aliquots which were stored at -80°C until the day of use. Aqueous stock solutions (ca. 1 mg/mL) of penicillin G, ampicillin, amoxicillin, cloxacillin, penicillin V, and/or cephapirin were prepared, combined to form mixed stock solutions if needed, and either stored refrigerated for up to two weeks (*5*), or for longer storage, were divided into aliquots and stored at -80°C. The β-lactam stock solutions were used to prepare fortified milk samples containing various concentrations of the β-lactams. These concentrations usually corresponded to one-half, one-times, and twice the regulatory target level (T, see Table I). Control and fortified milk samples were extracted and analyzed according to written procedure directions (either published paper or in Standard Operating Procedure [SOP] format provided by the developers). Control milk results were used to check for potential matrix interferences, and fortified milk results were used to estimate precision and accuracy.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

Table I. U.S. Regulatory Target Levels for β-Lactam Residues in Milk

Drug	*Type*[a]	*Milk Concentration*
Penicillin G	Safe Level	5 ppb
Ampicillin	Tolerance	10 ppb
Amoxicillin	Tolerance	10 ppb
Cloxacillin	Tolerance	10 ppb
Cephapirin	Tolerance	20 ppb

[a] "Tolerances" are residue concentrations defined in the U.S. Code of Federal Regulations. Where an official tolerance is either zero or does not exist, FDA may promulgate a "safe level," which is the residue concentration at which regulatory action will be taken. In the case of penicillin G, a drug with zero tolerance in milk, the safe level corresponds to the approximate detection limit of the official assay.

Results and Discussion

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

Charm Sciences, Inc., Receptorgram Procedure. The β-lactam procedure was supplied to us in detailed SOP format which has since been published (*2*). It has the potential to detect six different β-lactam residues in milk, including all five listed in Table I. Raw milk is initially identified as residue-positive using the Charm II Quantitative Assay. Positive raw milk is extracted using acidic buffer precipitation followed by solid phase extraction, concentration, and LC fractionation. Fractions corresponding to the elution times observed for the six β-lactams on a separate analysis of standards are collected. The six fractions (after concentration in the case of cloxacillin) are each diluted with control milk and assayed by the Charm II Quantitative Assay. Whichever fraction is positive (if any) identifies which of the six potential residues is present, i.e., penicillin G vs. cloxacillin vs. ampicillin. The identification of β-lactams in the fractions is not quantitative. (The procedure developers achieved some quantitation of the penicillin G LC fraction by employing ^{14}C-labeled penicillin G as an internal standard. Based on this internal standard, the absolute recovery of penicillin G was 37%, which was used to "correct" concentrations determined in that fraction. Utilization of specific radiolabeled β-lactams as internal standards may allow quantitation of other residues, but these internal standards are generally not commercially available.) For quantitation, the unfractionated raw milk is reassayed by the quantitative assay and the appropriate standard curve applied to derive the milk residue concentration from the observed sample counts divided by the control counts (B/B_0).

The B/B_0 response vs. concentration is different for each of the six beta-lactams. Violative milk samples must usually be diluted to bring their concentrations within the most sensitive part of the standard curve, which is not linear. Since the precision and accuracy of the whole procedure depend on the quantitative performance of this assay, we evaluated it first. Table II shows our results obtained when analyzing fortified raw milk samples with the Charm II Quantitative Assay using standard curves that we generated. While most of the accuracies (recoveries) at the target level (T) were acceptable, some of the CVs were high. The CVs can be reduced if the sample is assayed several times and the counts averaged before applying the standard curve. Generating standard curves uses numerous kits. This procedure is limited in that quantitation on the whole unfractionated raw milk sample will not work at all if there is more than one drug or an active metabolite present in the sample. For this reason, we did not pursue validation of the receptorgram procedure further.

Table II. Results Using Charm II Quantitative Assay

Residue	*Level*	*Fortified Conc. (ppb)*	*Found Conc. (ppb)*	*Recovery (%)*	*CV (%)*	*n*
Penicillin G	½ T	2.5	2.6	104	13	6
	T	5	5.5	110	37	6
Ampicillin	¼ T	2.5	3.0	120	12	3
	½ T	5	5.9	119	13	3
	T	10	8.4	84	21	3
Cloxacillin	½ T	5	6.1	122	41	4
	T	10	9.7	97	19	4
	2 T	20	22.9	115	29	4

Gas Chromatographic (GC) Determination of β-Lactams. The procedure developed by Meetschen and Petz is a traditional, lengthy, multistep liquid-liquid extraction procedure involving separatory funnels and several rotovap and centrifugation steps (*3*). The residues are derivatized using diazomethane and analyzed by capillary GC using cool on-column injection and a nitrogen-phosphorous detector. The procedure detects neutral and acidic penicillins, including penicillin G and cloxacillin, but not amphoterics such as ampicillin or cephalosporins such as cephapirin. We could prepare four samples in an 8.5 hour day for overnight analysis.

Figure 1 shows our chromatograms obtained from control milk and milk fortified with 5 ppb each penicillins G and V and 10 ppb cloxacillin. The sensitivity was quite good, with 2.5 ppb penicillin G and 5 ppb cloxacillin readily detectable and quantifiable. Quantitation was by comparison of the peak ratio to the triazine internal standard with an external standard curve ratio. There were no interferences in the control milk--penicillin G and V are both resolved from the endogenous milk peak--but these chromatograms do illustrate a potential problem with this procedure. Some extract preparations contained a large initial peak when chromatographed, which occasionally completely obliterated the peaks of interest. These chromatograms show just the tail of a moderately sized initial peak.

Table III. Results Using Meetschen & Petz GC Procedure

Residue	*Level*	*Fortified Conc. (ppb)*	*Found Conc. (ppb)*	*Recovery (%)*	*CV (%)*	*n*
Penicillin G	½ T	2.5	1.9	77	41	3
	T	5	3.3	65	25	4
	2 T	10	8.0	80	16	3
Penicillin V	N/A[a]	2.5	2.0	78	3	4
	N/A	5	3.3	66	10	3
	N/A	10	6.8	68	25	4
Cloxacillin	½ T	5	3.4	67	21	4
	T	10	6.1	61	15	3
	2 T	20	13.0	65	31	4

[a] Not approved for use in dairy cows; no tolerance or action level has been established in the U.S. in milk.

The quantitative results for the GC procedure are listed in Table III. The recoveries at the target level are all within acceptable limits, but again, some CVs are high. Most of the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

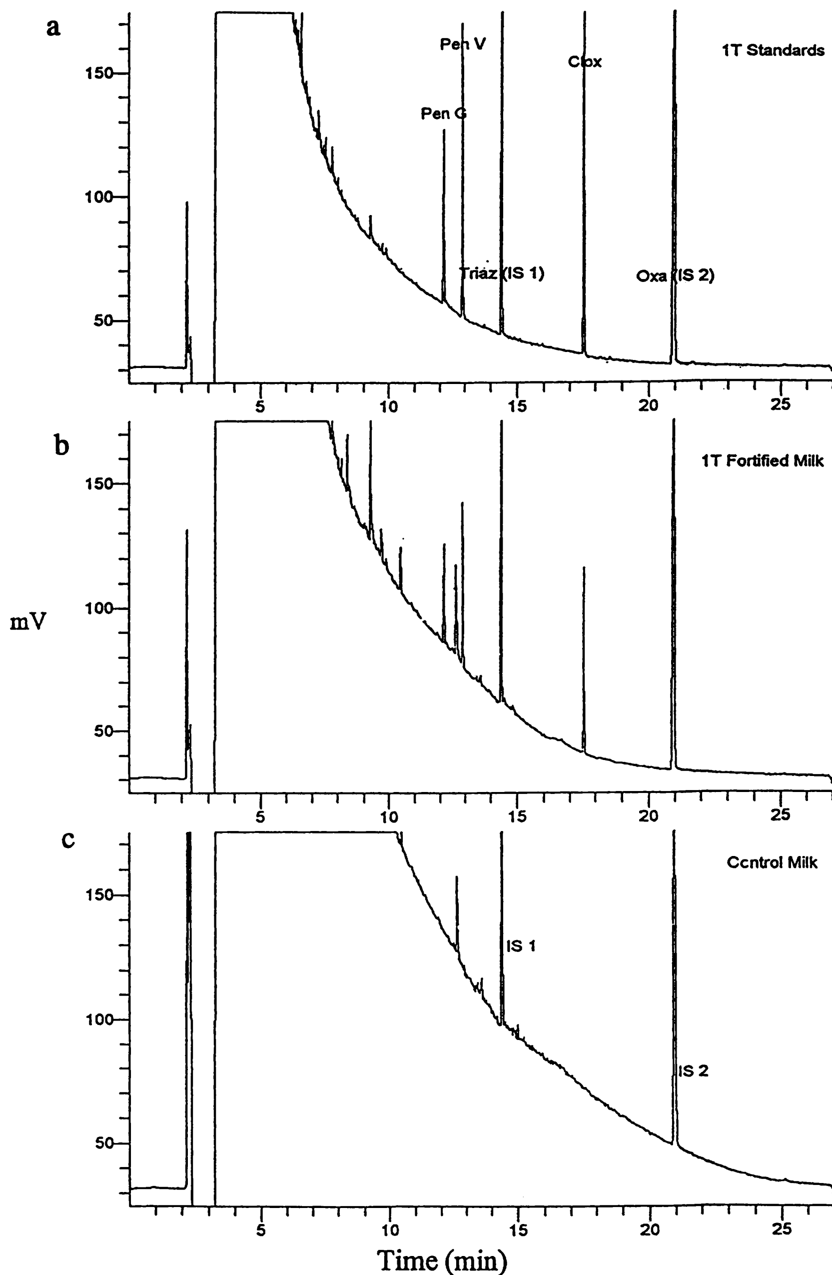


Figure 1. GC determination of diazomethane derivatized β-lactam residues. a) External standards equivalent to 5 ppb penicillins G and V and 10 ppb cloxacillin. b) Fortified milk extract. c) Control milk extract.

larger CVs were due to the occasional presence of the huge initial peak. Additional ruggedness evaluation or more experience using the procedure may bring the CVs within CVM guidelines.

LC Analysis of Triazole-Mercuric Chloride Derivatives. The SOP procedure we followed (*4*) was based on Agriculture and Agri-Food Canada's previously published procedure for penicillin G in tissue (*7*). Even though the procedure only detected one drug, we were interested in it because of its simplicity and speed. After adding penicillin V as an internal standard to 4 mL of milk, the milk was diluted with water and buffer and protein precipitated by acid-tungstate. The β-lactams were extracted and concentrated from the supernatant solution using a C-18 solid phase extraction (SPE) cartridge. The 1 mL eluate was derivatized with 2M 1,2,4-triazole containing 1 mM mercuric chloride. This allowed detection of neutral or acidic β-lactams at 325 nm, away from most milk interferences.

Our initial results were mixed. Figure 2 shows chromatograms of the same extract of milk fortified with 20 ppb of penicillin G and 100 ppb of the internal standard, penicillin V. The extraction and derivatization worked, but the derivatives were unstable. There was significant degradation after overnight refrigeration and the derivatives were completely gone after 4 hours at room temperature (Figure 2c). We also did not have the sensitivity required to detect 2.5 ppb of penicillin G, even when starting with 10 mL of milk.

We tried modifying the original procedure. The most significant change was to increase the starting milk volume to 25 mL and omit the dilution with water. More acid and tungstate were required to achieve the same pH and precipitate the proteins. We reluctantly tried doubling the mercuric chloride concentration to increase derivative stability. The stability improved, but we also decided to derivatize each sample immediately before LC injection. This was not difficult for analyses lasting only 8 to 10 minutes.

Figure 3 shows chromatograms obtained using the modified extraction procedure on control and fortified milk samples. We reduced the internal standard level to 20 ppb to be closer to the penicillin G target level. There were no interferences in the control milk (Figure 3a), and 2.5 ppb of Pen G was readily detectable (Figure 3b). However, we found, as recommended by the procedure developers, that a fortified milk standard curve was necessary to achieve accurate quantitation. This was likely due to the derivatization reaction being very sensitive to the amount of acetonitrile present in the SPE eluates. Table IV shows our results obtained with the modified procedure. Both accuracy and precision at the target level were very good. We regard these results as very preliminary, since they represent a single day's labor.

Table IV. Preliminary Results Using Modified Boison LC Procedure

Residue	*Level*	*Fortified Conc. (ppb)*	*Found Conc. (ppb)*	*Recovery (%)*	*CV (%)*	*n*
Penicillin G	½ T	2.5	2.8	112	3	3
	T	5	4.4	88	7	3
	2 T	10	9.2	92	11	3

LC Determination of β-Lactam Residues Using Automated LC Cleanup. Schematically, this recently published (5) procedure was quite simple. Protein was precipitated from the milk using buffer and acetonitrile, the filtrate was concentrated by evaporation, and a

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

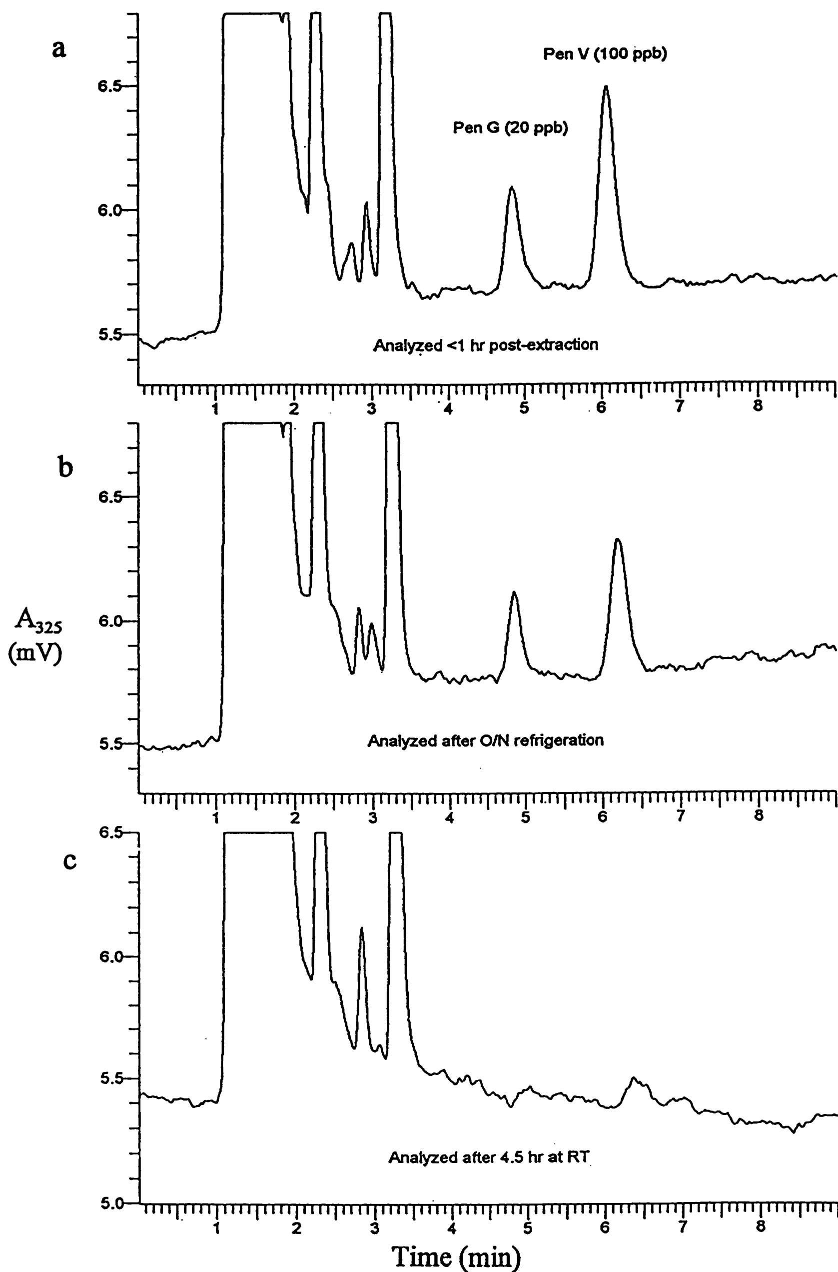


Figure 2. LC determination of 1,2,4-triazole/mercuric chloride derivatized β-lactam residues. a) Extract of milk fortified with 20 ppb penicillin G and 100 ppb penicillin V as internal standard. b) Same extract after overnight refrigeration and c) after 4.5 hours at room temperature.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

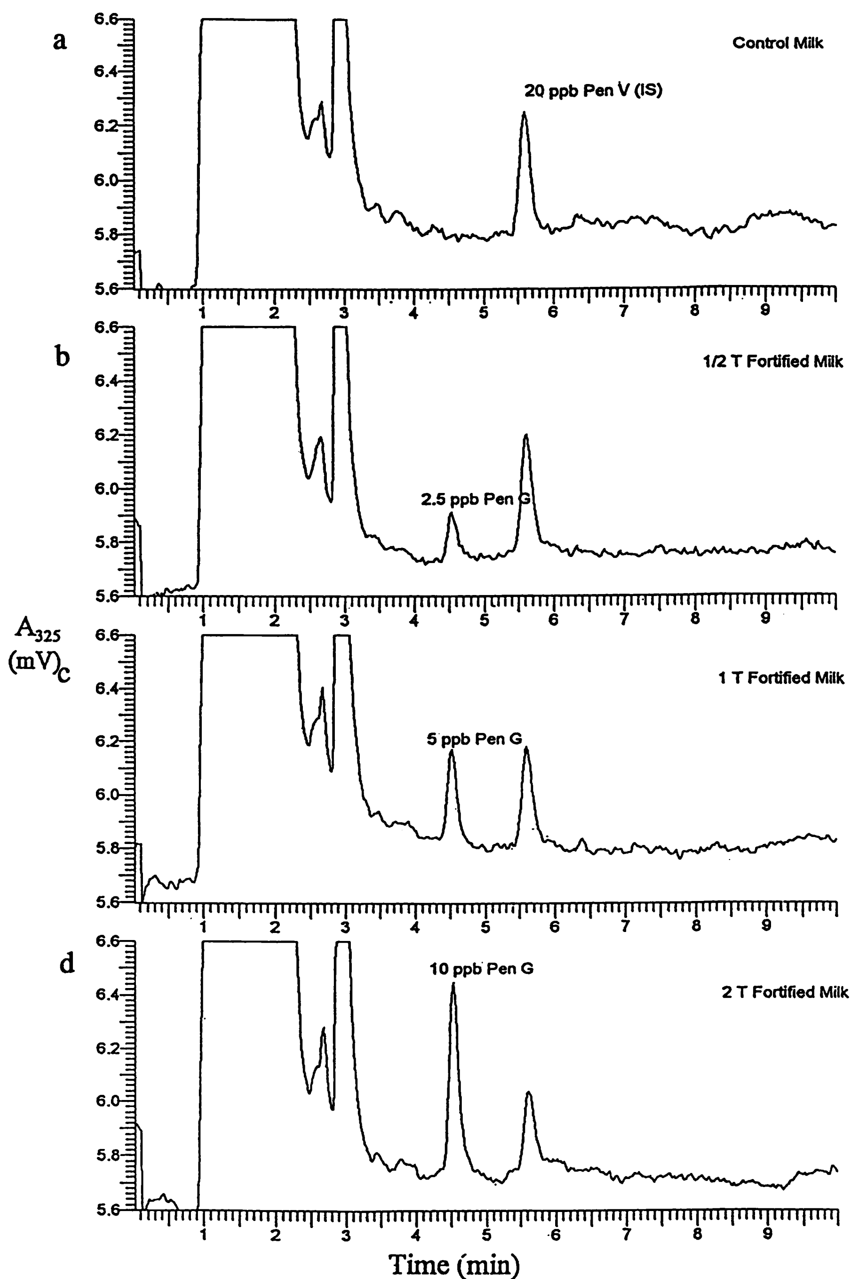


Figure 3. LC determination of derivatized β-lactam residues using modified procedure. a) Control milk with 20 ppb penicillin V as internal standard. b) Fortified milk, 2.5 ppb penicillin G. c) Fortified milk, 5 ppb penicillin G. d) Fortified milk, 10 ppb penicillin G.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

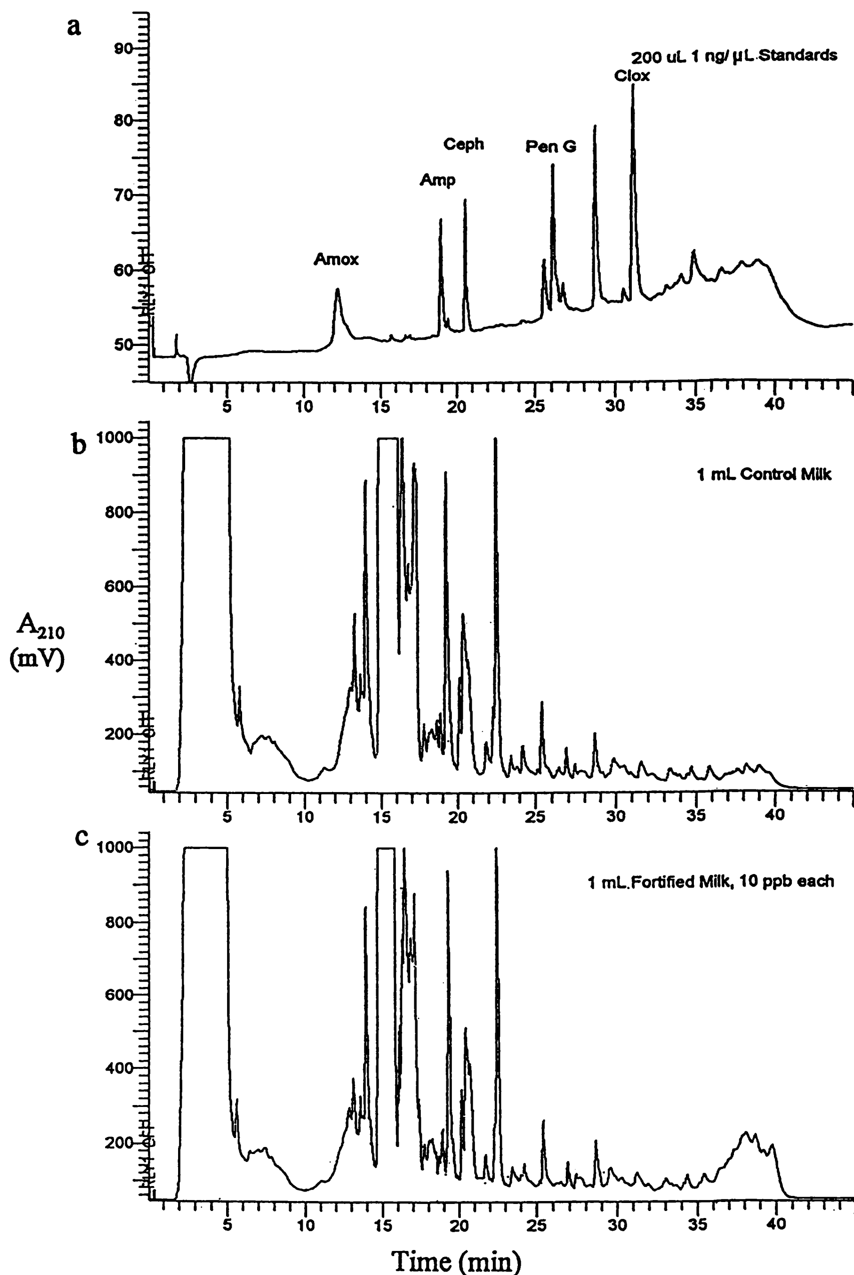


Figure 4. Cleanup HPLC chromatograms. a) External standards used to establish fraction collection windows. b) Control milk extract. c) Fortified milk (10 ppb each) extract.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

gradient LC system used to fractionate the extract. Fractions corresponding to each beta-lactam were collected, concentrated by evaporation, and rechromatographed on a second LC system specific to each residue. This procedure has the potential to determine six different β-lactams. We evaluated its performance for penicillin G, ampicillin, amoxicillin, cloxacillin, and cephapirin.

The initial gradient chromatography is shown in Figure 4. The chromatograms were quite "dirty" and it was impossible to distinguish the fortified milk sample (Figure 4c) from the control milk sample (Figure 4b). Notice also that the absorbance scale, at 210 nm, on these two chromatograms is at its maximum 1000 mV setting. The top chromatogram (Figure 4a), with a scale of 50 mV, was of the 1 ng/μL standards which were used to determine the windows for fraction collection, similar to the receptorgram procedure described above.

Figure 5 shows sample analytical chromatograms for penicillin G quantitation. We had some initial problems with an interference in control milk which prevented quantitation at or below 5 ppb, but these were solved by changing our water source and by changing the mobile phases frequently (Figure 5a). Figure 5b shows an analytical chromatogram of milk fortified at twice the target level, and Figure 5c depicts the 1 ng/μL standard used for single point quantitation in this procedure.

Our results using this procedure are summarized in Table V. The recoveries are all within CVM guidelines, but some of the CVs are high. Cloxacillin and cephapirin analyses were always successful, but amoxicillin's retention time sometimes shifted during the initial gradient chromatography, resulting in poor recoveries for those analyses. The results reported here do not reflect analyses where the recovery was below 20%, which were completely omitted from the statistical calculations.

Table V. Results Using LC Analysis with Automated LC Cleanup

Residue	*Level*	*Fortified Conc. (ppb)*	*Found Conc. (ppb)*	*Recovery (%)*	*CV (%)*	*n*
Penicillin G	½ T	2.5	2.5	99	27	4
	T	5	4.5	90	12	4
	2 T	10	9.5	95	33	4
Amoxicillin	½ T	5	5.4	108	25	2
	T	10	6.6	66	38	3
	2 T	20	11.1	56	7	3
Ampicillin	½ T	5	4.1	81	37	3
	T	10	7.2	72	39	3
	2 T	20	14.9	75	13	3
Cloxacillin	½ T	5	4.7	94	16	4
	T	10	9.8	98	18	4
	2 T	20	19.6	98	8	4
Cephapirin	½ T	10	6.7	67	15	3
	T	20	13.7	68	5	3
	2 T	40	29.3	73	14	3

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

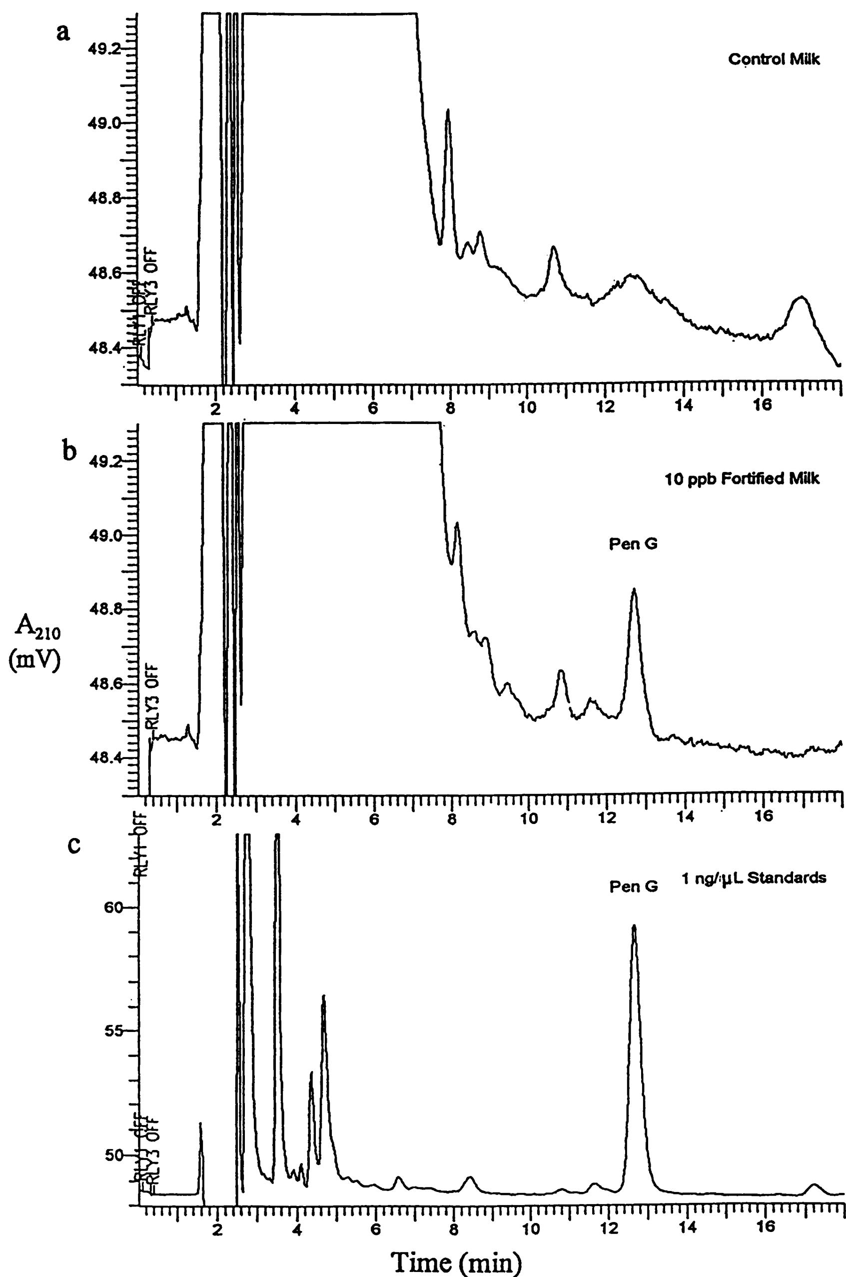


Figure 5. Analytical chromatograms for penicillin G quantitation following HPLC cleanup. a) Control milk. b) Milk fortified with 10 ppb penicillin G. c) Penicillin G external standard, 1 ng/µL, used for single point quantitation.

Conclusions and Recommendations

When choosing or developing an analytical procedure for a particular task, a chemist is ultimately limited to those procedures which can actually **perform** the task. In this instance, the task was to accurately quantitate β-lactam residues in raw milk. Within varying limitations, each of the procedures we evaluated was at least partially capable of performing that task. However, these four procedures varied strongly from each other in aspects that were not necessarily reflected in the performance parameters and statistics listed in Tables II through V. With multiple options for a potential procedure, the chemist should also consider collateral criteria, such as required time and equipment investments, ruggedness of the procedure, and amount and type of hazardous waste generated. Each of the procedures we evaluated had advantages and limitations. Some of the major ones (in several cases reflecting our subjective opinion) are listed in Table VI.

Table VI. Comparison of Collateral Considerations of the Four Procedures

Procedure	*Advantages*	*Limitations*
Receptorgram	Assays all 6 β-lactams Simple extraction Can have an answer on a single unknown on same day	Uses radioactivity Can not quantitate if more than one drug or metabolite present in milk
Meetschen & Petz	History of use in a regulatory setting Good sensitivity	Laborious: 4 samples per day Uses chlorinated solvents and diazomethane Ruggedness problems Can not do amphoterics or cephalosporins
Boison, Keng, & MacNeil	Simple Fast: 12+ samples per day Minimal organic waste	Uses mercuric chloride--generates some mixed waste Stability and sensitivity problems Only assays penicillin G Need to use fortified matrix standard curve
Moats & Harik-Khan	Assays all 6 beta-lactams Adequate sensitivity Some automation possible	Need at least 2 LC systems Must change columns & mobile phases frequently Can take as long as 3 days to get answer Not rugged--requires analyst experienced with procedure Quantitation based on one-point standard (1 ppm)

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

None of the procedures above is an ideal regulatory multiresidue determinative procedure for β-lactam residues in milk. All the procedures have drawbacks; in some instances the limitations are serious enough to eliminate the procedure's potential use in a regulatory setting. The choice is especially narrow for analysis of β-lactam residues other than penicillin G. CVM plans to continue work toward development and /or validation of a practicable multiresidue β-lactam determinative procedure in milk. Final evaluation of potential determinative procedures will include analysis of incurred residue samples (milk from treated cows), more extensive interference and ruggedness testing, and evaluation in a multilaboratory method trial.

Acknowledgments

The following people spent significant time on the phone or with visits from one or another of us while we learned their procedures: Robert Salter and Eliezer Zomer, Charm Sciences, Inc.; Michael Petz, University of Wuppertal, Germany; Joe Boison, Agriculture and Agri-Food Canada; William Moats, U.S. Department of Agriculture.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch012

Literature Cited

(*1*) General Principles for Evaluating the Safety of Compounds Used in Food-Producing Animals. V. Guideline for Approval of a Method of Analysis for Residues. Revised July **1994**. U.S. Food and Drug Administration, Center for Veterinary Medicine.
(*2*) Zomer, E.; Quintana, J.; Saul, S.; Charm, S. E. *J. AOAC Int.* In press.
(*3*) Meetschen, U.; Petz, M. *J. Assoc. Off. Anal. Chem.* **1990**, *vol. 73*, pp. 373
(*4*) Boison, J. O. K.; Keng, L. J.; MacNeil, J. D. *J. AOAC Int.* **1994**, *vol. 77*, pp. 565
(*5*) Moats, W. A.; Harik-Khan, R. *J. AOAC Int.* **1995**, *vol. 78*, pp. 49

Chapter 13

Immunoaffinity Chromatography as a Tool for the Analysis of Antibiotics and Sulfonamides

E. Märtlbauer, R. Dietrich, and E. Usleber

Institute for Hygiene and Technology of Food of Animal Origin, Veterinary Faculty, University of Munich, Schellingstrasse 10, 80799 Munich, Germany

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

Monoclonal antibodies against streptomycin, sulfamethazine, and sulfadiazine were used for the preparation of immunoaffinity chromatography (IAC) columns. The capacity of the resulting columns was approximately 250, 1050, and 1050 ng per column for sulfadiazine, sulfamethazine (sulfadimidine), and streptomycin, respectively. Artificially contaminated milk samples were used for the performance testing of the columns under practical conditions. Between 84.2 and 96.7% of sulfadiazine, sulfathiazole, and sulfamerazine were bound by the column containing the monoclonal antibody against sulfadiazine. 89.8% of the applied sulfamethazine was retained by the respective column. The streptomycin column bound 80.4% and 88.7% of milk samples containing 100 ppb streptomycin and dihydrostreptomycin, respectively. A multi-immunoaffinity chromatography (MIAC) column for sulfonamides bound between 78.6 and 98.7% of a mixture of sulfadiazine, sulfathiazole, sulfamerazine, and sulfamethazine (100 ng each).

Next to the risks for our food supply by the contamination with pathogenic microorganisms, food hygiene is increasingly concerned with risks arising from natural contaminants (bacterial enterotoxins, mycotoxins, seafood toxins) and "man-made" residues of, e.g., pesticides or veterinary drugs (*1*). Particularly due to the frequent use of antibiotics and sulfonamides in veterinary medicine the carry over of these substances into food of animal origin is not unusual. Studies on the occurrence of veterinary drugs (*2, 3*) revealed that penicillines, chloramphenicol, sulfonamides, aminoglycosides and tetracyclines are among the substances most often found in food. For the protection of the consumer, maximum residue limits (MRL) were established for these chemotherapeutics according to EU regulations. These legal requirements and the consumers demand for safe food still cause analytical problems and

0097–6156/96/0636–0121$15.00/0
© 1996 American Chemical Society

require alternative and complimentary methods such as immunochemical techniques.

During the last decade immunoaffinity chromatography (IAC) has become a versatile tool not only for the analysis of contaminants but also for the detection of residues of veterinary drugs. The broad application of this method, however, is still prevented by the limited availability of specific antibodies. This paper describes the properties of monoclonal antibodies against streptomycin, sulfamethazine and sulfadiazine, and their use in immunoaffinity chromatography, particularly for the analysis of milk samples.

Materials and Methods

Standards. Sulfadiazine, sulfamethazine, sulfathiazole, sulfamerazine, streptomycin, and dihydrostreptomycin were purchased from Sigma-Aldrich, Germany.

Monoclonal Antibodies. Monoclonal antibodies against sulfadiazine (SDA III 2G6), sulfamethazine (SMA 14 DP), and streptomycin (STM 4E2) were used throughout this study. The properties of these monoclonal antibodies have been described elsewhere (*4*).

Preparation of Immunoaffinity Columns. Two types of sepharose were employed as the column matrix. CNBr-activated Sepharose 4B (Pharmacia, Germany) was used to bind the monoclonal antibodies against sulfadiazine, sulfamethazine and streptomycin. In addition, activated CH Sepharose 4B (Pharmacia) was tested for coupling the monoclonal antibody against streptomycin. The instructions of the manufacturer were followed throughout the coupling procedure. The amount of antibody used in the coupling procedure corresponds to the amount of antibody bound per ml of sepharose as shown in Table I. Portions (200 μL) of gel were dispensed into disposable minicolumns (Mobitec, Göttingen, Germany).

For the preparation of the multi-immunoaffinity columns for sulfonamides 400 and 150 μL of the sepharose preparations containing the antibodies against sulfamethazine and sulfadiazine, respectively, were mixed and loaded into one minicolumn.

Immunoreagents for Enzyme Immunoassay (EIA). Sheep anti-rabbit immunoglobulin G (Anti-rabbit IgG), purified by immunoaffinity chromatography, was used as described earlier (*5*). The rabbit antiserum against streptomycin and the streptomycin-horseradish peroxidase (HRP) conjugate were used as described previously (*6*). This EIA is specific for streptomycin and dihydrostreptomycin, having relative cross-reactivities of 100% and 150%, respectively. The rabbit antisera against sulfamethazine and sulfadiazine, respectively, and the corresponding enzyme conjugates have been described in detail (*7*). The EIA for sulfamethazine showed relative cross-reactivities of 100% and 56% for sulfamethazine and sulfamerazine, respectively. Therefore, determination of sulfamerazine was possible with the sulfamethazine EIA. The EIA for sulfadiazine was used for the detection of both sulfadiazine and sulfathiazole having cross-reactivities for these substances of 100% and 11%, respectively. The microtiter plates (Maxisorp) were from Nunc GmbH, Wiesbaden, Germany.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

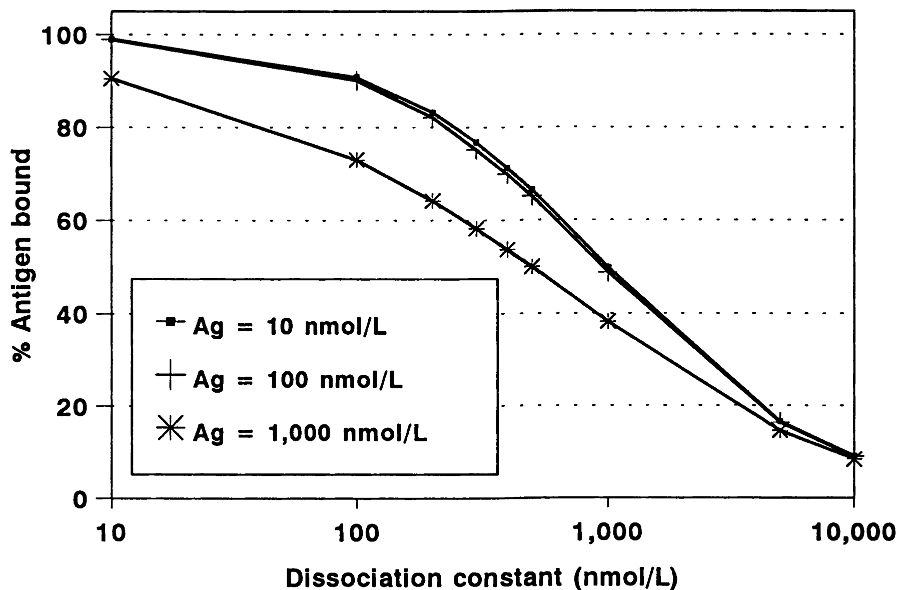
Enzyme Immunoassay for Streptomycin. Microtiter plates were coated with sheep anti-rabbit IgG (10 μg/mL carbonate-bicarbonate buffer [0.05 mol/L, pH 9.6]; 100 μL/well) overnight in a humid chamber. Free protein binding sites of the plate were blocked with phosphate buffered saline (PBS, 0.01 mol/L phosphate buffer containing 0.1 mol/L NaCl, pH 7.3) containing sodium caseinate (20 g/L) for 30 min at room temperature, then the plate was washed three times with Tween 20 solution (0.25 mL per liter of 0.15 mol/L sodium chloride solution). To each well, 35 μL of streptomycin standard or sample extract solution in PBS was added. Then, antiserum dilution (1:6000 in PBS; 35 μL/well) and streptomycin-HRP solution (1:5000 in 1% sodium caseinate/PBS; 35 μL/well) were added and incubated for 2 h at room temperature. The plate was washed again, and substrate/chromogen solution (1 mmol 3,3',5,5'-tetramethylbenzidine and 3 mmol H_2O_2 per liter potassium citrate buffer, pH 3.9 [*8*] was added (100 μL/well). After 15 min, the color development was stopped with 1 mol/L H_2SO_4 (100 μL/well) and the absorbance at 450 nm measured. The tests were evaluated using a competitive EIA calculation software (*9*).

Enzyme Immunoassays for the Sulfonamides. The respective ammonium sulfate-precipitated antiserum was diluted in carbonate-bicarbonate buffer and dispensed (100 μL) into the wells of a microtiter plate. The reciprocal of the dilution was 5000 and 2000 for the antisera against sulfadiazine and sulfamethazine, respectively. The plate was incubated for 18 h at room temperature. Free protein binding sites were blocked with PBS containing 1% casein by incubation for 30 min at room temperature. The plate was washed and made semidry. A 50-μL portion of drug standard solution (in PBS) and 50 μL of the respective hapten-HRP conjugate (0.17 μg sulfadiazine-HRP or 0.34 μg sulfamethazine-HRP, respectively, per mL of PBS containing 1% casein) were added to each well, mixed and allowed to react for 2 h at room temperature. The plate was washed, substrate/chromogen solution was added, and the results evaluated as described above.

HPLC-Procedure for Sulfonamides. HPLC was performed using Waters pumps (model 510), gradient programmer (Waters automated gradient controller model 680) and an UV-detector (LKB 2141, Bromma, Sweden). A column (250 x 4 mm) containing Lichrospher RP-8 (5 μm) material and gradient elution were used for the separation of the sulfonamides. The mobile phase was 10% acetonitrile in phosphate buffer (0.05 mol/L, pH 5.0; solvent A) and pure methanol (solvent B). The run started with 80% solvent A and 20% B for 5 minutes. The flow rate was 1 mL per minute. Then, using a linear gradient for additional 15 minutes, 70% A and 30% B were reached after a total running time of 20 minutes.

Determination of Capacity. For the determination of the capacity of the IACs, 20 mL of PBS containing sulfadiazine, sulfamethazine or streptomycin at a concentration of 100 ng/mL were applied to the respective column equilibrated with PBS, then the column was then washed with 10 mL PBS. The bound antigen was eluted as shown in Table II. The neutralisation buffer for these eluates was carbonate-bicarbonate buffer, 0.05 mol/L, pH 9.6. Quantitative determinations were performed by using the respective EIA.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013



Concentration of antibody binding sites = 1,000 nmol/L

Figure 1. Theoretical binding curves calculated using the equation described by Halfman and Schneider (*11*). Ag is the total concentration of antigen, the total concentration of antibody binding sites is 1,000 nmol/L. The concentration of bound antigen, which is presented in the figure as percentage of the total antigen concentration, is plotted against the dissociation constant. The curves show the results for three different total antigen concentrations.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

Performance Testing. In order to test the applicability of the IACs, all substances listed in Table II and III were diluted in milk. The milk samples were defatted by centrifugation at 4°C (4000 × g for 20 min). The samples were then diluted 1 + 2 with PBS and warmed up to 37°C using a water bath. The disposable minicolumns containing the sepharose-bound antibodies were equilibrated with 10 mL PBS. The diluted milk samples (3−20 mL) were applied to the minicolumns at a flow rate of approximately 3 mL per minute. Then the columns were washed with another 10 mL of PBS. The bound antibiotic or sulfonamide was eluted with either 1.5 mL of glycine/HCl buffer (0.1 mol/L, pH 2.5) or 15% acetone in water (v/v) as outlined in Table II. The multi-immunoaffinity columns were eluted with pure methanol (1.5 mL).

Results and Discussion

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

According to the IUPAC Nomenclature for Chromatography 1993 (*10*), affinity chromatography is characterized as "the particular variant of chromatography in which the unique biological **specificity of the analyte and ligand interaction** is utilized for the separation". If the ligand is a specific antibody, the classification according to the mechanism of separation is "Immunoaffinity Chromatography" and the analyte and ligand interaction represents the reversible association between antibodies and their corresponding antigens. The binding forces involved are weak molecular interactions like Coulomb and Van der Waals forces as well as hydrogen and hydrophobic binding. The antigen-antibody reaction is based on the law of mass action. The most important reaction parameter is the dissociation constant K_d. For the antibodies used in this study the K_d ranged between $10^{-7}-10^{-9}$ mol/L. If K_d is known, theoretical binding curves may be calculated according to the equation described by Halfman and Schneider (11). Figure 1 shows that at a given concentration of antibody binding sites of 1 μmol/L more than 80 % of the antigen are bound, if the dissociation constant is about or below the antigen concentration. The same percentage of 1 μmol of antigen is only bound, if the dissociation constant is tenfold lower. This underlines the importance of antigen binding capacity for IACs and in view of this only monoclonal antibodies were used in this study.

Besides affinity the specificity of the antibodies is another important parameter which is usually determined by testing the binding of substances of similar structure (Figure 2) under the conditions of the competitive EIA. The antibody against streptomycin showed quite good reactivity also towards dihydrostreptomycin (cross-reactivity 85.7%, Table II).

Interesting to note is also the specificity pattern of the antibodies against sulfonamides. The results of the competition studies (Table II) showed that the specificity is determined by the variable heterocyclic residue of the sulfanilamide backbone as the aromatic amino group (N^4) was used for the coupling reaction. The monoclonal antibody against sulfamethazine showed reaction with sulfamerazine, which lacks only one methyl group. Whereas the sulfadiazine antibody showed broader specificity, also binding with sulfathiazole, sulfamerazine and sulfapyridine, which makes this antibody quite useful for MIAC.

For the preparation of the IA-columns we used two types of sepharose as column matrix. The cyanogen bromide activated form, which provides a 1−atom

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

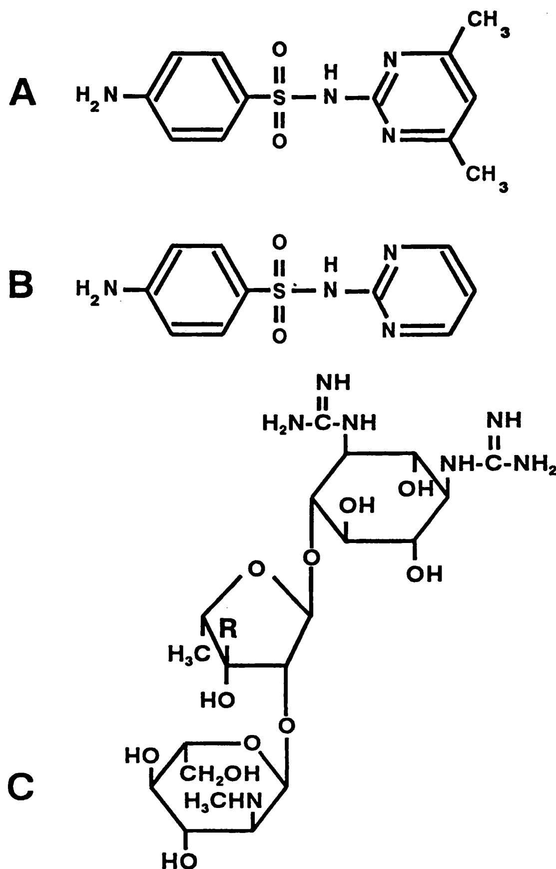


Figure 2: Structures of antimicrobials: (A) Sulfamethazine, (B) Sulfadiazine, (C) Streptomycin (R=CHO) and Dihydrostreptomycin (R=CH_2OH).

spacer, was used for all antibiotics. In the case of streptomycin, also the CH sepharose containing N-hydroxysuccinimide as active ester was tested. Between 1.43 and 6.1 mg of monoclonal antibody were bound per mL of sepharose. The capacity of the columns was measured by EIA and the results are presented in Table I. The capacity range was between 1.27 and 5.25 μg of antigen per mL of gel which corresponds to a calculated specific activity of the antibody between 14.2 and 44.8%. A remaining specific activity of 44.8% represents a good result, but even 14% are acceptable, if the availability of the antibody is not limited. The main reason (14) for the reduced activity is probably inactivation of the antibodies during the coupling procedure due to steric hindrance (coupling near the antigen binding site).

Table I: Capacity of IAC-Columns

Monoclonal antibody for	*Gel*	*Antibody bound per mL gel*	*Antigen bound per mL gel*	*Specific activity*
Sulfadiazine	CNBr-activated Sepharose 4B	1.43 mg	1.27 μg	27.5 %
Sulfamethazine	CNBr-activated Sepharose 4B	3.37 mg	5.25 μg	44.8 %
Streptomycin	CNBr-activated Sepharose 4B	5.09 mg	5.25 μg	14.2 %
	activated CH Sepharose 4B	6.11 mg	4.47 μg	15.5 %

To determine the capacity of the IACs all substances were diluted in buffer (PBS) and applied to the columns. When, however, the substances were added to milk and these milk samples were applied directly to the column less then 60% of the expected amount of analyte was retained (Figure 3). Binding of analyte from milk was, however, similar or even better than from buffer when milk was diluted. This demonstrates the influence of the sample matrix on antigen-antibody binding, a common problem in immunoassays. As expected from the specificity studies, it was found that dihydrostreptomycin was also retained on the column. This result is important for practical reasons since dihydrostreptomycin is preferable used in most therapeutic treatments rather than streptomycin. Based on these experiments all the following trials were performed according to the procedure described under materials and methods. This procedure worked well also with the sulfonamide columns, as presented in Figure 4, when HPLC was used as detection method. The chromatogram shows the analysis of an IAC purified artificially contaminated milk sample containing sulfadiazine, sulfathiazole, sulfamerazine, and sulfamethazine. No matrix peaks have been found under the HPLC-conditions used at the retention time window of the sulfonamides.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

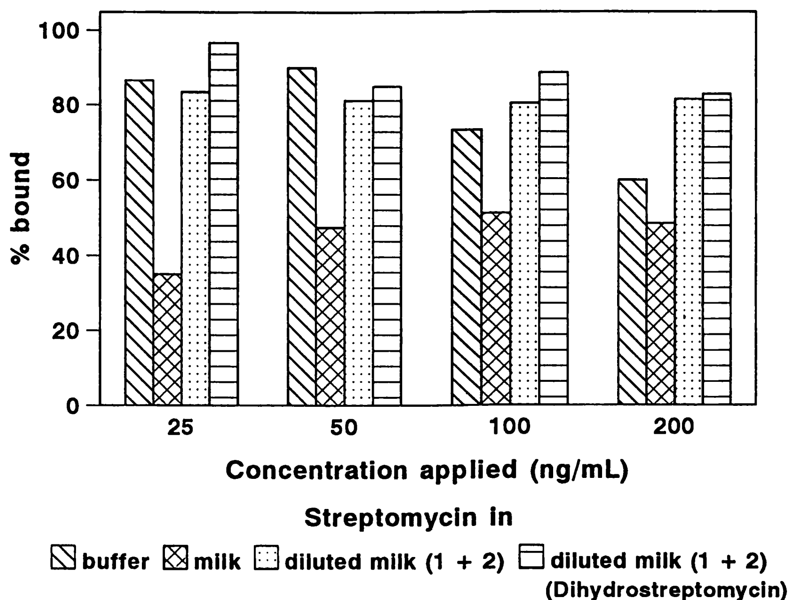


Figure 3. Comparison of the recovery studies using the IAC-column for streptomycin. The x-axis shows the concentrations of streptomycin and dihydrostreptomycin which were applied to the column. The y-axis shows the percentage of antigen retained on the column when buffer, milk or diluted milk were used as "mobile phase".

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

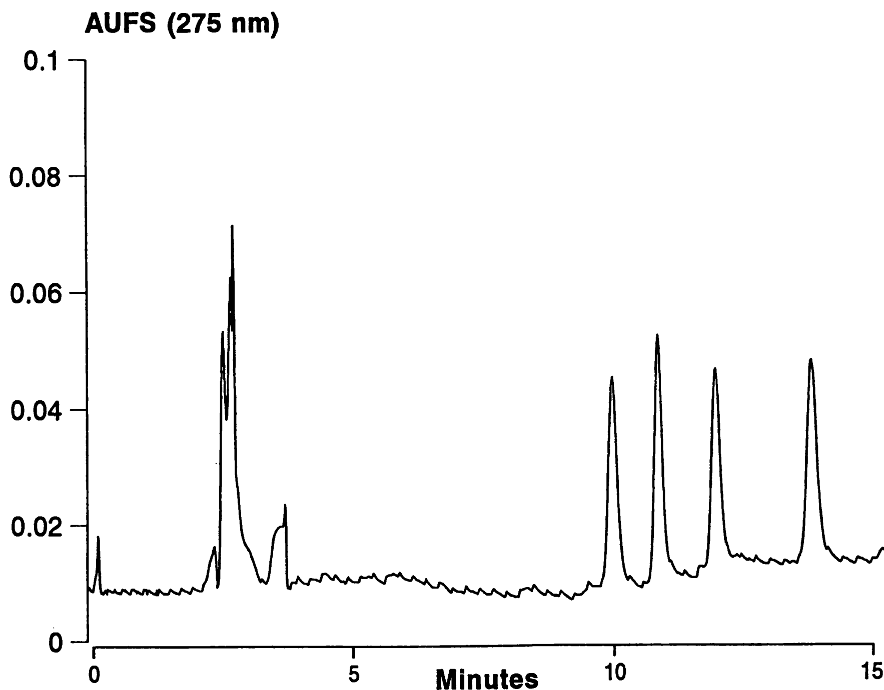


Figure 4. HPLC chromatogram of an artificially contaminated milk sample after IAC-cleanup. The sample contained sulfadiazine, sulfathiazole, sulfamerazine, and sulfamethazine (100 ng/mL each). UV-absorbance was monitored at 275 nm with the detector set to 0.1 AUFS. Retention times were 10.1, 11.05, 12.2, and 14.0 minutes for sulfadiazine, sulfathiazole, sulfamerazine, and sulfamethazine, respectively.

Table II summarizes the performance of IAC-columns if milk samples were analysed. Usually more than 80 % of the single substances were bound with a coefficient of variation below 10 % (n = 3−5).

Table II: Specificity of the Monoclonal Antibodies and Performance of IAC-Columns

IAC-Column	*% Cross-Reactivity*	*Bound (%)*	*Eluted with*
Sulfadiazine	Sulfadiazine 100	96.7	0.1 mol/L glycine/HCl (pH 2.5)
	Sulfathiazole 18.3	84.2	
	Sulfamerazine 16.1	92.3	
Sulfamethazine	Sulfamethazine 100	89.8	15 % acetone in distilled water
Streptomycin	Streptomycin 100	80.4	0.1 mol/L glycine/HCl (pH 2.5)
	Dihydrostreptomycin 85.7	88.7	

Eluting by using either glycine-hydrochloric acid buffer or 15 % acetone enabled the repeated use − up to 30 times − of the columns. Combining both sulfonamide antibodies in one column gave a MIAC for the purification of at least 4 sulfonamides with similar results as for the single columns (Table III).

Table III: Performance of MIAC-Columns for Sulfonamides

Monoclonal antibodies against	*% Cross-Reactivity*	*Bound (%)*	*Eluted with*
Sulfadiazine/ Sulfamethazine	Sulfadiazine 100	96.2	MeOH (100 %)
	Sulfathiazole 18.3	87.0	
	Sulfamerazine 16.1	78.6	
	Sulfamethazine 100	98.7	

Another recent example for the effective use of IAC was the determination of streptomycin in honey. The details of this method have been presented by Usleber et al. (*12*).

The use of immunoaffinity chromatography for sample purification and/or analyte enrichment, combined with an immuno- or physicochemical detection method represents a promising approach in analytical chemistry (*13−16*). If two or more different antibodies are combined, multi-immunoaffinity columns can be produced which have the potential to select groups of substances out of complex samples or sample extracts. This technique may be applied when a group of structurally different substances, which cannot be covered by a single antibody, has to be detected. Considering the number and variety of drugs used in veterinary medicine,

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

this approach my in practice even be the only possibility to fulfill legal requirements in the future. If a high capacity of the columns is desireable, monoclonal antibodies offer certain advantages also in terms of reproducibility.

What is needed for the future is more effort from the commercial side in the development of IACs or MIACs for residues of veterinary drugs in food, in order to improve and simplify our analytical methods.

Acknowledgments

We thank B. Minich and M. Straka for excellent technical assistance.

Literature Cited

1. Großklaus, D. In *Rückstände in von Tieren stammenden Lebensmitteln*, ed. Großklaus, D., Paul Parey, Berlin, **1989**.
2. Bergner-Lang, B.; Edelhäuser, M.; Klein, E.; Maixner, S.; Malisch, R.; Pletscher, D. *Fleischwirtsch.* **1989**, *69*, 524–528.
3. Suhren, G.; Hoffmeister, A.; Reichmuth, J.; Heeschen, W. *Milchwiss.* **1990**, *45*, 485–490.
4. Märtlbauer, E.; Usleber, E.; Schneider, E.; Dietrich, R. *Analyst* **1994**, *119*, 2543–2548.
5. Märtlbauer, E.; Gareis, M.; Terplan, G. *Appl. Environ. Microbiol.* **1988**, *54*, 225–230.
6. Schnappinger, P.; Usleber, E.; Märtlbauer, E.; Terplan, G. *Food Agric. Immunol.* **1993**, *5*, 67-73.
7. Märtlbauer, E.; Meier, R.; Usleber, E.; Terplan G. *Food Agric. Immunol.* **1992**, *4*, 219–228.
8. Gallati, H.; Pracht, J. *J. Clin. Chem. Clin. Biochem.* **1985**, *23*, 453–460.
9. Märtlbauer, E. In *Enzymimmuntests für antimikrobiell wirksame Stoffe.* Ferdinand Enke Verlag, Stuttgart, **1993**; pp 206–222.
10. IUPAC, Nomenclature for Chromatography. *Pure Appl. Chem.* **1993**, *4*, 819–872.
11. Halfman, C. J.; Schneider, A. S. *Anal. Chem.* **1981**, *53*, 654–658.
12. Usleber, E.; Dietrich, R.; Märtlbauer, E.; Unglaub, W. BTEC 073, 209th ACS National Meeting, Anaheim, USA, April 2-6, **1995**
13. Dietrich, R.; Usleber, E.; Terplan, G. *107th Annual AOAC International Meeting*, **1993**, Washington, D.C.; p 143.
14. Katz, S. E.; Siwierski, M. *J. Chromatogr.*, **1992**, *624*, 403–409.
15. Van de Water, C.; Haagsma, N. *J. Chromatogr.*, **1991**, *566*, 173–176.
16. Haasnot, W.; Schilt, R.; Hamers, A. R. M.; Huf, A.; Farjam, A.; Frei, R. W.; Brinkman, U. A. T. *J. Chromatogr.*, **1989**, *489*, 157–160.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch013

Chapter 14

Particle Concentration Fluorescence Assays for Rapid Detection of Trace Levels of Antibiotics

Marjorie B. Medina

Eastern Regional Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

New approaches for detection of spectinomycin and penicillin G are presented in these studies. An aminoglycoside-binding protein (ABP) immobilized on polystyrene latex particles and spectinomycin labeled with fluorescein isothiocyanate (FITC) were utilized for the spectinomycin assay. A penicillin binding protein (PBP) labeled with FITC, and a betalactam covalently bound to particles were used for detection of penicillin G. The antibiotics in the samples were pre-incubated with the binding proteins prior to addition of spectinomycin-FITC or betalactam-particles. The excess reagents were drained and after washing the particles, the fluorescent labeled compounds captured by the particles were measured. The assays were designed for detection of spectinomycin at 0-50 ppb (parts per billion) and 0-25 ppb for penicillin G. These techniques can provide rapid and sensitive biochemical methods to detect antibiotics in foods of animal origin.

Rapid methods are needed to screen for the presence of trace levels of specific antibiotics or a class of antibiotics in biological fluids and tissues. Enzyme immunoassay techniques have been used only to a limited extent for detection of veterinary drugs due to lack of sensitivity needed for detection at action levels. The high specificity of immunoassays also limits the number of compounds that can be analyzed compared to a broad spectrum detection obtainable using microbial inhibition assays. Production of antibodies with desired specificities and affinities is time consuming and requires specialized facilities not available to most analysts. Due to these difficulties, the use of proteins with binding properties to antibiotics were explored and characterized. The binding proteins used in this research had been utilized for affinity chromatography (*1*), a rapid assay (*2*) and for screening of betalactams using an enzyme tracer in SNAP test (Idexx Laboratories). In general, the sensitivity of assays can be improved by increasing the surface area for the capture molecules and by improving the signal generating tracer. The use of latex particles provide a larger

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

surface area than microliter wells. In addition, the capture molecule can be covalently attached to latex particles in contrast to passive adsorption onto microliter wells. Higher signals are generated by fluorescence labels than chromophores generated by enzymes and the fluorescent signals are measured directly, thus, eliminating the development step necessary for enzyme labels. In addition, high sensitivity fluorescence detectors for microliter wells have recently become available.

There are very few methods for the analysis of spectinomycin in foods. Chromatographic methods utilizing an ion pair solid phase extraction, HPLC separation followed by post column derivatization and fluorescent detection of its 2-naphthalenesulfonyl chloride (NSCl) derivatives were reported for quantification of spectinomycin. The sensitivity of the liquid chromtographic method was 4 ng per sample load (*3*) and 50 ppb spectinomycin was detectable in swine and chicken plasma (*4*). The official method (*5*) for detection of spectinomycin is a microbial turbidimetric assay (*6*) with an LDL (lowest detectable level) of 2.8 ppm in all tissues of all species. The tolerance level for spectinomycin residue is 100 ppb in uncooked edible tissues in chickens (*7*). Therefore, a rapid method with higher sensitivity than the microbiological turbidimetric assay is needed to rapidly screen for the presence of spectinomycin at ppb levels.

The standard methods for screening and quantitation of betalactams in milk were described by Bishop *et al.* (*8*). The radiolabeled receptor assay and the microbial inhibition disc assay using *Bacillus stearothermophilus var. calidolactis* were used for quantitation of betalactams. The presence of betalactams was confirmed by the hydrolysis of the betalactams with penicillinase. Most regulatory laboratories confirm the presence of betalactams by the *Sarcina lutea* cylinder plate and *Staphylococcus epidermidis* assays. These microbial inhibition assays are slow and cumbersome. A rapid quantitative assay is needed to analyze betalactams in biological samples such as fluid or dry milk and tissue samples. This research presents a new approach for a rapid, sensitive and quantitative method for detection of betalactams.

The objectives of this study were to utilize aminoglycoside binding protein (ABP) and penicillin binding protein (PBP), polystyrene latex particles and fluorescein isothiocyanate as a tracer for the development of biochemical assays for spectinomycin and penicillin G (Figure 1).

Materials and Methods

Materials and Equipment. Fluorescein isothiocyanate, Isomer I, 98% (FITC), Penicillin G and spectinomycin, bovine serum albumin (BSA), Brij and N-ethyl-N[3] (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), were purchased from Sigma. Microtiter wells with membrane bottoms, Fluorescent Concentration Analyzer (FCA) and reader were purchased from Idexx, Inc. (Westbrook, Maine). FITC-labeled penicillin binding protein (PBP-FITC), 5 and 6 membered rings betalactams attached to bovine serum albumin coated on latex particles (BLS-BSA-particle), carboxylated polystyrene latex particles (0.87 μm), sample diluent (phosphate buffered saline) and wash buffer were synthesized and prepared by Idexx, Inc. Tomy Microcentrifuge was obtained from Peninsula Laboratories (Belmont, CA) and the Sonicator was from Heat Systems-Ultrasonics (Plainview, NY);.Buchler vortex evaporator was from Labconco (Kansas City, Missouri); Ultraturrax T25 Homogenizer was from IKA-Works

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

(Cincinatti, Ohio). The swine serum samples were obtained from Hatfield Company (Hatfield, PA).

Spectinomycin Assay. The reagents for spectinomycin assay were prepared using procedures described below. The optimum assay conditions were determined for detection below 50 parts per billion.

Preparation of Reagents. The manufacturer's procedure for ligand immobilization onto the latex particles was modified for the covalent attachment of ABP to the particles. The carboxylated latex particles were dispersed by sonication (10 pulses of 75% duty cycle using power #3). Aliquots of 0.2 ml particles were transferred into 10 ml conical polypropylene tubes. The carboxyls were activated by adding 150 mg EDC and allowed to stand for 15-20 min at room temperature. Two ml of 0.1 mg/ml aminoglycoside binding protein in pH 7, 0.1M phosphate buffer was heated at 37°C for 30 min and added to the activated particles drop by drop while mixing gently. The mixture was incubated at 37°C for 1.5 hr. The derivatized latex (latex-ABP) was centrifuged at 8225 x g (10,000 rpm) for 5 min at 4°C. The supernatant was assayed for protein content and compared with initial protein concentration using a Biorad assay. The latex-ABP was washed twice with 2 ml of pH 7 phosphate buffer and centrifuged at 3000 x g for 10 min. Phosphate buffer (4 ml) containing 0.1% sodium azide and 0.015% Brij surfactant was added to the latex-ABP and the mixture was sonicated with 10 pulses prior to storage at 4°C.

Biorad Assay. Samples and standards (20 µl) were transferred to microtiter wells and 100 ul of Biorad reagent was added. The absorbance was determined after 5 and 10 min at 595 nm. The sample concentration were determined from 0, 0.125, 0.025 0.05, and 0.1 mg/ml of lysozyme standard.

FITC Labeling. Spectinomycin base (0.01 mM) and FITC (0.02 mM) were dissolved separately in 0.5 ml of 10 mM dibasic phosphate buffer adjusted to pH 8.8 with 10 mM monobasic phosphate buffer. The mixture was mixed gently for 2 hours at room temperature prior to storage at 4°C. The labeling was carried out in brown vials or in tubes wrapped with aluminum foil to prevent photodegradation of the fluorescent compounds. Dilutions were made such that 20 µl contained approximately 5 ng spectinomycin-FITC. Completion of derivatization was screened by thin layer chromatography using 10 ml of developing solvent (methanol:chloroform:acetone: ammonium hydroxide; 3:3:3:1). The reaction mixture was diluted in distilled water (1:100) and applied one microliter to TLC plates and developed for 10 min in the round glass bottle (118 ml capacity). The derivatized compound was compared to a 0.1 µM FITC. These TLC conditions were also used to purify spectinomycin-FITC derivatives when derivation was not complete using Whatman Channeled TLC plates. The spectinomycin-FITC bands were scraped and extracted with phosphate buffer, pH 6.

Latex Particle Assay. Binding and competition interactions were assessed. The ABP-particles containing 0.6 µg protein in 10 ml were evaluated for binding with 20 µl spectinomycin-FITC, diluted at 1:10,000 (245 ng/ml), 1:20,000 (122 ng/ml), and 1:40,000 (61 ng/ml) for 30 min. The effects of 2 binding modes (1 step and 2-step

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

binding) were compared. In a 1-step equilibrium binding assay, the labeled and unlabeled spectinomycin were incubated (30 min) simultaneously with the ABP-particle. In a 2-step saturation binding assay, the displacement of the bound spectinomycin-FITC was determined by adding unlabeled spectinomycin (20 µl of 100 ng/ml) after the 30 min incubation with spectinomycin-FITC then the sample was further incubated for an additional 30 min.

Dose response in buffer and biological samples were determined. Spectinomycin standards were prepared in 10 mM phosphate buffer with 0.1 % BSA, pH 6.5 containing 0, 5, 10, 25, 50 and 100 ppb spectinomycin solid. The swine serum samples (1 ml) were deproteinized with an equal volume of acetonitrile and centrifuged at 3000 x g. The acetonitrile in the supernate was evaporated using a vortex evaporator for 20 minutes. The volumes were adjusted to 0.5 ml with deionized water and 0.5 ml of 10-100 ppb of spectinomycin standards in phosphate buffer were added. Twenty microliters of standards and spiked serum were transferred to the Idexx microtiter wells. The latex ABP-particles were added and the mixtures were pre-incubated for 20-30 min. The tracer, spectinomycin-FITC, (20 µl) was added and the mixture was incubated for 30-45 min. The excess reagent was evacuated with 25 mm Hg for 30 sec. The wash reagent, 50 µl of phosphate buffer, pH 6.5 containing 0.015% Brij was manually dispensed onto the wells and the buffer was evacuated in the FCA analyzer. All the wells must be free from liquid prior to reading at 485 nm emission and 520 nm excitation. This procedure is illustrated in Figure 2.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

Penicillin G Assay. The dose of penicillin G that resulted in a minimum and maximum binding in buffer and biological samples was determined using the procedure described below.

Preparation of Standards and Samples. Penicillin G standards were prepared at 0, 6.25, 12.5, 25, 50, 100, 1000 ppb in Idexx sample diluent with an added 1% BSA. Raw milk samples were centrifuged at 3000 x *g* (15 min) and penicillin G standards were added to the skimmed milk at 0, 2.5, 5, 10, 20 and 50 ppb. A calf kidney was macerated with a mini food processor and a 5 gram aliquot was suspended in the Idexx diluent buffer (PBS) and homogenized by Ultraturax for 2 min. An additional 5 ml diluent buffer was added and the sample was further homogenized for 30 s, and centrifuged at 3000 x *g* for 15 min. The clarified kidney extracts were spiked at 0 - 25 ppb.

Latex Particle Assay Protocol. The spiked milk samples, kidney extracts and buffer standards (20 µl) were transferred into duplicate sample wells. Twenty microliter of PBP-FITC were added to each well and the plate was mixed by gently tapping horizontally. The penicillin G in the buffer standards and milk samples were allowed to bind to the fluorescent labeled penicillin binding protein (PBP-FITC) for 10 min. Ten microliters of the betalactams-latex particle complex (BLS-BSA-particle) were added to all wells and the mixture was allowed to incubate the second time for 10 min at room temperature. The BLS-BSA-particle bound to the available PBP-FITC and the excess analytes and other sample components were evacuated from the wells using the vacuum for 30 s. Deionized water (50 ml) was added to wash the latex particle complex and the

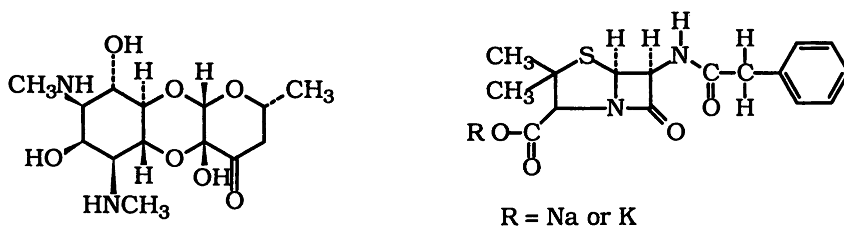


Figure 1. Structures of spectinomycin and penicillin G.

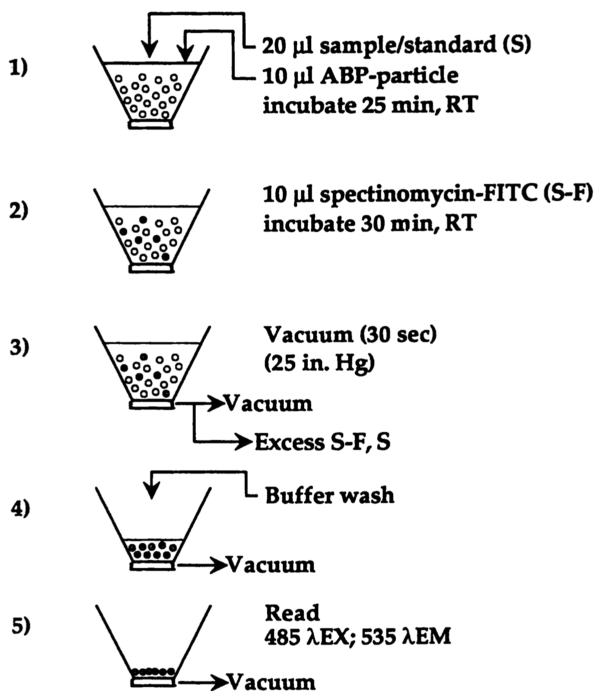


Figure 2. Spectinomycin assay protocol.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

water was evacuated (30 s vacuum). The fluorescence signals of the dried wells were measured at 485 nm excitation and 520 nm emission with a gain setting of 25. This protocol is illustrated in Figure 3.

Results

Spectinomycin. The spectinomycin assay reagents prepared and synthesized at ERRC were evaluated for the development of a competitive binding assay. The evaluation of the binding properties of the ABP-particles and spectinomycin-FITC demonstrated that the FITC-spectinomycin had fluorescent signals that proportionally decreased as the concentration was reduced. The spectinomycin-FITC at 61 ng/ml (1:40,000 dilution) was detectable at 25 x gain. These responses were linear from 4.9 ng (20 µl of 245 ng/ml) to 1.2 ng (20 µl of 61 ng/ml) and indicated that less FITC-spectinomycin was captured by the APB-particles when present at lower concentrations. The addition of 20 ng of spectinomycin to the ABP-particle and spectinomycin-FITC complex resulted in the displacement of the bound FITC-spectinomycin at the higher concentrations of 4.9 ng and 2.45 ng. This displacement or competition for the binding sites was greater (96 %) in a 2-step saturation assay compared to a 1-step equilibrium assay (34%). However, the dilute amount of spectinomycin-FITC (1.22 ng) was not displaced by the unlabeled spectinomycin in a 2-step assay but the unlabeled drug competed for 78% of the binding sites in the 1-step equilibrium assay. These interactions suggest that this

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

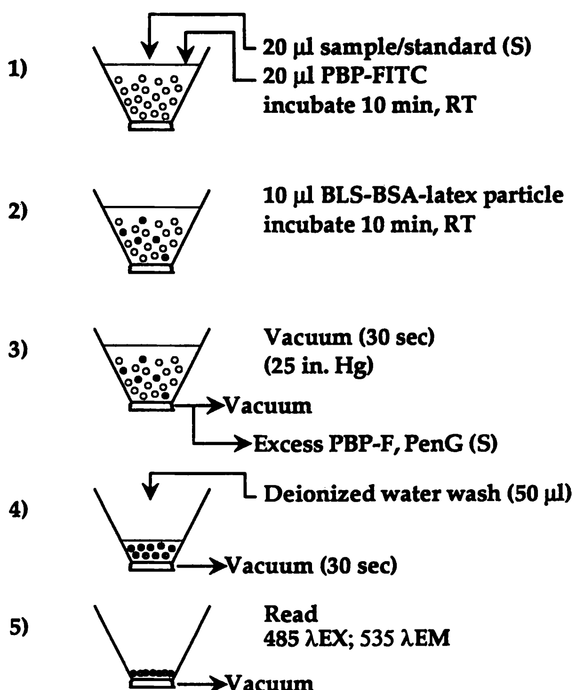


Figure 3. Penicillin G assay protocol.

assay can be used for trace level analysis (below 10 ppb) of spectinomycin using a 2-step assay and utilizing tracer concentrations of 2.5 ng or greater, per test.

The conditions for a spectinomycin assay were optimized. The pH of the binding and wash buffers was optimum between 6 - 6.5. For detection in a range of low parts per billion, each test had a calculated amount of 5 ng spectinomycin-FITC and 60 mg protein equivalent of ABP-particle. When these conditions were used to determine the binding competition of the unlabeled spectinomycin at 0 - 250 ppb, the dose response was curvilinear and typical of saturation binding curves (Figure 4A). A near linear response was indicated at 0 - 50 ppb (Figure 4B). The hyperbolic or curvilinear response of the ligand-binding assays indicated multiple binding sites or heterogeneous binding between spectinomycin and the ABP (9). The hyperbolic plots show that saturation of the binding sites occurred at a concentration where the signal plateau. Therefore, the detection range of the assay was adjusted below the saturation point. Munson and Rodbard (10) developed versatile computer programs for the analysis of nonlinear data generated from ligand-binding interactions. However, a simple linear transformation of the binding assay data was reported by Chase (11, 12, 13), plotting the ratio of total over bound signals (T/B) vs concentration of the analyte. This approach was utilized in radioimmunoassay analysis of estradiol (14) and a mean regression correlation (R^2) of 0.989 was reported. Likewise in this study, the linear transformation of the data in Figure 4A had an R^2 of 0.922 which resulted from plotting T/B vs 0-50 ppb spectinomycin. In Figure 4B, the plot of T/B vs 0-50 ppb had an R^2 of 0.981. The simple approach of data reduction using this linear transformation was suitable when handling small number of samples and when computer programs or software are not available to the analyst.

These ligand-binding assays were carried out in 2 binding modes and again, the results of a 2-step incubation assay showed higher sensitivity and linearity than a 1-step

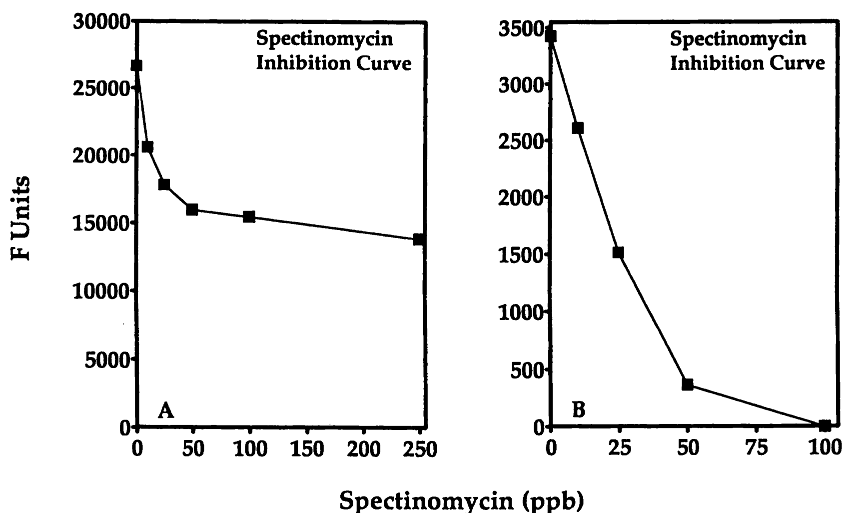


Figure 4. Dose-response curve of spectinomycin in phosphate buffer. A: 0-250 ppb. A second power polynomial line fit resulted an $R^2 = 0.918$ (0 - 100 ppb) and $R^2 = 0.777$ (0 - 250 ppb). B: 0 - 100 ppb. A second power polynomial line fit had an $R^2 = 1.000$ (0 - 100 ppb).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

equilibrium assay. The assay was applied to biological samples. A deproteinized serum spiked at 1-50 ppb had a near linear response with a correlation coefficient (square root of R^2), r = 0.962. The analysis of the homogenate of a liver tissue extracted with buffer, centrifuged and prefiltered was not successful due to clogging of the membranes. The membrane with 1.2 μm pore size and particles with a diameter of 3.6 micron were also utilized. The filtered extracts still resulted in clogged membranes. Therefore, it was concluded that in future studies, the tissue extracts should be deproteinized for analysis by this assay.

Penicillin G. The conditions for the betalactam assay were optimized for minimum and maximum detectability in buffer, spiked milk and kidney tissue extract. The dose response curve of 0-100 ppb penicillin G in diluent buffer resulted in a curvilinear saturation curve (Figure 5) and indicated an analytical capability from 0 - 50 ppb with a linear response from 0 - 25 ppb. An R^2 = 0.951 resulted from a linear transformation of the fluorescence signals into T/B vs 0 - 25 ppb concentration. Initial studies indicated that an addition of 1 % BSA to the sample diluent buffer was necessary as an assay reagent. Without the added BSA, the fluorescent readings were erratic and showed no competition trend. The fluorescence signals of the same concentration of penicillin G in diluent buffer resulted in 30-40% higher fluorescence signals than in a spiked defatted milk. The lower fluorescent signals in the milk sample indicated that compounds

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

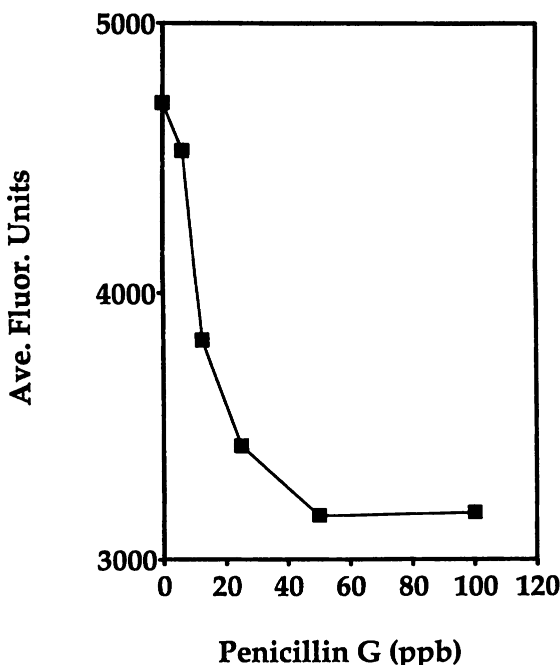


Figure 5. Dose-response curve of penicillin G in phosphate buffer.

blocked the binding of the BLS-BSA-latex with the PBP-FITC. These compounds could either bind with the PBP or the immobilized betalactam groups. The interference by the sample matrix can be corrected by using spiked milk or incurred milk with a known betalactam concentration as a calibration standard. This phenomenon is not unique to this assay as this matrix effect had been reported for the detection of sulfamethazine by ELISA assays in milk (15).

Raw milk was centrifuged to remove the particulates and milk fat which interfered with the membrane filtration. The defatted and clarified milk was analyzed after the addition of penicillin G. The fluorescence signals from the particle bound FITC-PBP was plotted against 0 - 50 ppb penicillin G in phosphate buffer. A typical calibration curve is shown in Figure 6. Results from 6 analyses (Table 1) showed that the calibration lines (fitted with a second power polynomial equation) had a mean regression correlation (R^2) of 0.945. The linear transformation of these data, plotted as T/B vs concentration showed regression correlations of R^2 = 0.911 for 0 - 50 ppb and R^2 = 0.950 for 0 - 20 ppb concentrations. As typical of ligand-binding assays, the linear fit improves with the detection range selected below the saturation points of the binding sites, i.e. the plateau in the calibration curve. In this study, the range of 0 - 50 ppb is the concentration range of interest for detection of these antibiotics.

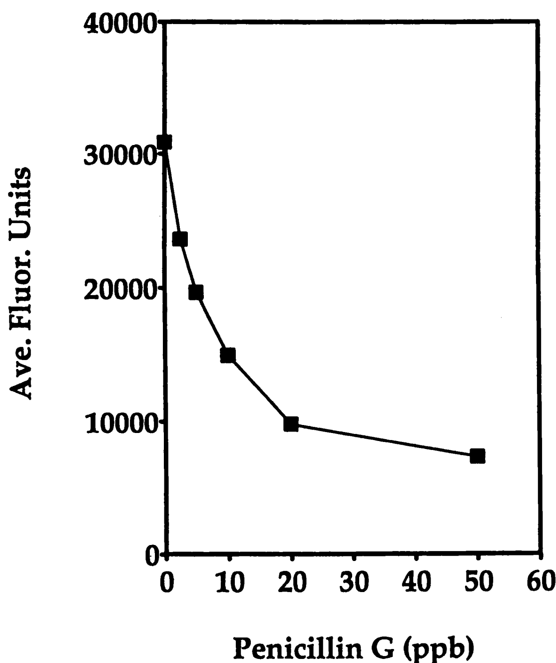


Figure 6. Dose-response curve of penicillin G added to defatted milk.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

Table 1. Linear Transformation of the Dose-Response Data Plotted as the Ratio of Total Over Bound Fluorescence (T/B) vs Dose[a]

Trial Number	R^2 Curvilinear Fit	T/B vs 0 - 50 ppb	R^2	T/B vs 0 - 20 ppb	R^2
1[b]	0.909	Y=1.115 + 0147X	0.853	Y=1.058 + 0.0198X	0.892
2	0.966	Y=0.671 + 0.1099X	0.982	Y=0.704 + 0.1046X	0.881
3	0.977	Y=0.890 + 0.1370X	0.981	Y=0.875 + 0.1346X	0.968
4	0.956	Y=1.289 + 0.0640X	0.932	Y=1.021 + 0.1062X	0.999
5	0.888	Y=1.515 + 0.0774X	0.882	Y=1.095 + 0.1437X	0.976
6	0.976	Y=1.327 + 0.0622X	0.834	Y=0.909 + 0.1283X	0.982
Mean	0.945		0.911		0.950

[a]Penicillin G was added to defatted milk at 0, 2.5, 5.0, 10, 20 and 50 ppb.
[b]This assay utilized the first batch of PCFIA (Particle Concentration Flourescence Immunoassay) reagents. Pencillin G was added at 0, 6.25, 12.5, 25, 50 and 100 ppb.
R^2 = regression correlation.

The analysis of spiked kidney extracts resulted in a quantifiable detection of 1.25 - 25 ppb penicillin G (Figure 7). This dose-response curve fit had a correlation coefficient of 0.888. With further refinement of the sample preparation, this correlation can be increased. The kidney extract contained colloidal proteins which blocked the membranes, thus, preventing drainage of the wells and resulted in erratic results. As in the spectinomycin assay, there is a need to deproteinate the samples prior to analysis. This process might entrap the drug with the precipitate or release the drug from their weakly bound drug-protein complex in the biological system, thus, making the drug available for analysis. The effect of this precipitation process will be assessed in future studies.

Conclusions

These studies showed that the particle concentration fluorescent assay utilizing binding proteins with selectivity for some drugs can be used for trace level analysis of veterinary drug residues. The assay showed a sensitivity below 5 ppb (ng/ml) and can be utilized for quantitative analysis. Forty sample extracts can be analyzed in duplicate in 30-60

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

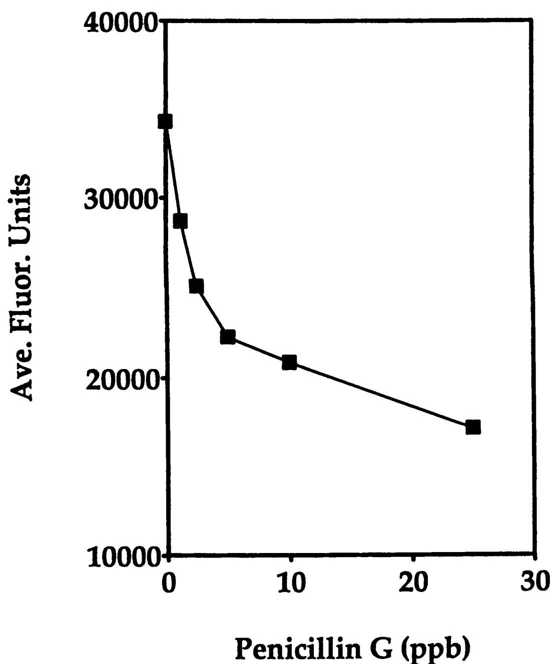


Figure 7. Dose-response curve of penicillin G added to kidney extract.

min. These assays are simple and rapid with a high throughput and can bridge the gap between microbial inhibition assays and chromatographic analysis.

Literature Cited

1. Campbell, N.F.; Medina, M.B. Affinity Clean-Up of Hygromycin B for Chromatographic Analysis. 1993. Abstract. Paper No. 27. 205th American Chemical Society Meeting, Denver, Colorado.
2. Medina, M. B. "A Competitive Protein Binding Assay for Spectinomycin Using Particle Concentration Fluorescent Assay". Abstract paper No. 28-024. 108th AOAC International Meeting, Portland, Oregon, **1994**.
3. Tsugi, K.; Jenkins, K.M. "Derivatization of Secondary Amines With 2-Naphthalene-Sulfonyl Chloride For High Performance Liquid Chromatographic Analysis Of Spectinomycin". *J. Chromatography,* **1985,** *333,* 365-380.
4. Haagsma, N; Keegstra, J.R.; Scherpenisse, P. "High-Performance Liquid Chromatographic Determination of Spectinomycin in Swine, Calf and Chicken Plasma". *J. Chromatography, Biomedical Applications.* **1993,** *615,* 289-295.
5. FSIS, Compound Evaluation and Analytical Capability National Residue Program Plan. Science and Technology, Food Safety and Inspection Service, U. S. Department of Agriculture, 1993. Washington D.C.
6. NADA 47-244, New Animal Drug Application, Upjohn Company, Kalamazoo Michigan.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

7. Code of Federal Regulation. 1993. Title 21, Section 556.600
8. Bishop, J.R.; Senyk, G.F.; Duncan. S.E. "Detection of Antibiotic/Drug residues in milk and Dairy Product," In: *Standard Methods for the Examination of Dairy Products.* Marshall, R.T., Ed.; 16th Edition; 1992; pp. 347-395.
9. Attie, A.D.; Raines, R.T. Analysis of Receptor-Ligand Interactions. *J. Chemical Education.* **1995,** *72,* 119-124.
10. Munson, P.J.; Rodbard, D. Ligand: A Versatile Approach for Characterization of Ligand-Binding Systems. *Anal. Biochem.* **1980,** *107,* 220-239.
11. Chase, G. Some Concepts of RIA Theory, Data Reduction, and Quality control. I. Basic Approaches to Data Reduction. Ligand Quarterly. **1979,** *2(3)* 25-28.
12. Chase, G. Some Concepts of RIA Theory, Data Reduction, and Quality Control. II. The "Ideal" RIA. Ligand Quarterly. **1979,** *2(3),* 29-22.
13. Chase, G. Ligand Quarterly. Some Concepts of RIA Theory, Data Reduction, and Quality Control. I. The Scatchard Plot and The Equilibrium Model. Ligand Quarterly. **1979,** *2(34),* 30-33.
14. Medina, M.B.; Direct radioimmunoassay of 17β- estradiol in ether extracts of bovine sera. J. Agricultural and Food Chemistry. **1986,** *34,* 1046-1049.
15. Medina, M.B.; Barford, R.A.; Palumbo, M.S., Rowe, L.D. Evaluation of Commercial Immunochemical Assays for Detection of Sulfamethazine in Milk. J. Food Protection. **1992,** *55,* 284-290.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch014

Chapter 15

Determination of Veterinary Drugs in Dry Milk Powder by Supercritical Fluid Extraction–Enzyme-Linked Immunosorbent Assay

Viorica Lopez-Avila and Janet Benedicto

Midwest Research Institute, California Operations, 625-B Clyde Avenue, Mountain View, CA 94043

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch015

The objective of this study was to establish whether supercritical fluid extraction (SFE) coupled with enzyme-linked immunosorbent assay (ELISA) is a viable technique for the determination of sulfamethazine in dry milk powder at concentrations as low as 2.5 ng/g. Extraction and analysis of organic compounds in various matrices by SFE-ELISA has been under evaluation in our laboratory since it results in greater sample throughput, allowing rapid screening of environmental samples. In this study, we demonstrated that sulfamethazine could be extracted quantitatively (average recovery at 5-ng/g spike was 91.7 percent) from spiked dry milk powder at 450 atm and 80°C using supercritical carbon dioxide modified with 10 percent methanol as the extraction fluid. The extracted material was collected in methanol, concentrated to dryness, and redissolved in phosphate buffer for analysis by a competitive ELISA. The precision of the ELISA technique, as established over a period of 17 days, was 15 percent or better for sulfamethazine concentrations ranging from 5 to 15 ng/mL of extract in phosphate buffer.

Analytical scale SFE has gained popularity among the conventional sample preparation techniques such as Soxhlet and sonication extraction because (a) it requires much less solvent for extracting the compounds of interest from the solid matrix, (b) is fast, (c) is selective, and (d) allows easy removal and disposal of the extraction solvent. The most common fluid in SFE is carbon dioxide, but small amounts of modifiers (e.g., methanol, acetonitrile, methylene chloride) can be added to supercritical carbon dioxide to make it more polar. Recently, we have seen a continuous increase in the number of publications dealing with analytical SFE and the number of compounds that can be extracted by SFE. For these reasons, we have undertaken this study to evaluate SFE as a potential extraction technique for sulfamethazine from powdered milk.

0097–6156/96/0636–0144$15.00/0
© 1996 American Chemical Society

A study by Malik et al. (1) concluded that sulfamethazine could be extracted from 10-g samples of dry milk powder with supercritical carbon dioxide at 50°C and 390 atm; however, to achieve quantitative extraction efficiency the extraction time had to be extended to 2 hrs (the concentration of sulfamethazine in dry milk powder was 163 ng/g). Extraction of sulfonamides from food animal products (e.g., liver, swine muscle), fortified chicken tissue, and various solid supports (e.g., diatomaceous earth, silica gel) using carbon dioxide and carbon dioxide modified with methanol have been reported (2-4). To enhance the yield of sulfonamides extracted from solid supports with supercritical carbon dioxide, Tena and coworkers (4) used 0.1 M trimethylphenylammonium hydroxide in methanol, as ion-pair reagent, and extraction with supercritical carbon dioxide at 40°C and 280 atm.

Another technique that is also gaining momentum, especially in environmental analysis, is ELISA. The most common format in enzyme immunoassays is competitive ELISA. In this type of assay, the antibodies specific to the analyte to be detected are immobilized (coated) onto a solid phase, either a plastic tube, a well on a microtiter plate, or paramagnetic particles. The enzyme-hapten conjugate and the target analyte are added to the antibody-coated tube or plate for a short incubation (15 to 30 min) during which time the target analyte and the enzyme-hapten conjugate compete for antibody binding sites. After incubation, the unbound material is removed, and a substrate-chromogen solution is added. After another short incubation period during which the enzyme converts the substrate-chromogen to a colored product, the reaction is terminated and the absorbance is measured with a spectrophotometer.

The ELISA is attractive because it (a) allows high sensitivity and high degree of selectivity in antibody binding, (b) is relatively cheap, and (c) has potential for field use. There are, however, disadvantages to ELISA that need to be presented. The development of the ELISA technique is lengthy (i.e., takes 8 to 12 months), (b) the ELISA specificity limits its use to analysis of only one or a few closely related compounds, (c) the detection range is very narrow, and (d) occasionally ELISA shows too many false positives or fails to detect the analyte (5).

This paper will present the extraction of sulfamethazine from dry milk powder with supercritical carbon dioxide modified with methanol and detection of sulfamethazine by ELISA.

Materials and Methods

Reagents. All immunological reagents used in this study—including reaction tubes coated with anti-sulfamethazine antibody, horseradish peroxidase—sulfamethazine conjugate (traces), phosphate buffer (20 mM, pH 6+0.2) 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide in citrate buffer (color developer), and dilute sodium dodecyl sulfate (stopping solution) were obtained from Idetek, Inc. (Sunnyvale, CA). Details on the composition of the various reagents are not available (proprietary information). Sulfamethazine was obtained form Sigma Chemical Co. (St. Louis, MO). The dry milk powder used in this study was Carnation Nonfat milk and was purchased from a local supermarket.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch015

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch015

Table I. Optimization of SFE Conditions for Sulfamethazine

Parameter	*Percent Recovery*
300 atm/60°C/30 min dynamic/carbon dioxide	0
450 atm/80°C/30 min dynamic/carbon dioxide	0
450 atm/120°C/30 min dynamic/carbon dioxide	Recovery not determined due to high background
450 atm/80°C/30 min dynamic/carbon dioxide with 10 percent methanol (spiked at 5 ng/g)	91.7 ± 17 (no TEA) (n=4) 105[a] (with 20 µL TEA) 68.1[a] (with 40 µL TEA)
350 atm/80°C/30 min dynamic/carbon dioxide with 10 percent methanol (spiked at 5 ng/g)	21 (n=2)
250 atm/80°C/30 min dynamic/carbon dioxide with 10 percent methanol (spiked at 5 ng/g)	13 (n=2)

[a]Single determinations.

Table II. ELISA Performance (Sulfamethazine)

Standard conc. (ng/mL)	*Measured concentration (ng/mL)*									*Percent RSD*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*Ave. ± SD*	
0	0	0	0	0	0	0	0	0	0	—
5	4.96	4.27	4.72	4.87	4.95	5.14	5.22	5.04	4.9 ± 0.3	6.0
7.5	—	—	5.91	6.96	7.18	8.72	7.54	6.07	7.1 ± 1.0	15
10	11.3	8.67	12.0	8.64	10.6	9.38	10.2	9.75	10.1 ± 1.2	12
15	12.7	11.3	12.2	—	—	—	—	—	12.1 ± 0.7	5.9

SFE Procedure. The SFE extractions were performed with an Isco SFX 3560 (Isco, Inc., Lincoln, NE) automated extractor (24 vessels) at 450 atm and 80°C for 30 min in the dynamic mode. The flow rate of the carbon dioxide (SFE/SFC grade carbon dioxide, Air Products, Allentown, PA) modified with 10 percent methanol was approximately 1.5 mL/min. This flow was maintained using a stainless steel coaxially heated capillary restrictor (temperature 80°C). The extracted material was collected in methanol (1 mL initial volume with 0.5 mL additional volume added at 10 min intervals during extraction). After extraction, the methanol extracts were concentrated to dryness, and the extract residue was redissolved in 1 mL phosphate buffer immediately prior to ELISA. All SFE experiments were performed with 2-g portions of the powdered milk, which was spiked with sulfamethazine at 2.5 or 5 ng/g. The spike was added to the powdered milk in 50 or 100 µL of 100-ng/mL solution of sulfamethazine in methanol.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch015

ELISA Procedure. For ELISA, 250 µL of the dry milk powder extract and 250 µL of the tracer solution were added to each reaction tube and were incubated for 3 to 4 min at room temperature (the tubes were kept on a mechanical shaker during the incubation period). Following incubation, the reaction tubes were washed at least 6 times with the wash solution (saline and surfactant) provided with the Idetek kit. Solution 2 (color developer, 500 µL) was then added to each tube and the tubes incubated for an additional 3 to 4 min. The reaction was terminated by the addition of 500 µL of the stopping solution (sodium dodecyl sulfate). Spectrophotometric analysis of the final colored product was performed using the Ohmicron RPA-1 photometric analyzer (Ohmicron Corporation, Newtown, PA) set at 405 nm. The observed sample results were compared to a linear regression line using a log-logit standard curve prepared from analysis of calibration standards at 0, 5, 7.5, and 10 ng/mL. An additional standard at 15 ng/mL was evaluated for calibration; however, it was found to exceed the linear range of the assay.

Results and Discussion

Preliminary experiments conducted with supercritical carbon dioxide at 350 atm/60°C and 450 atm/80°C using a 30-min extraction time indicated that sulfamethazine could not be extracted from the dry milk powder when spiked at 5 ng/g. Extraction with supercritical carbon dioxide at 450 atm/120°C resulted in a high background and, thus, inconclusive results by ELISA. Use of carbon dioxide modified with 10 percent methanol resulted in quantitative extraction (average recovery of 91.7 ± 17 percent) of sulfamethazine from dry milk powder spiked at 5 ng/g (Table I). Additional experiments performed with modified supercritical carbon dioxide at lower pressures (e.g., 350 atm and 250 atm) indicated poor recovery of sulfamethazine when spiked at 5 ng/g. Furthermore, addition of triethylamine (TEA), as a matrix modifier, had no effect upon recovery when 20 µL of neat TEA were added, but lowered the recovery to 68 percent when 40 µL of neat TEA were used in the extraction; therefore, we concluded that there was no need to use a matrix modifier.

The linearity of the ELISA was verified with standards at 0, 5, 7.5, 10, and 15 ng/mL. As shown in Table II, the 15-ng/mL standard exceeded the linear range.

The calibration standards were analyzed over a period of two weeks; the percent RSDs shown in Table II indicate that method precision is within 15 percent.

The data presented in Table III indicate no background interferences at 1 ng/g in the method blank and acceptable recoveries for the blowdown evaporation step. Also shown in Table III is method performance for sulfamethazine spiked into dry milk powder at 2.5 ng/g. Based on these data, we concluded that sulfamethazine can be recovered from dry milk powder by supercritical fluid extraction and that analysis by ELISA allows detection of sulfamethazine concentrations as low as 2.5 ng/g.

Work in progress in our laboratory is addressing other veterinary drugs including oxytetracycline and penicillin G. Preliminary experiments indicate that the former compound cannot be extracted from the spiked dry milk powder by SFE with supercritical carbon dioxide alone or supercritical carbon dioxide modified with methanol using in-situ derivatization with trifluoroacetic anhydride. The latter compound is a β-lactam and is not stable in methanol; therefore, another modifier is being investigated for this purpose.

Table III. Verification of SFE/ELISA Procedure

Step	*Percent recovery*
Method blank	ND (detection limit 1 ng/g)
Methanol spiked at 10 ppb, blowdown evaporation, and reconstitution in buffer	96.1 (n=2)
Nonfat powdered milk spiked at 2.5 ng/g	63.7 ± 2.8 (n=3)

Acknowledgment

The authors wish to thank Les Myers of Isco, Inc., for making the Isco SFE-3560 available for use in this study, and Idetek Corporation for donating the ELISA test kits.

Literature Cited

1. Malik, S., Duncan, S. E., Bishop, J. R., and Taylor, L. T., *J. Dairy Sci.* **1994** *77*, 418.

2. Cross, F. R., Ezzell, J. L., and Richter, B. E., *J. Chromatogr. Sci.* **1993** *31*, 162.

3. Parks, O. W., and Maxwell, R. J., *J. Chromatogr. Sci.* **1994** *32*, 290.

4. Tena, M. T., Luque de Castro, M. D., and Valcarcel, M., *Chromatographia* **1995** *40*, 197.

5. Lopez-Avila, V., Charan, C., and Beckert, W. F., *TRAC* **1994** *13*, 118.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch015

Chapter 16

High-Performance Liquid Chromatography–Receptorgram: A Comprehensive Method for Identification of Veterinary Drugs and Their Active Metabolites

E. Zomer, J. Quintana, J. Scheemaker, S. Saul, and S. E. Charm

Charm Sciences Inc., 36 Franklin Street, Malden, MA 02148

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

The HPLC-receptorgram combines the advantages of HPLC separation with the multiresidue detection of the Charm II tests (CT II). The HPLC-receptorgram was first introduced in 1987 to fill a gap in determinative methodology that was discovered by the introduction of the microbial receptor assay for sulfonamides, a Charm II Test. The procedure has been tested for identification and quantitation of the most common veterinary drugs at regulatory levels or lower. It has been validated for over 40 individual drugs from 7 antibiotic families: 10 ß-lactams, 13 sulfonamides, 8 tetracyclines, 4 macrolides, 3 amphenicols, and other miscellaneous drugs.

The procedure combines a simple aqueous extraction buffer, solid phase clean-up and concentration step with HPLC fractionation of individual drugs. Final identification and quantitation is achieved with the CT II . In less than 3 hours a drug contaminant can be identified. The method provides identification of new drugs, drug components or their metabolites. For example, when wide spread contamination with sulfa drugs in milk was detected in 1987, sulfamethazine was the most common contaminant identified by this method. In the next few years, five other sulfonamides appeared although widespread sulfa drug contamination disappeared (1). Ceftiofur and its active metabolite were among the most frequently identified residues in suspected bulk milk samples in 1994. When chlortetracycline (CTC) medicated feed was used, an active metabolite was detected in milk in addition to chlortetracycline. Similarly, in an incurred study with pigs fed chlortetracycline, the HPLC-receptorgram identified CTC and the active metabolite in urine, serum, and tissue. In chloramphenicol - suspected milk from Europe, chloramphenicol and the glucuronide metabolite were confirmed.

0097–6156/96/0636–0149$15.00/0
© 1996 American Chemical Society

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

Introduction

In recent years, regulatory agencies in Europe and the USA have been mandating precise measurements to detect veterinary drug residues at the levels of concern or regulatory levels. Countries around the world are developing policies known as "Integrated Residue Monitoring Programs".

Broad spectrum microbial inhibition tests with improved detection sensitivities for various antimicrobials have been introduced for veterinary drug residue screening. The CT II tests which are capable of detecting compounds belonging to the same drug family at or below their defined maximum residue levels (MRL), can provide the desired selectivity and sensitivity, as well as characterization of antimicrobials detected by the microbial inhibition tests. However, CT II on its own cannot discriminate between two drugs belonging to the same drug family.

A multiresidue confirmatory system is urgently needed to accommodate regulatory needs. HPLC systems and protocols are available for a limited number of drugs (2-7). For most drugs, including ß-lactams, clean up and fractionation procedures are available. However, sensitivity is a major problem, as many drugs have no characteristic absorption spectrum and can not be monitored with adequate sensitivity or selectivity. The combination of the HPLC and CT II sensitivity can provide the specificity, sensitivity and selectivity required from a multiresidue identification and quantitation system.

This paper describes a procedure in which interfacing the CT II Test with HPLC separation provides an excellent method for the detection and identification of individual veterinary drugs and their metabolites in milk, urine, serum, and animal tissue at concentrations of interest to regulatory agencies.

Experiments and Results

The HPLC system includes LKB 2150 HPLC pump, Waters 990 photodiode array detector, 200 µl injection loop, Foxy 200 fraction collector and Charm 7600 system. For fractionation of ß-lactams and sulfonamides an Alltech Lichrosorb RP-8 column (10 µm, 4.6 x 250 mm) was used with an isocratic buffer system containing 50 mM phosphate buffer, pH 6.0 and methanol (65:35) for ß-lactams and 10 mM ammonium acetate pH 4.6 and acetonitrile (78:22) for sulfonamides. For tetracyclines, a Polymer Laboratories' PLRP-S column (5 µm, 4.6 x 250 mm) was used with mobile phase consisting of 5 mM ammonium oxalate pH 3.5, acetonitrile and methanol (75:10:15).

McIlvaine/EDTA buffer (25 mM sodium citrate/phosphate in 0.1 M EDTA, pH 4.5) was used for the extraction and cleanup procedure . Samples of 20 ml milk or 5 gm tissue were homogenized with 20 ml McIlvaine/EDTA buffer. Homogenates were heated for 20 min at 80°C, cooled, and centrifuged for 10 min at 3000 xg to remove coagulated proteins. The supernatant solutions were further purified and concentrated using C8 Bond Elut cartridges for ß-lactams and sulfonamides (8, 9) or C18 cartridges for tetracyclines. Analytes were eluted with 2.5 ml methanol, dried under nitrogen, resuspended in 200 µl HPLC buffer, and injected into the HPLC. Fractions were collected according to retention time of standards and directly analyzed by CT II Test (figure 1). Retention times and window set-up for 8 tetracyclines are given in Table 1. Quantitation of analytes was done using the CT II dose response curve of the identified drug. The detection levels are summarized in Table 2.

All drugs tested were stable for several hours at room temperature and remained bioactive for at least 24 hours when stored at 4°C. Thus, fractions may be refrigerated and tested the next day.

Recovery: Radiolabelled tracers of ^{14}C penicillin, ^{3}H sulfamethazine and ^{3}H tetracycline have been used routinely as internal standards to verify that the fraction collector is programmed properly and also to calculate the recovery for the extraction method. Overall recovery has been consistent at 30-40% with 8-10% CV of the maximum theoretical value of 40% calculated from the relative volumes used at each steps (this is equivalent to 75-100% recovery).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

Case studies with the HPLC-receptorgram in Milk

Incurred study of ß-lactams in dairy cows

The HPLC-receptorgram was used to detect residues in incurred milk. Healthy cows were individually injected with the six common ß-lactams drugs. The milk, collected at 12, 24, 36 and 48 hours, was monitored on the HPLC-receptorgram. The results for 12 and 24 hours indicate a high level of parent drug in the sample (data not shown). At 36 hours postinjection, antibiotic levels were negative or near the safe level. The HPLC-receptorgram was negative at 48 hours postinjection. Active metabolites (desacetylcephapirin and cysceftiofur) were detected when cephapirin or ceftiofur was administered intramammary (Figure 2-3). With intramuscular injection of ceftiofur the metabolite was the main active peak (Figure 4). The method has been shown to be applicable for the detection of monobasic, acidic and amphoteric ß-lactams and could be used to identify both penicillins and cephalosporins at safe levels or below.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

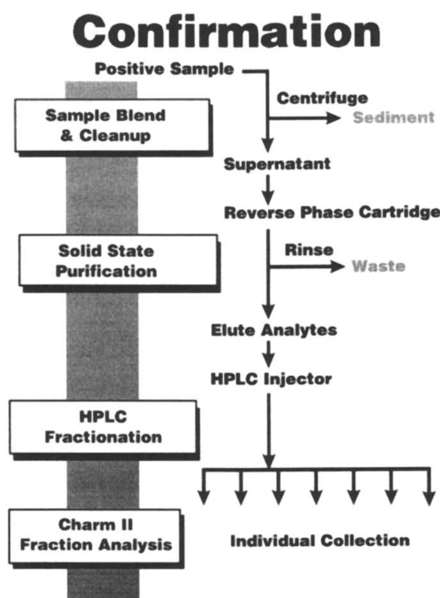


Figure 1. Schematic presentation of the HPLC-receptorgram used to identify drugs in a sample that tested positive by a screening method

Table 1. Calibration for the HPLC-receptorgram and time windows for collection of eight tetracyclines.

Drug	Retention time (min)	Time windows Start (min)	End (min)
MINOCYCLINE (MIN)	6.18	5.48	6.48
OXYTETRACYCLINE (OXT)	7.18	6.48	7.48
TETRACYCLINE (TET)	9.22	8.58	9.30
ROLITETRACYCLINE (ROL)	9.42	9.30	10.20
DEMOCLOCYCLINE (DEM)	13.54	12.54	15.00
CHLORTETRACYCLINE (CTC)	22.54	21.24	24.24
METHACYCLINE (MTC)	26.50	25.02	29.00
DOXYCYCLINE (DOX)	34.19	32.19	37.19

Table 2. Detection Levels of Antimicrobial drugs in Milk by HPLC-receptorgram , Charm II Tests and AIM-96 *(Representative antimicrobial drugs and detection levels in ppb)*

ANTIBIOTICS	AIM-96 [a] (FARM TEST)	CHARM II [b]	HPLC RECEPTOGRAM	EC MRL [c]	U.S. SAFE LEVEL / TOLERANCE
β-LACTAM					
Amoxicillin	4 / (4) ♦	10 / (1.5) ♦	8	4	10
Ampicillin	4 / (4) ♦	9 / (1.5) ♦	2	4	10
Cephapirin	10 / (10) ♦	4.5 / (1) ♦	3	—	20
Cloxacillin	30	50 / (10) ♦	10	30	10
Dicloxacillin	25	45 / (5) ♦	10	30	—
Naxcel (Ceftiofur)	50 / (12) ♦	25 / (2) ♦	10	100	50
Oxacillin	25	80 / (10) ♦	15	30	—
Penicillin G	3 / (2.5) ♦	4.8 /(1.5)♦	1	4	5
SULFA DRUGS					
Sulfachloropyridazine	5	3	3	100	10
Sulfadiazine	5	4 ♦	3	100	10
Sulfadimethoxine	10	4 ♦	3	100	10
Sulfamerazine	10	4	3	100	10
Sulfamethazine	15	10 ♦	5	100	10
Sulfamethoxazole	5	2	2	100	10
Sulfaquinoxaline	5	3	3	100	10
Sulfisoxazole	5	4	3	100	10
Sulfathiazole	5	8 ♦	4	100	10
Dapsone	5	2	1	0	—
TETRACYCLINE					
Chlortetracycline	150	28 ♦	15	100	30
Democlocycline	150	30	15	100	—
Doxycycline	50	100	30	100	—
Minocycline	50	30	15	100	—
Oxytetracycline	100	30 ♦	15	100	30
Rolitetracycline	80	10	10	100	—
Tetracycline	80	10 ♦	10	100	80
MACROLIDE					
Clindamycin	100	75	50	—	—
Erythromycin	100	25	25	40	50
Lincomycin	—	75	50	—	—
Pirlimycin	100	50	50	—	400
Spiramycin	500	50	50	200	—
Tylosin	20	50	50	50	50
AMINOGLYCOSIDE					
Gentamycin	30	24/12	Under Development	100	30
Neomycin	50	5	Under Development	500	150
Streptomycin	500	12.5	Under Development	200	125
AMPHENICOL					
Chloramphenicol	>1000	0.5	0.2	0	0
Flurophenicol	>1000	10	4	—	—
Thiaphenicol	>1000	5	2	—	—
OTHERS DRUGS					
Novobiocin	>1000	100	Under Development	—	100
Spectinomycin	>1000	30	Under Development	200	30
Trimethoprim	100	50	25	50	—

(a) Broad spectrum microbial inhibition assay using Charm Sciences AIM-96 or the Farm test format with the programmable incubator. (b) Charm II tests using U.S. safe level standards. ß-lactam levels are for the Sequential Assay and in brackets forthe Quantitative Assay methods . (c) MRL - Maximum Residue Levels. ♦ Levels (ppb) Detectected 90% of the time with 95% confidence (by the confirmation procedure) for US Safe Levels and approved by the FDA/AOAC RI indpendent test kit evaluation program, 1993. -- Not tested.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

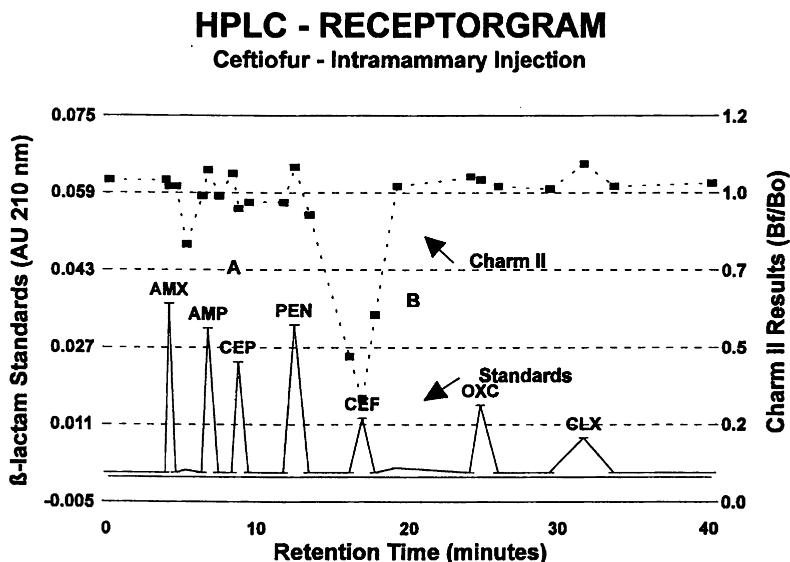


Figure 2. HPLC-receptorgram identification of ceftiofur (peak B) and its active metabolite (peak A) in milk, 36 hour after intramamary injection (dashed line). Peaks 1-7 are the elution profile of 7 ß- lactam standards (solid line): amoxicillin (AMX), oxacillin (OXC), penicillin PEN), cephapirin (CEP), cloxacillin (CLX), ceftiofur(CEF), ampicillin (AMP)

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

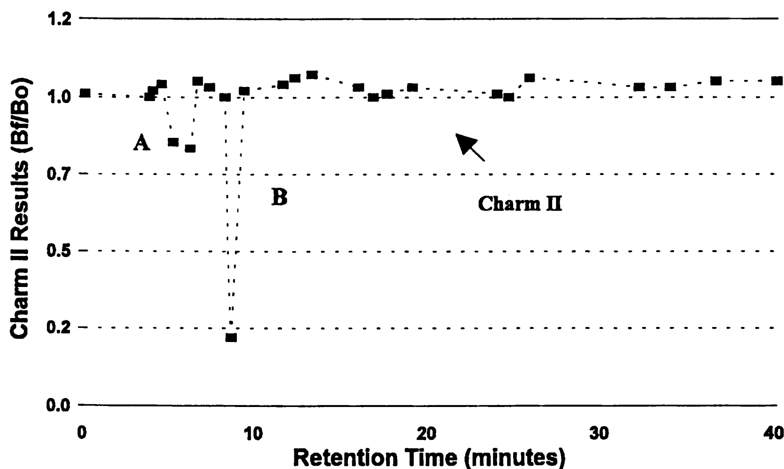


Figure 3. HPLC-receptorgram identification of cephapirin (peak B) and its active metabolite (peak A) in milk, 36 hour after intramamary injection (dashed line).

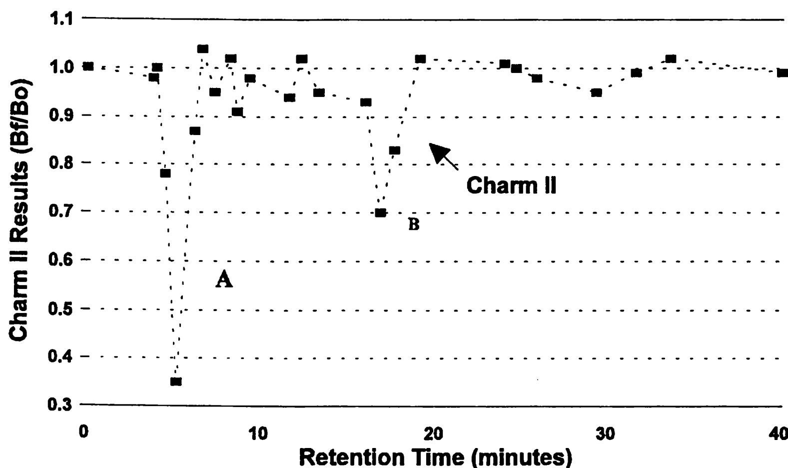


Figure 4. HPLC-receptorgram identification of ceftiofur (peak B) and its active metabolite (peak A) in milk, 12 hours after intramuscular injection (dashed line).

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

ß-lactam in bulk fluid raw milk

Positive milk samples from across the US (56 in total) indicate that penicillin G was the major ß-lactam residue in milk (41%). Ceftiofur, which was only recently introduced into the dairy industry, was the second most frequently detected residue (29%). Cephapirin and ampicillin were detected in 26% and 4% of the samples, respectively. Ceftiofur residues appeared to be in most cases from administration by means other than intramuscular injection.

Tetracycline incurred study in dairy cow

Low levels of CTC and metabolite have been detected in the milk supply. When CTC-medicated feed was investigated, a second major active component was detected in addition to CTC with a retention time similar to that of tetracycline. Similar results were observed in pig urine (Figure 5).

Field survey of off the shelf milk in Europe

The HPLC-receptorgram was used to identify the few positives of off the shelf milk samples found in this survey of 337 samples from 6 countries (10). Chloramphenicol and chloramphenicol glucuronide were confirmed in 2 milk samples by using retention time and enzymatic degradation by glucuronidase. Also sulfamerazine was confirmed at non violative levels in 2 milk samples (lower than 10 ppb) and 1 milk sample contained a low level (lower than 50 ppb) of minocycline.

Multicomponents in a single drug application

Several antibiotics are known to contain more than one active component. Gentamicin for example contains up to 4 related active components (C1, C2, C1a and A) which further challenge a determinative method as all 4 compounds have a cumulative effect in an inhibition assay or receptor assay (CT II). When the CTII was used to detect active macrolide antibiotics in HPLC fractions, 3 active components were detected in spiramycin and tylosin fortified milk. An HPLC-receptorgram for tylosine is given as an example (Figure 6). Similar results were obtained by UV monitoring of the standards, example for tylosine is presented (Figure 6). These active components need to be detected at levels at least 5 times lower than the MRL in order to qualify as a determinative method. The HPLC-receptorgram

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

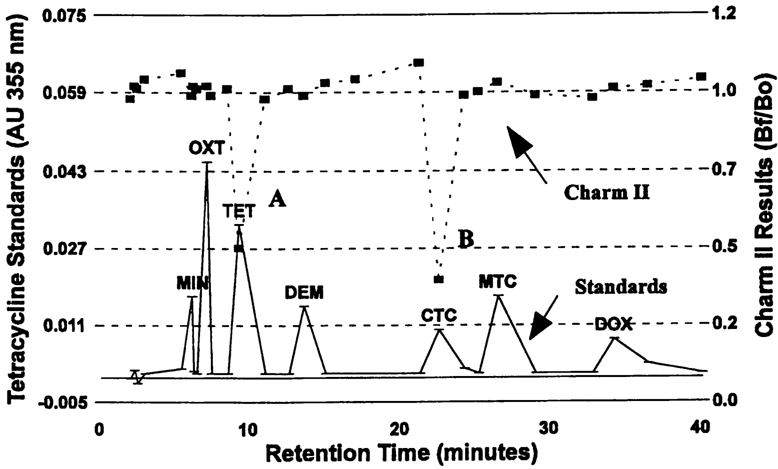


Figure 5. HPLC-receptorgram identification of chlortetracycline (peak B) and an active metabolite (peak A) in pig urine, after oral administration of medicated feed (dashed line).

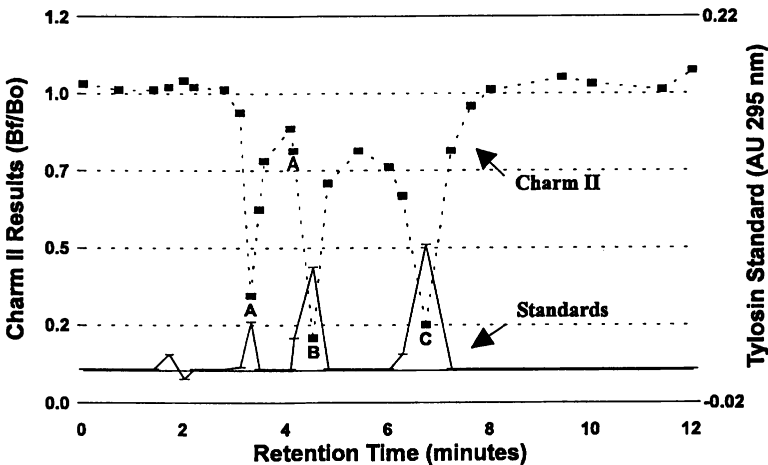


Figure 6. Multiple active tylosine peaks (A, B and C) detected by HPLC receptorgram and Ultraviolet monitoring of USP tylosin standard.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

TABLE 3: Total Tetracyclines levels In Pig Tissue And Fluid After Oral administration of Chlortetracycline Medicated Feed

Pig urine were screened on the Charm II at 2000 ppb CTC as cut off for positive. The results for the negative group and positive group are presented below*:

Table 3A: Pig urine samples with CTC concentrations less than 2000 ppb

	Muscle	Kidney	Liver	Serum	Urine
Results	(ppb)	(ppb)	(ppb)	(ppb)	(ppb)
Pig 3	neg	neg	neg	<40	neg
Pig 4	neg	neg	neg	<40	400
Pig 8	neg	120	48	220	1500
Pig 9	neg	296	48	220	1800
Pig 10	neg	neg	neg	neg	neg
Pig 11	neg	neg	neg	neg	neg
Pig 12	neg	88	64	60	450
Pig 13	neg	80	<40	<40	1050

* Samples with less than 40 ppb could not be quantitated with accuracy.

Table 3B: Pig urine samples with CTC concentrations greater than 2000 ppb

	Muscle	Kidney	Liver	Serum	Urine
Results	(ppb)	(ppb)	(ppb)	(ppb)	(ppb)
Pig1	148	350	100	280	2700
Pig 2	90	440	120	265	2250
Pig 5	48	400	232	260	10500
Pig 6	50	200	120	225	6000
Pig 7	60	560	320	270	6000

Table 3C: Summary table for Pig urine samples with CTC concentrations greater than 2000 ppb (STD - Standard Deviation)

	Muscle	Kidney	Liver	Serum	Urine
Summary					
CTC level (ppb)	79	390	178	260	5490
CTC level STD	38	118	85	19	2963
Ratio to Muscle	1.0	5.8	2.9	3.9	96.4
Ratio STD	NA	2.9	2.1	1.4	81.8
Tolerance Equivalent (ppb)	100	578	292	385	9640

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

was able to detect both spiramycin and tylosin at their respective MRLs of 200 and 50 ppb.

Case studies with the HPLC-receptorgram in Pig Tissues

Field study of pigs fed CTC medicated feed

A predictive method for measuring antimicrobial drug levels in tissue was tested using CTC levels found in urine or serum. Urine and muscle samples from a group of pigs administered standard CTC medicated feed (200gm/ton) diet which require 1 day withdrawal, were analyzed using the HPLC-receptorgram . Initial results indicate an average urine level of 2000 ppb of combined CTC and its active metabolite, is equivalent to an average of 50 ppb in muscle. This level was used as a cut off to screen a second group of pigs. Urine, serum, liver, kidney and muscle were tested for total tetracyclines (Table 3a,b). In the negative group, muscle levels of total tetracycline were all below the minimum detection levels of the assay. However, the positive group muscle levels ranged between 48-140 ppb. The study established the ratio of the combined tetracycline level in urine, serum, kidney, and liver to muscle tissue at 96.4, 3.9, 5.8 and 2.9 respectively (Table 3c). Serum has emerged as a better predictive fluid for tissue levels with CV of less than 10% as compared to over 30% for urine.

Conclusion

Since the introduction of HPLC-receptorgram in 1987 it has fulfilled the gap in determinative methodology created by new and improved screening methods for identification and quantitation of antimicrobial drugs in milk and other livestock products, accommodating all regulatory requirements. It is cost-effective and simple enough to be effectively integrated into any existing or new residue-monitoring programs. The combination of the CT II assay and HPLC provides high selectivity and excellent sensitivity for veterinary drug residue analysis.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

References

1. Charm S.E, Zomer, E and Salter, R. J. Food Protection. **1989**, 51,920-924
2. Smedley, M.D. AOAC International. **1994**, 77,1112-1118
3. Modin, R. and Schroder-Nielsen, M.S. Acta Pharm. Suec. **1971**, 8, 573-584
4. White, C.R., Moats, W.A. and Kotula, K.I. AOAC International **1993**, 76(3),549-554
5 Ting, S., J. Assoc. Off. Anal. Chem. **1988**, 71 (6), 1123-1130.
6. Moats, W.A. J. AOAC Int. **1993**, 76 (3), 535-539.
7. Fletouris, D.J., Psomas, J.E., and Mantis, A.J. J. Agric. Food Chem. **1992**, 40 (4), 617-621.

8. Zomer, E., Saul S., and Charm S.E. J. AOAC International **1992**, 75 (6), 987-993.
9. Zomer, E., Quintana, J. Saul S., and Charm S.E. J. AOAC International. **1995**, 78 (5), 1165-1172.
10. Suhren, G., Hoffmeister, A., Reichmuth, J., and Heeschen, W.; "Milchwissenschaft, **1990**, 45 (8): 483-550.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch016

Chapter 17

Determination of Diuretic Drugs Used in Food-Producing Animals

Badar Shaikh

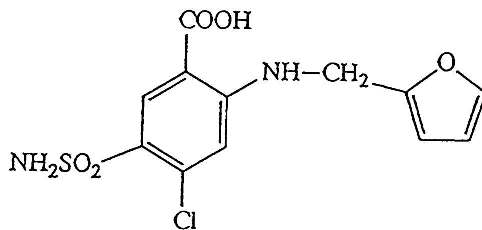
Center of Veterinary Medicine, U.S. Food and Drug Administration, BARC-East, Building 328A, Beltsville, MD 20705

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

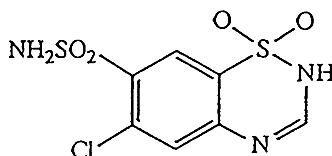
Thiazide diuretics (chlorothiazide, hydrochlorothiazide, and trichlormethiazide) and a loop diuretic (furosemide) are used in dairy cattle for the treatment of post-parturient edema of the mammary gland and associated structures. The potential misuse of these diuretic drugs in the cattle may lead to harmful residue concentrations in meat and milk destined for human consumption. Therefore, analytical methods which are sufficiently sensitive to monitor residue concentration levels remain an urgent need for these diuretics. This article reviews various research approaches described in the literature for the extraction, isolation, and quantitation of diuretics in biological matrices with emphasis on the liquid chromatographic determinative procedures.

Diuretics are chemically heterogenous compounds used as therapeutic agents in certain pathological conditions to eliminate bodily fluids. They not only promote renal excretion of water and salt but also affect the renal reabsorption and excretion of other ions, e.g., potassium, calcium, and magnesium *(1)*. The diuretics, due to their variety of chemical structures, have different pharmacological properties and, accordingly, are classified into four groups *(2)*: carbonic anhydrase inhibitors, loop, thiazide and thiazide type, and potassium sparing diuretics. The carbonic anhydrase inhibitors e.g., acetazolamide, decrease the conversion of bicarbonate ion to carbonic acid resulting in an increase in sodium, potassium, and bicarbonate renal excretion; this also increases the pH of the urine. Furosemide has been reported to be a most potent and short acting loop diuretic. It is widely used to treat edematous states of hepatic, cardiac, and renal origin *(3)*. It is also a common drug of abuse in livestock shows since it reduces tissue water resulting in improved muscle tone appearance *(4)*. The thiazide diuretics, chlorothiazide (CTZ), hydrochlorothiazide (HCTZ), and trichlormethiazide (TCMTZ) increase the excretion of potassium and can cause hypokalemia during long-term maintenance therapy. Therefore, these diuretics are often given with potassium-sparing diuretics such as amloride, in order to maintain electrolyte balance in the body *(1)*.

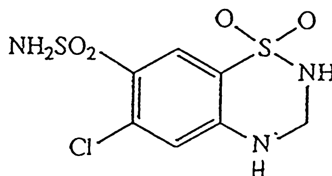
This chapter not subject to U.S. copyright
Published 1996 American Chemical Society



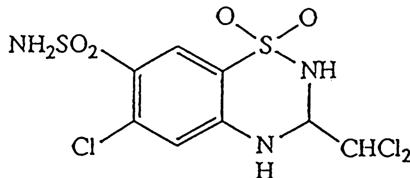
Furosemide (FUR)



Chlorothiazide (CTZ)



Hydrochlorothiazide (HCTZ)



Trichlormethiazide (TCMTZ)

Figure 1. Chemical structures of diuretics used in food Producing animals.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

Use of diuretics by athletes has been banned since the 1988 Olympic Winter and Summer Games. They are misused by athletes to reduce the body weight and to increase the urine flow leading to a reduction in the concentration of other doping agents *(5)*. The use of carbonic anhydrase inhibitor diuretics results in an alkaline urine that reduces the urinary excretion of basic doping agents leading to a negative test result. The chemical structure of furosemide is similar to thiazides but it is a more potent diuretic. Thiazide, loop, and potassium sparing diuretics, alone or in combination, are used widely in the treatment of hypertension, congestive heart failure, and some types of edema. The diuretics have been reported to cause potential toxic effects such as bone marrow depression, hyperbilirubinemia, altered carbohydrate metabolism, and elevated blood levels of urea, uric acid, and sugar *(6)*.

Furosemide and thiazide diuretics, CTZ, HCTZ, and TCMTZ, are approved for use in dairy cattle for the treatment of post-parturient edema of the mammary gland and associated structures *(7)*. The unauthorized use of these diuretics, or the failure to follow label directions for approved use in the cattle, could lead to unacceptable residues in meat or milk destined for human consumption. Therefore, monitoring of the residue of these diuretic drugs in food is a part of a general policy to prevent unapproved uses of diuretics. This article covers the review of analytical chemical methods for the determination of diuretic drugs in biological matrices, particularly in milk, plasma, and urine with emphasis on liquid chromatographic methods.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

Diuretics Approved for Use in Food Producing Animals

The chemical structures of the diuretics approved for use in dairy cattle (7,8) are given in figure 1. Furosemide is a strongly acidic ortho-chlorosulfonamide compound but, it has an additional carboxyl group that differentiates it from the weakly acidic thiazide diuretics. Table I summarizes safe milk concentration levels, milk withdrawal times, etc. of diuretics used in dairy cattle (7). While there are no official tolerances for these drugs in milk, U.S. Food and Drug Administration has established the safe levels provided in Table I. The withdrawal time of TCMTZ is not listed in the Code of Federal Regulations (CFR), but, it is considered to be 72 h, similar to other thiazides *(8)*. The safe level is the amount of total residue permitted in the milk from treated animals. The withdrawal time is the period required for the residue of toxicological concern to reach safe concentration in food intended for human consumption. Milk must not be used for consumption or animals must not be slaughtered for food before the respective withdrawal times are observed after the treatment. Furosemide and HCTZ are administered intra-muscularly (IM) or intravenously (IV) and CTZ and TCMTZ are administered orally (OR). Their dose levels are shown in Table I.

In determining diuretics, or other drugs, in various matrices, it is often necessary to know their physical-chemical characteristics, such as solubility, stability, pKa, etc. Table II shows general physical-chemical characteristics of diuretics reported in various literature sources *(9, 10, 21)*. In general, the diuretics are soluble in alcohol and water with the exception of HCTZ which is insoluble in water. Furosemide is the least stable and is a strongly acidic compound among these diuretics; it has natural fluorescence with excitation and emission wavelengths at 272 and 410 nm, respectively *(11)*. The UV absorption spectra of the thiazide diuretics in methanol were determined in our

laboratory. They gave two absorption bands, the most intense being at 225 nm (Table II).

Table I. Safe Milk Levels, Dose, and Milk Withdrawal Times

Diuretic	*Species*	*Safe Level ng/mL*	*Dose mg/Animal*	*Withdrawal Time Hours*
Furosemide	Cattle	10	500*	48
CTZ	Cattle	67	2000**	72
HCTZ	Cattle	67	125-250*	72
TCMTZ	Cattle	7	200**	72

*IM or IV, **Oral

Table II. General Characteristics of Diuretics

Diuretic	*Solubility*			*Stability*			*pKa*	*UV, nm*
	Wa	*Me*	*Et*	*Light*	*Acid*	*Base*		*(methanol)*
CTZ	S	SS	S	+	+	-	8-10	225,280
HCTZ	IS	S	S	+	+	+	8-10	225,270
TCMTZ	S	S	S	+	+	+	8.6	225,270
FUR	SS	S	S	-	-	-	4-5	272ex, 410 em

Wa = water, *Me* = methanol, *Et* = ethanol, S = soluble, IS = insoluble,
SS = slightly soluble, EX = exitation, EM = emission, + = stable, - = unstable

Rowbothen, et al. *(12)* reported that the photochemical degradation of furosemide is a hydrolysis and a photochemical oxidation process. The major product generated is 4-chloro-5-sulphamoylanthranilic acid (CSA) which is further converted into 4-chloro-5-sulphoanthranilic acid. Acid hydrolysis of the furosemide also gives CSA and furfuryl alcohol. Bach, et al. *(13)* reported that furosemide modifies the thermotropic properties of phospholipids, an important constituent of kidney plasma membranes; it also causes a decrease in the electrical resistance of planar lipid membranes, facilitating the transport of ions. Macheras, et al. *(14)* reported that the fat content of milk had no significant effect on the binding of CTZ and HCTZ to milk. However, during the binding study at various temperatures (5-37 °C), higher binding was observed at lower temperatures than that at higher temperatures for both drugs, resulting in enhanced availability of the free drugs at physiological temperatures.

Methods of Analysis

In the last decade automated instrumentation has revolutionized analytical methods development including extraction, clean up, determinative, and con-firmatory procedures. Such instruments have also increased sample throughput and shown a several-fold improvement in sensitivity with reasonable cost per analysis. The present review deals with liquid chromatographic (LC) methods of analysis for diuretic drugs used in food producing animals.

Loop Diuretics. Three compounds namely furosemide, ethacrynic acid, and bumetanide fall in this category and act by affecting the resorption of sodium primarily in the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

ascending limb of the loop of Henle. The use of furosemide has become wide spread in veterinary medicine. Ethacrynic acid is reported to be equally potent, while bumetanide has been reported to be about 40 times more potent than furosemide *(15)*. Among the loop diuretics, only furosemide is approved for use in cattle.

Almost all reported analytical methods for the analysis of furosemide employ LC. These methods have been limited to assaying furosemide in plasma and urine and have been extensively reviewed *(1,11)*. Only recently, an LC method for the determination of furosemide in milk has been reported *(11)*. The method involved defatting of the milk by centrifugation followed by deproteination with acetonitrile. The acetonitrile was evaporated and the aqueous layer was analyzed directly by LC. The LC conditions employed a reversed phase column, an isocratic phosphate/acetonitrile mobile phase, and a fluorescent detector with excitation and emission wavelengths set at 272 and 410 nm, respectively. The average recovery and C.V. for fortification levels at concentration ranges between 5-20 ng/mL was 95 and 9%, respectively. The method is rapid and sensitive and the limits of quantitation and detection were 5 and 3 ng/mL, respectively. The method is quite suitable for routine monitoring of furosemide. Because of a close resemblance of the structure of the furosemide with thiazides, the derived extraction and LC conditions have a good potential for use in the determination of these diuretics in the milk matrix.

Thiazide Diuretics. Thiazide diuretics used in food producing animals include chlorothiazide, hydrochlorothiazide, and trichlormethiazide. Trichloromethiazide is given in combination with dexamethasone *(8)*. Trichlormethiazide is an orally administered, highly effective diuretic agent of the benzothiazide series *(16)*. Studies in man and experimental animals show that TCMTZ presents a favorable pattern of less potassium excretion than CTZ or HCTZ. The clinically determined saluretic potency of TCMTZ is estimated to be 10-20 times that of HCTZ and 100-200 times that of CTZ with a decrease in the incidences of hypokalemic manifestations. Among thiazide diuretics, hydro-chlorothiazide is the most used and studied diuretic. As in the case of furosemide, almost all of the work on thiazide diuretics involves the analysis of plasma and urine. A detailed review of LC methods for diuretics in plasma and urine, has been provided *(1)*; therefore, only additional developments since 1992 will be reviewed.

There is only one method in the earlier literature that was used for the determination of chlorothiazide in human milk *(6)*. The method involves acidification of the milk followed by ethyl acetate extraction. The ethyl acetate layer is evaporated to dryness and re-extracted with NaOH and chloroform. The basic aqueous layer is hydrolyzed, acidified, and assayed at 520 nm wavelength. The limit of detection was 1 ug/mL. The procedure is not sensitive and probably not suitable for the residue analysis. However, some of the clean up steps used in this procedure may be adopted for the isolation of thiazides from bovine milk prior to the LC analysis.

Jumppanen et al. *(17)* screened diuretics in the blood serum and urine by capillary zone electrophoresis (CZE) with UV detection. Serum was deproteinated with methanol prior to solid phase extraction (SPE) clean up. The diuretics were eluted from the SPE column with methanol. They were analyzed in a fused silica column and with a 3-(cyclohexylamino)-1-propanesulfonic acid buffer at pH 10.6. Baseline separation of the four (furosemide and the three thiazides) and a number of other diuretics was achieved in less than 20 minutes. The authors proposed that detection limits at the low

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

fentomole concentration levels could be achieved by this technique. For urine analysis, the methanol deproteination step was not required. This procedure has a potential for the determination of diuretics in milk at low residue concentration levels; however, milk matrix may create some problems if proteins are not completely removed from the sample.

Love and Fett *(18)* directly analyzed urine containing diuretics including furosemide and hydrochlorothiazide by using a micellar chromatographic procedure. The non-ionic surfactant, polyoxyethylene lauryl ether (Brij 35) at pH 5.5-7.5 was found to provide the best separation of the diuretics from the urine background. UV and fluorescence detection was used for the HCTZ and the furosemide, respectively. The limits of detection for HCTZ and furosemide were 300 and 30 ppb, respectively. In this procedure, the retention of the sample matrix rather than that of diuretics was optimized and the procedure may be applied for use in the milk matrix.

Ventura et al. *(19)* obtained optimal extraction of the diuretics in urine by utilizing alkaline extraction at pH 9.5 with ethyl acetate and salting out with sodium chloride. The LC analysis was carried out by using a reversed phase column, diode array detector, and a mobile phase of acetonitrile and ammonium acetate, pH 3, with gradient elution. The detection limits for HCTZ and furosemide were 100 and 50 ppb, respectively.

Domingo et al. *(20)* also used a micellar mobile phase of 0.05M SDS-5% methanol for direct urine analysis of diuretics on a reversed phase column with UV detection set at 275 nm. Although some other diuretics separated adequately from the matrix, the diuretics of veterinary interest, HCTZ and furosemide, were overlapped by the urine matrix. Additional work would be required to achieve satisfactory separation of the two diuretics from the matrix.

Campins-Falco et al. *(21)* evaluated a number of solid-phase extractions (SPE) cartridges (C18, C8, C2, CN, CH, and phenyl) to clean up urine samples during the analysis of diuretics before injecting into the LC column. The results obtained were compared with those obtained with a classical or normally used liquid-liquid extraction employing ethyl acetate under both acidic and basic conditions. The average recovery of HCTZ with liquid-liquid extraction was 67% as compared to 34% for furosemide. However, SPE techniques gave excellent recoveries for furosemide with all cartridges. The recovery range was 70-92% as compared to HCTZ with recovery range of 27-53%. It should be noted that when acetonitrile extraction was employed without acidic or basic conditions *(11)*, the furosemide recoveries from milk matrix were in the 90% range. Nevertheless, this liquid-liquid extraction procedure has a great potential for use in the determination of thiazide diuretics in milk matrix.

Heraez-Hernandez et al. *(22)* optimized an LC procedure for the analyses of diuretics including furosemide and hydrochlorothiazide in urine matrix. The optimal conditions included a mobile phase of acetonitrile/phosphate buffer (pH 3) in a gradient elution mode and UV detection at 230 nm. The time of analysis was short (under 10 min) and limits of detection for furosemide and HCTZ were 0.02 and 0.01 ug/mL.

Conclusion

With the exception of furosemide, the analytical methods for the determination of the residues of HCTZ, CTZ, TCMTZ in milk are not available in the literature. Among the

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

thiazide diuretics, only methods for HCTZ have been studied extensively in plasma and urine but not in milk. The optimization of extraction (liquid-liquid and SPE) and LC procedures, including mobile phase and detection techniques, are described. These procedures have a good potential for application in the determination of loop and thiazide diuretics in the milk matrix. Micellar and CZE procedures provide new challenges for the analysis of diuretics in milk. The former affords direct analysis and the latter provides greater sensitivity.

Literature Cited

1. Herraez-Hernandez, R.; Campins-Falco, P.; Sevillano-Cabeza, A. *Chromatog.* **1992**, 33, 177-185.
2. Cooper, S.F.; Masse, R.; Dugal, *J. J. Chromatogr.* **1989,** 489, 65-88.
3. Smith, D.E.; Lin, E.T.; Bent, L.Z. *Drug Metab. and Dispos.* **1980,** 8, 337-342.
4. Ray, A.C.; Tanksley, T.D.; LaRue, D.C.; Reager, J.C. *Am. J. Vet. Res.*, **1984,** 45, 1460-1463.
5. Ventura, R.; Fraisse, D.; Becchi, M.; Paisse, O.; Segura, J. J. *Chromatogr.* **1991,** 562, 723-736.
6. Werthmann, Jr., M.W.; Krees, S.V. J. *Pediatrics* **1972** , 81, 781-783.
7. *Code of Federal Regulations; Food and Drugs*; **1993,** parts 520.420; 520.1010; 522.1010; 522.1150
8. Talbot, R.B.; Fernandez, A.H.; Melendez, L.V. Eds; *List of FDA Approved Animal Drug Products*; Laboratory of Veterinary Medical Informatics, Information Series 87-1, VA-MD Regional College of Veterinary Medicine, Blacksburgh, VA, 1987, 2.47.
9. Croo, F.DE.; Bossche, W.V.D.; Moerloose, P.DE. *J. Chromatogr.* 1985, 325, 395-411.
10. *The Merck Index*, 11th edition, Editor, Budavari, S., Merck and CO., Rahway, N.J., 1989.
11. Shaikh, B.; *J. Agr. & Food Chem.* **1995,** In Press.
12. Rowbotham, P.C.; Stanford, J.B.; Sugden, J.K. *Pharm. Acta Helv.* **1976,** 51, 304-307.
13. Bach, D.; Vinkler C.; Miller, I.R.; Caplan, S.R. *J. Membrane Biol.* **1988,** 101, 103-111.
14. Macheras, P.E.; Koupparis, M.A.; Antimisiaris, S.G. *J. Pharm. Sci.* **1988,** 77, 334-336.
15. Gross, D.R.; *In Veterinary Pharmacology and Therapeutics,* Editors, Booth, N.H. and McDonald, L.E., "Drugs Affecting Fluid and Electrolyte Balance", The Iowa State University Press, Ames, IA, 1988, Chapter 31, 551-562.
16. *Compendium of Veterinary Products*; North American Compendiums, Inc.; Port Huron, MI, 1993, pp 601.
17. Jumppanen, J.; Siren, H.; Riekkola, M.-L. *J. Chromatogr.* **1993,** 652, 441-450.
18. Love, L.J.C.; Fett, J.J.; *J. Pharmaceut. Sci.* **1991,** 9, 323-333.
19. Ventura, R.; Nadal, T.; Alcade, P.; Pascual, J.A.; Segura, J. *J. Chromatogr.* **1993,** 655, 233-242.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

20. Domingo, E.B.; Hernandez, M.J.M.; Ramos, G.R.; Alvarez-Coque, M.C.G. *J. Chromatogr.* **1992,** 582, 189-194.
21. Campins-Falco, P.; Herraez-Hernandez, R.; Sevillano-cabeza, A. *J. Liq. Chromatogr.* **1991,** 14, 3575-3590.
22. Herraez-Hernadez, R.; Campins-Falco, P.; Sevillano-Cabeza, A. *J. Liq. Chromatogr.* **1992,** 15, 2205-2224.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch017

Chapter 18

Dye Residues in Foods of Animal Origin

J. E. Roybal, A. P. Pfenning, S. B. Turnipseed, J. A. Hurlbut, and A. R. Long

Animal Drugs Research Center, U.S. Food and Drug Administration, Denver Federal Center, P.O. Box 25087, Denver, CO 80225–0087

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

Dyes are used for numerous purposes, including animal husbandry and aquaculture. Food destined for human consumption could contain residues of these compounds and unnecessary exposure to dye residues has the potential for human health hazards. This chapter examines methodology used to detect and identify several commonly used dye, their residues and metabolites. Discussion focuses on matrices and methods used and developed in our laboratory as well as work of other investigators. The metabolism, analysis and confirmation of Gentian Violet (GV), Malachite Green(MG) and Methylene Blue(MB) by HPLC, LC/MS and GC/MS are covered. Some information generated in our laboratory includes: •GV metabolized in chickens, yielding demethylated and reduced products. •Analysis of GV-incurred chicken tissue, 3hr post-dosing, revealing 20-105ppb GV total residues. • Chicken fat yielding 49.3ppb of Leucogentian Violet (LGV), the main metabolite. •Analysis of MG-incurred catfish, 24hr post-dosing, produced average residues of 289ppb Leucomalachite Green(LMG), as the predominant metabolite. •MB in milk was metabolized to the completely demethylated product, Thionin, 26.6ppb, 72hr post-dosing.

Harvey W. Wiley, pioneer and father of the Pure Food and Drug Act, in response to the public demand, initiated by Sinclair's book "The Jungle", convinced Congress in 1906 *"to investigate the character of food preservatives, coloring matters, and other substances added to foods, to determine their relation to digestion and health, and to establish the principles which should guide their use"* (*1*).

Dyes have always played an important role in the human experience. When it was discovered that some dyes have medicinal qualities, their use extended into controlling human and veterinary diseases. Some dyes have very specific activity in

This chapter not subject to U.S. copyright
Published 1996 American Chemical Society

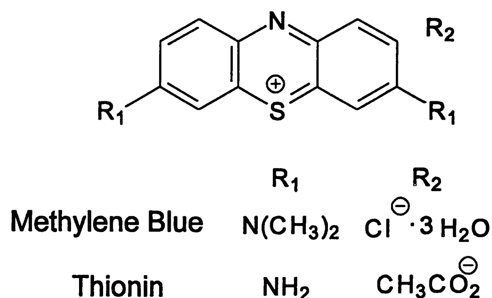
combatting bacterial infections. Animal drugs, as with all drugs, leave residues, and foods derived from treated animals have the potential to contain these residues. These residues could consist of either the parent drug or a metabolite and their presence in food products could prove detrimental to human health. Since banning the use of therapeutic drugs in animal husbandry is not feasible or practical from a scientific or economic standpoint, methods are vital to monitor our food supply to assure a drug-free and wholesome product in the market place.

Four categories of dyes having antibacterial activity or some medicinal benefit are: Triphenylmethanes(rosaniline series), azo derivatives, acridines and phenothiazine congeners. Methyl derivatives of the triphenylmethane(TPM) dyes comprise a series of basic dyes, the ones active in basic medium, are specific bacterials against gram-positive microbes. Gentian violet(GV), Malachite green(MG) and Methyl violet are example of TPM's. Azo dyes such as Congo red and Sudan IV(Scarlet red) are specifically active against gram-negative bacteria due to their high activity in acid medium. The derivatives of acridine, coal tar based, are yellow dyes that have been designated as flavines. These dyes have either basic or neutral properties. They are excellent antiseptics and exhibit strong activity against trypanosome assault(trypanocide). Acriflavine/Proflavine/Quinacrine are examples of the acridine group which have demonstrated a utility for bactericidal action. Acriflavine is neutral in character and has been shown to be an effective treatment for bovine mastitis. As an antiseptic it is used as an agent against local and urinary infections. Proflavine differs from acriflavine by the absence of a methyl group and is particularly effective against *Proteus*, enterobacterial infections. Quinacrine is categorized as a therapeutic agent in veterinary medicine as an antiprotozoal/teniacide. It destroys all types of asexual plasmodia making it an effective combatant against malaria(*2,3*). Methylene blue(MB) and Toluidine blue O are dyes in the phenothiazine category. While these do not exhibit antibacterial qualities as such, they are useful dyes for certain medical situations (antidotal and antiseptic). Methylene blue is valuable as an antidote in cyanide, nitrate and/or alcohol poisoning. Toluidine blue O has been used clinically as an antiheparin agent to control idiopathic (of unknown cause) uterine bleeding(*3,4*).

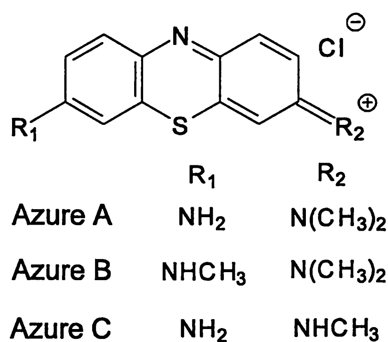
In 1989 we published our first work in the area of dye analysis(*5*). Since then we have developed methods for several dyes in different matrices. Although often studied and with the possible implication for use in animal husbandry and aquaculture, essentially no methods have been developed for the detection and determination of acridine residues in any matrix other than pharmaceuticals. Methods for detection or analysis of residues and metabolites of these compounds are underway but specifics are not available at this time. Although comprising one of the larger groups of the synthetic dyes(>50%), evidence of use of the azo dyes in animal husbandry does not currently exists. This report will deal with the metabolism, analysis and confirmation of two dyes from the TPM category: GV and MG; and with MB from the phenothiazine category, Figures 1 and 2.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

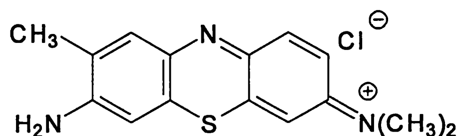
September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018



	R1	R2
Methylene Blue	$N(CH_3)_2$	$Cl^{\ominus} \cdot 3H_2O$
Thionin	NH_2	$CH_3CO_2^{\ominus}$



	R1	R2
Azure A	NH_2	$N(CH_3)_2$
Azure B	$NHCH_3$	$N(CH_3)_2$
Azure C	NH_2	$NHCH_3$



Toluidine Blue O

Figure 1. Chemical Structure of selected Phenothiazine dyes discussed in text.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

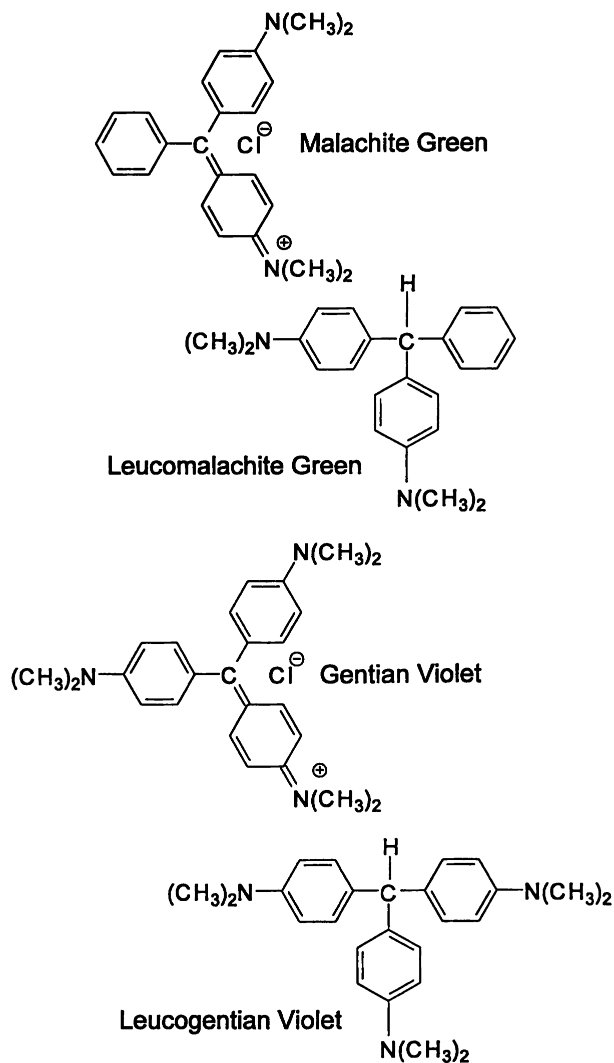


Figure 2. Chemical Structure of selected Triphenylmethane dyes discussed in text.

Metabolism

Gentian Violet.

Gentian violet(GV) was utilized as a feed additive to inhibit mold and fungal growth in poultry feed before 1990. In the mid-1980's, the National Center for Toxicological Research(NCTR) reported on their considerable research into the toxicity of GV in mice and its metabolism in chickens(*7,10*). Chronic toxicity testing displayed evidence that gentian violet has a tumorigenic effect in several organs of mice. At 240 hours after the final dose, residue equivalents in male chicken livers remained at 20.9 ppb.

The biotransformation, in uninduced liver microsomes, of gentian violet in treated animals results in demethylated metabolites. The metabolic profile for GV in mice, rat, hamster, guinea pig and chicken was found to be the same regardless of gender(*6*). In addition reduction of the parent dye by the intestinal microflora, in an anaerobic state led to the leuco entity, Leucogentian violet(LGV)(*7*). The metabolism was confirmed in chickens administered GV. Chicken detoxification of GV resulted in several demethylated metabolites in liver, thigh and breast muscle besides GV: pentamethylpararosaniline(PENTA), N,N,N',N'-1-tetramethylpararosaniline(N'-1-TETRA), N,N,N',N"-2-tetramethylpararosaniline(N'-2-TETRA) and the reduced form, LGV. The major metabolite of GV in chicken fat was LGV (*8,9*).

In 1991, regulations were amended banning the use of up to 8 ppm of GV in poultry feed as a mold inhibitor. This amendment to the Code of Federal Regulations, 21CFR, stated that GV is not generally recognized as safe (GRAS) or sanctioned as a food additive when added to animal feed for any nondrug use. The regulations were further amended to declare that GV is not generally recognized as effective(GRAE), or "grandfathered" under the Drug Amendments of 1962, and is therefore a new animal drug when used for any veterinary drug purpose in food animals.

Malachite Green.

Malachite green(MG) is primarily employed in the aquaculture industry as an antifungal/antiparasitic agent. It is normally administered as a flush at 1ppm concentration. Because MG is of the TPM family, many investigators have raised questions about pathology of it when present in food products. In late 1992, FDA Chemical Selection Working Group, placed MG as FDA's priority chemical nomination for FY 1993 to the National Toxicological Program(NTP). The chairman Dr. Allaben, concluded that MG had a high probability of abuse due to its effectiveness for combatting fungal infections in aquatic species, even though it is not approved for food fish. After an exhaustive literature review of MG, Culp(*11*) stated, "*The potential for consumer exposure to malachite green exists most notably through its use as an antifungal agent in the commercial fish hatcheries. With the exception of an U.S. Fish and Wildlife Investigational New Animal Drug exemption*

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

for treating specific threatened aquatic species, malachite green is not approved for use on any aquatic species by the FDA or the Environmental Protection Agency".

The metabolism of MG in trout and catfish has been documented(*12-14*). MG is rapidly absorbed through the gills. It is reduced to the leuco metabolite, leucomalachite green(LMG) and deposited in the fatty tissue of the fish. It was noted that trout excreted intact MG relatively rapidly, yet LMG slowly. Fat content influenced the excretion rate, the higher the fat content the greater deposition of LMG. This effect appeared to be cumulative with repeated treatments. Bauer(*13*) reported the half-life of LMG to be about 40 days. No evidence of systematic demethylation of MG has been observed in aquatic species.

Methylene Blue.

The principle use of methylene blue(MB) is for treating nitrate and/or cyanide poisoning in ruminants. While not as potent and effective as other agents, MB does exhibit antiseptic properties. In fish farming the potential exists that MB could become an alternative to MG as an antifungal/antiparasitic. MB was tested and found to be mutagenic, active with and without Aroclor induced rat liver microsome preparations, S9(*15*).

Prior to 1970 little had been reported on the metabolism and elimination of MB. In mammals, MB is absorbed from the GI tract and rapidly reduced to leucomethylene blue(LMB). It is excreted in the urine and bile, primarily as LMB(*16*). DiSanto and Wagner(*17*), reported the reduction of MB *in vivo* and eliminated as LMB, MB and one or more "chromogenic" substances in urine. A follow-up study about the ability of MB to permeate the blood/milk barrier, indicated that the transport mechanism required conversion of MB to LMB(*18,19*).

When MB is administered to lactating cattle, strong evidence is found for a metabolic profile, in milk, consisting of several demethylated products. Milk contained various metabolites at different stages of demethylation, in addition to a MB-organic complex. The principle metabolites, besides unchanged MB(tetra-methyl), were azure B(tri-methyl), azure A(di-methyl) and azure C(mono-methyl)(*20*). Further investigation demonstrated the MB-complex to be a protein-thionin conjugate(*21*). Thionin, the completely demethylated product of MB, was the residue having the longest residence time in milk. While toluidine blue O was not observed as a metabolic product, it has been known to be used in place of thionin and methylene blue as a nuclear stain. Its use in veterinary medicine has not been documented. It's mentioned here as a suggested internal standard in the LC analysis for MB.

Analysis

TPM dyes, GV and MG, as well as the phenothiazine, MB, have recently been studied at some length. Methods have been developed and are available for residue analysis in a variety of matrices due to the knowledge or suspicion of unapproved or extra-labeled use. Metabolic data show that residues may be of either the "chromic" or "leuco" form. Procedures used for monitoring food destined for human consumption should be capable of detecting both forms. Table I is provided as a quick reference for methods discussed in section "Analysis" and "Confirmation".

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

Feeds

Gentian Violet. Methodology is available for the determination and quantitation of GV in feeds(*22-24*). The Rushing and Bowman, 1980, technique consisted of an acidified solvent extraction followed by size exclusion chromatography(*22*). The feed was mechanically shaken for 1 hour with a mixture of MeOH/1 N HCl-(99+1). An aliquot of the filtered supernatant was evaporated to near dryness. This was transferred to a Sephadex LH-20 cleanup column with several aliquots of benzene/MeOH. The column was washed continually with benzene/MeOH. The GV-containing fraction was collected and the eluant evaporated. The sample was redissolved in MeOH for LC analysis by visible mode detection at 588nm. The extractions and isolations (cleanup) used reflected that era. As dangers of benzene and halogenated hydrocarbons became evident changes in this method were needed. In 1989, Martinez and Shimoda(*23*) modified the Rushing /Bowman method to eliminate the use of benzene. Recoveries using this modified procedure remained high with excellent CV values. The elution profile, from a reverse phase C_{18} column, of GV and its demethylated metabolites, PENTA, N-1-TETRA AND N-2-TETRA under the LC conditions described by these investigators was in decreasing polarity. In 1989, we evaluated and reported the use of electrochemical detection(ED) for these compounds. Because of the requirements of ED, LC chromatographic parameters were designed such that the elution profile is reversed(*5*). Applying this LC system to feeds produced a method which eliminated the use of the Sephadex column. It limited solvent usage to acetonitrile and methanol and increased sample output due to its "dilute and shoot" approach. Reduction in solvents, type and volume, decreased the hazardous waste stream requiring disposal, creating a more economical and efficient method. It also provides the option of two detection modes, ED or Visible. Analysis of GV from poultry feed fortified at 5 levels(2-10ppm) produced an overall mean recovery of 86%(n=25); RSD=3.8% with ED detection and 94%(n=25); RSD=2.7% using VIS detection(*24*).

Malachite Green/Methylene Blue. Because of the mode of MG and MB administration, no procedures exist for their analysis in any kind of feed matrix.

Water

Gentian Violet. In addition to feed and urine, Rushing/Bowman(*22*) analyzed waste water for GV. Waste-water is passed through a Sep-Pak C_{18} to trap any GV present. The dye is then eluted with 10% phosphate buffer, pH3/methanol. Triplicate analysis of fortified waste-water at 10ppb yielded average recovery of 60.5%; RSD=2.1%.

Malachite Green. In 1991, Allen and Meinertz(*26*) developed and incorporated a post-column PbO_2 oxidation chamber to detect both chromic and leuco forms of GV and MG simultaneously in the visible range, 588 and 618nm, respectively.

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

TABLE I. Summary of Methods of Analysis of Dyes Discussed in Text.

MATRIX	DYE	DETECTION	DETECTION LIMIT	OBSERVATION	REFERENCE
FEED	GV	LC, C_{18}, MeOH/phosphate buffer, pH3, 588nm	0.01ppm	use of benzene and size exclusion column(Sephadex)	22) Rushing and Bowman, 1980
FEED	GV	LC, C_{18}, MeOH/phosphate buffer, pH3, 588nm	not given, fortified at 2.5, 5 and 10ppm	eliminated use of benzene with Sephadex column	23) Martinez and Shimoda, 1989
FEED	GV	LC, Cyano(CN), ACN/acetate buffer, pH4.5, ED(1V) & 588nm	not given, fortified at 4-8ppm	No Sephadex column used	24) Roybal,*et al.*,1992
WATER	GV	LC, C_{18}, MeOH/phosphate buffer, pH3, 588nm	0.01ppm	preconcentration on Sep-Pak C_{18}	22) Rushing and Bowman, 1980
WATER	MG	LC, Cyano(CN), MeOH/acetate buffer, pH4.5, ED(1.2V)	0.07mg/L(ppm)	adjust pH to 5, preconcentrate on SPE-CN	25) Sagar,*et al.*,1994
WATER	GV/LGV MG/LMG	LC, C_{18}, MeOH/acetate buffer, GV(588nm) and MG(618nm)	2ppb	PbO_2 post-column oxidation	26) Allen and Meinertz,-1991 27) Allen,*et al.*,1992
URINE	GV	LC, C_{18}, MeOH/phosphate buffer, pH3, 588nm	0.001ppm	adjust to pH7, extract into DCM	22) Rushing and Bowman, 1980
TISSUE (chicken)	GV and metabolites	LC, Cyano(CN), ACN/acetate buffer, pH4.5, ED(1V)	5ppb	describes metabolic profile in tissue and fat	8) Roybal,*et al.*,1990 9) Munns,*et al.*,1990
TISSUE (chicken)	LGV	LC, Cyano(CN), ACN/acetate buffer, pH4.5, UV(265nm)	2ppb	applied to fat only	28) Heller,1992
TISSUE (trout)	MG/LMG	LC, C_{18}, MeOH/acetate buffer, PbO_2 oxid., MG(618nm) and scintillation counter	not given, fortified at 0.5, 0.65 and 1ppm	applied to fish eggs, fry and adult tissue of trout	29) Allen,*et al.*,1994

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

TABLE I. continued

MATRIX	DYE	DETECTION	DETECTION LIMIT	OBSERVATION	REFERENCE
TISSUE (catfish)	MG/LMG	LC, Cyano(CN), ACN/acetate buffer, pH4.5, PbO_2, VIS(618nm)	1ppb	applied to MG-incurred catfish	14) Roybal,*et al.*, 1995
TISSUE (trout)	MG	HPTLC, silica gel, ACN/0.01M naphthalenedisulfonic acid	10ppb	Screening test with HPLC /PDA as confirmation	30) Muñoz,*et al.*, 1993
MILK	MB and metabolites	LC, Cyano(CN), ACN/acetate buffer, pH4.5, VIS(627nm)	Lowest fortification at 2.5ppb	preliminary depletion study, MB, azure A,B,C, thionin and MB-organic complex detected.	20) Munns,*et al.*, 1992
MILK	Thionin: metabolite of MB	LC, Cyano(CN), ACN/acetate buffer, pH4.5, VIS(603nm)	1ppb	Depletion of thionin from MB-incurred milk	21) Roybal,*et al.*, 1993
TISSUE (chicken)	LGV	MS confirmation, EI, DB-1 (100% methylsilicone), SIM m/z 126, 237, 252, 253, 372 and 373(molecular ion)	10ppb	confirmatory only for LGV in chicken fat, sample work-up by Munns, et al,(9),1990	32) Wilson,*et al.*, 1994
TISSUE (catfish)	LMG	MS confirmation, EI, 12.5M HP-1 (cross-linked poly[dimethylsilicone), SIM, m/z 330, 329, 253, 210, and 165	10ppb	confirmatory only for LMG in catfish, sample work-up by Roybal, *et al.*,(*14*),1995 with LMG isolation	34) Turnipseed,*et al.*, 1994

This detection approach was applied to the analysis of MG in pond water(*27*). Average recoveries of MG/LMG at the 2ppb fortification level were 95% and 59%, respectively. Sagar, *et al.*(*25*), 1994, analyzed drinking and river water for MG residuals. After adjustment of water samples to pH 5, it was passed through an SPE column for concentration of MG, which is then eluted with mobile phase. ED is used for detection using similar LC parameters described by Roybal, *et al.*(*5*).

Urine

Gentian Violet. Analysis of urine for GV was accomplished by pH adjustment to 7.0 with 6N NaOH. Solution was partitioned with dichloromethane(DCM). The DCM layer was dried with Na_2SO_4, evaporated and the residue was redissolved in MeOH for LC analysis(*22*). This approach could likewise be applied for MG residues in urine.

Tissue

Gentian Violet. In 1990, investigators Roybal and Munns, working concurrently, developed procedures for the analysis of chicken for GV and its metabolites (*8,9*). Both used the LC with ED as the determinative step. For analysis of chicken tissue, liver, thigh muscle and breast muscle, the analytes are extracted overnight from tissue with acetonitrile/buffer. The supernatant is partitioned into DCM. Polar lipids are removed with an alumina column followed by partitioning into DCM from a citrate buffer. The compounds are isolated using a disposable cation exchange column(CBA). GV, LGV and demethylated metabolites are eluted from a CBA column with 0.02% HCl/MeOH. Liquid chromatography(LC/ED) is performed by isocratic elution with a buffered mobile phase from a cyano(CN) column. Amperometric ED detection is performed at +1.000V, glassy carbon vs Ag/AgCl. Average recovery of GV, LGV plus the pentamethyl and tetramethyl metabolites from fortified chicken liver, at 20ppb(n=5) of each was 80.0%(%CV=9), 76.2% (%CV=4), 83.4(%CV=7) and 75.6(%CV=10), respectively. GV-incurred chicken liver yielded residues of GV, pentamethyl and tetramethyl metabolites with mean values(n=10) of 31.2ppb(%CV=9), 34.2ppb(%CV=8) and 39.6ppb(%CV=6), respectively, for an average total residue of 105ppb(%CV=5) with no LGV detected. LGV was detected in the thigh and breast muscle, in addition to the other metabolites found in liver. In thigh, LGV ranged from 2.1ppb-4.6ppb and in breast 0.4ppb-3.7ppb.

Munns, et al(*9*), on the other hand found that chicken fat contained predominantly LGV. The procedure involves extraction with warm DCM and partitioning into acidified aqueous to remove the fat. Buffer to neutral with citrate buffer and extract into DCM. This extract is then analyzed by LC/ED. Average levels of LGV in fat were found to be 49.3ppb(%CV=2). In 1992, Heller(*28*) modified Munns' procedure by using UV detection at 265nm. He reported comparable results.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

Malachite Green. The main concern for MG is its use in aquaculture. To this end, the methods developed have been for analysis of fish for its residues. Allen, *et al.*(*29*), published a method for the analysis of fish eggs, fry and adult rainbow trout for MG. They extracted the sample with 1% acetic acid in either acetonitrile or methanol. This extract is then partitioned with chloroform. The organic layer is drained and dried through Na_2SO_4, evaporated and the residue is redissolved in mobile phase for LC/PbO_2 post-column oxidation/VIS determination. The method was monitored using [C^{14}]MG. Average recoveries, based on total [C^{14}]MG, were: in eggs, 85% and 98% at 0.5ppm and 1.0ppm, respectively; in fry, 68% at 0.65ppm and in muscle, 66% at 1.0ppm.

We recently reported on a method for determining MG/LMG in catfish(*14*). In the method, analytes are extracted from tissue with acetonitrile/acetate buffer, pH 4.5. The extract is partitioned into DCM. Cleanup and isolation is accomplished using a neutral alumina SPE and propylsulfonic acid cation exchange SPE columns. Interconversion of MG to LMG when exposed to fish tissue matrix had been reported(*29*). We suspected that this was most likely being caused by enzyme activity. Hydroxylamine hydrochloride was introduced into the extraction step to inhibit enzyme activity on the fate of MG. Isolation of both MG and LMG was accomplished using a cation exchange solid phase extraction column, propylsulfonic acid (PRS). The compounds are completely retained and efficiently eluted from the PRS-SPE. Recovery of MG/LMG without matrix (standard solution) from the PRS-SPE was 103% and 98.1%, respectively. MG/LMG added to tissue matrix, applied to and eluted from the PRS-SPE, demonstrated average recoveries of 74.2% (CV=8%,n=6) and 88.7%(CV=8%,n=6), respectively. The PRS-SPE column provides retention of MG/LMG utilizing ion exchange properties while minimizing the strong non-polar interactions exhibited by benzenesulfonic acid, SCX-SPE column. We used this characteristic to arbitrarily differentiate and classify PRS as a median range cation exchanger. Hydroxylamine hydrochloride also provided improved elution of MG from the PRS column and better stability of LMG in the LC portion of the assay. The LC is an isocratic elution with a buffered mobile phase from a cyano column. LC analysis coupled with PbO_2 post column oxidation, was performed monitoring the effluent at 618nm. Average recoveries of MG and from fortified catfish tissue at 23, 11 and 5.7ppb were 72.9%(CV=2%), 75.5% (CV=7%), 70.0%(CV=6%), respectively and LMG at 21, 10, and 5.3ppb were 87.4%(CV=3%), 88.1%(CV=6%), 82.6%(CV=12%), respectively. The method was applied to MG-incurred catfish at depuration times of 0, 2, 4, 8 and 24 hours. The average levels of residual MG and LMG in the 24 hour depuration catfish tissue were 73.4ppb and 289ppb, respectively.

Muñoz, *et al.*(*30*), developed a screening for detecting MG in fish. Their method involves extraction of fish tissue, pH4 adjusted with citrate buffer, with acetonitrile. A liquid-liquid partitioning of the extract with salted acetonitrile/DCM is performed. The organic layer is drained and evaporated. The residue is redissolved in acetonitrile and applied to an SPE-silica gel column. MG is eluted with 50/50 methanol/mobile phase. The eluate is defatted with hexane for HPTLC.

HPTLC is done on a silica gel plate with acetonitrile/0.01M naphthalenedisulfonic acid(70+30) solvent system. This screening technique has a reportable detection limit of 10ppb. Recoveries, as monitored by HPLC, were 55-70% at levels of 10, 25 and 50ppb.

Milk

Methylene Blue. The transport of MB through the blood-milk barrier was reported to be rapid in lactating cattle(*19*). Formation of the leuco form of MB appeared to be the mechanism involved. While the presence of LMB in urine had been described(*17*), Munns, *et al.*(*20*), could not verify its presence in milk. The Munns' method involved extraction of milk with acetonitrile by shaking and centrifugation. The supernatant is partitioned with NaCl and $CHCl_3$. The organic layer, containing any MB and azures residues, is evaporated to dryness. The aqueous layer, containing any thionin residue, is adjusted to pH10 with NaOH and extracted with $CHCl_3$. This organic layer is also taken to dryness. Each residue is treated in a like manner from this point. The residues are redissolved in acetonitrile, applied to an CBA-SPE column and eluted with 1N HCl/MeOH(1 + 99). Eluates are evaporated to dryness, dissolved in 0.2 mL acetonitrile and injected into the LC. LC monitoring of the column effluent was done at 627nm, due to the fact that each component had a different UV/VIS maximum based on the degree of demethylation (see ref. *31*, Roybal, 1992).

Munns(*20*) contended "*MB had undergone complex chemical changes resulting not only in demethylated metabolites, i.e., the azures and thionin but also in MB-organic complexes that complicated the clean-up procedure.*" It was also stated "*Complexed MB may account for some of the color in the frozen milk, but this could not be determined because no method presently exists for assaying complexed MB. The methodology presented here for MB and its metabolites in milk indicates that additional research is required to develop a method for the assay of complexed MB.*"

Based on the findings of Munns, *et al.*(*20*), we proceeded to investigate analysis of this MB-organic complex. In 1993, we presented and published a method for the analysis of Thionin in milk(*21*). From our investigation, it was determined that thionin was the major metabolite with the longest residence time, generated in milk from dairy cattle treated with MB. The organic complex appeared to be a thionin-protein conjugate. Our method involves extraction of milk with acetonitrile and hydrolysis of the supernatant to release the thionin-protein complex. The hydrolyte is made basic with NaOH and partitioned with DCM. The organic layer is evaporated to dryness and redissolved in mobile phase for LC analysis. LC is achieved by isocratic elution with a buffered mobile phase using a Phenomenex CN (cyano) column with detection at 603nm. Average recovery of thionin from fortified control milk at 5, 11, 21 and 54ppb was 60.9%(CV=7%), 54.3% (CV=12%), 57.0% (CV=5%) and 51.3%(CV=10%), respectively. The overall recovery was 56.0%(CV=10%). MB-incurred milk at post-dosing times of 8, 24,

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

32, 48, 56 and 72 hours was analyzed by this procedure. The average residual thionin in the 72 hour post-dosing milking was 27ppb.

Confirmation

Gentian Violet. Currently only one method exists for the structural confirmation of GV residues, as the LGV metabolite, in animal product tissue matrix. Wilson, *et al.*(*32*), developed and published a GC/MS method for LGV in chicken fat. The method uses the sample solution prepared for the LC determinative procedure of Munns, *et al.*(*9*), described earlier. The LC solution is partitioned with toluene. The organic phase is evaporated to dryness and reconstituted in acetonitrile. This solution is analyzed by GC/MS. Using EI/MS, six of the most abundant ions are monitored by SIM. These ions are m/z 126, 237, 252, 253, 372 and 373(molecular ion). The requirements of a minimum of three ions and a 10% relative abundance compared to standard material are satisfied for positive structural confirmation(*32*). This procedure was validated through the use of control, fortified and incurred chicken fat samples at the 10ppb level for LGV.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

Malachite Green. Turnipseed, *et al.* in 1994, reported on the comparison of GC/MS and particle beam LC/MS methods for confirming MG and LMG in catfish tissue(*33*). The LC/MS involves modification of the LC determinative method(*14*). The modifications consist of 1) use of ammonium acetate in place of the nonvolatile sodium acetate for the buffer preparations and 2) eliminating hydroxylamine hydrochloride from the SPE-PRS elution scheme. For the LC/MS a high carbon load semi-micro C_{18} column with an acetonitrile/ ammonium acetate buffer (80+20) mobile phase produced the desired chromatography. Use of the C_{18} column allowed for an increase in modifier needed to elute the compounds for obtaining the best performance from the particle beam interface. For GC/MS no modifications were necessary. After the LC determination, the extract is retained on SPE-CN column. The column is selectively eluted with mobile phase without counter ion, to isolate LMG. The eluate is extracted into DCM, dried through Na_2SO_4, evaporated to dryness and reconstituted in methanol for GC/MS.

The LC/MS confirmation will identify both MG and LMG. The GC/MS procedure is used to identify only LMG, the major metabolite, in the sample. Both the particle beam LC/MS and the GC/MS take advantage of selected ion monitoring (SIM), using ion at m/z 330, 329, 253, 210, and 165 with a dwell time of 50-75 ms/ion(LC) and a dwell time of 50 ms/ion(GC). Turnipseed demonstrated that either Particle Beam LC/MS or GC/MS can be used to confirm residues of MG in catfish. The advantage of LC/MS is the ability to identify both the chromic and leuco forms of MG. The drawback to LC/MS is the limit of detection for residual MG is 20 ppb or higher in tissue. Additionally, with particle beam, larger variation in the relative abundances of the ions is noted in the chromic dye due to reactions occurring in the mass spectrometer source. The GC/MS can only identify the leuco form, but LMG appears to be the marker residue for MG usage in catfish. The

detection limits are reported to be 5 ppb. The GC/MS procedure was applied to MG-incurred catfish(*34*). This positive structural identification approach was selected as the method of choice for confirmation of suspected use of MG in catfish.

Future Work

In the area of dyes used in animal husbandry, method development work continues. The need for quantitative as well as confirmatory methods exists to determine the fate of dye when administered to animals. Our current work is directed at 1) Methylene blue in aquatic species, 2) Methylene blue in bovine tissue, 3) Acriflavine/proflavine in aquatic species and 4) Malachite green in trout, salmon and shrimp. Other investigators are concerned and are working on Gentian violet in aquatic species. Concurrently with a quantitative methods, confirmatory procedures will be developed for MB/azures/thionin and Acriflavine/proflavine.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

Literature Cited

1) Reese, K.(1994). *Today's Chemist at Work*, **3**(1), 45-46.
2) Huber, W.G.(1982). in *Veterinary Pharmacology and Therapeutics*, Editors Booth, N.H. and McDonald, L.E.,"Antiseptics and Disinfectants", The Iowa State University Press, Ames, IA, Chp.47, 693-716.
3) Sollmann, T.(1964). *A Manual of Pharmacology*, Eighth Ed., W.B. Saunders Company, Philadelphia, PA., 570 and 828-837.
4) Harvey, S.C.(1975). in *The Pharmacological Basis of Therapeutics*, Editors Goodman, L.S.and Gilman, A.,"Antiseptics and Disinfectants; Fungicides; Ectoparasiticides", MacMillan Publishing, New York, NY, Chp.50, 987-1017.
5) Roybal, J.E., Munns, R.K., Hurlbut, J.A., Shimoda, W.(1989) *J. Chromatog.*, **467**(1), 259-266.
6) McDonald, J.J., Breeden, C.R., North, B.M., Roth, R.W.(1984) *J. Agric. Food Chem.*, **32**(3), 596-600.
7) McDonald, J.J.(1985) "Metabolism of Gentian Violet in Chickens" NCTR Technical Report for Experiment No. 6040.
8) Roybal, J.E., Munns, R.K., Hurlbut, J.A., Shimoda, W.(1990) *J. Assoc. Off. Anal. Chem.*, **73**(6), 940-946.
9) Munns, R.K., Roybal, J.E., Hurlbut, J.A., Shimoda, W.(1990) *J. Assoc. Off. Anal. Chem.*, **73**(5), 705-708.
10) Littlefield, N.A.(1984) "Chronic Toxicity and carcinogenicity studies of gentian violet in mice" NCTR Technical Report for Experiment No. 304.
11) Culp, S.J.(1994) "Malachite Green: A Literature Review", Reviewed for the NCTR/TSSRC/NTP, August 1994, Jefferson, AK 72079
12) Alderman, D.J., Clifton-Hadley, R.S.(1993) *J. Fish Dis.* **16**(4), 297-311.
13) Bauer, K., Dangschat, H., Knoeppler, H.O., Neudegger, J.(1988) *Arch. Lebensmittelhyg.* **39**(4), 97-102.

14) Roybal, J.E., Pfenning, A.P., Munns, R.K., Holland, D.C., Hurlbut, J.A., Long, A.R.(1995) *J. AOAC Int.* **78**,(2), 453-457.

15) Chung, K-T, Fulk, G.E., Andrews, A.W.(1981) *Appl. Environ. Microbiol.*, **42**(4), 641-648.

16) Plumb, D.C.(1991) *Veterinary Drug Handbook*, Pocket Edition, 634-635.

17) DiSanto, A.R. and Wagner, J.G.(1972) *J. Pharm. Sci.*, **61**(4), 598-602.

18) DiSanto, A.R. and Wagner, J.G.(1972) *J. Pharm. Sci.*, **61**(7), 1086-1090.

19) Ziv, G. and Heavner, J.E.(1984) *J. Vet. Pharmacol. Ther.*, **7**(1), 55-59.

20) Munns, R.K., Holland, D.C., Roybal, J.E., Meyer, J.G., Hurlbut, J.A., Long, A.R.(1992) *J. AOAC Int.,* **75**(5), 796-800.

21) Roybal, J.E., Munns, R.K., Holland, D.C., Hurlbut, J.A., Long, A.R.(1993) *Residues of Veterinary Drugs in Food*, Vol. 2, Haagsma, N., Ruiter, A.,Czedik-Eysenberg, P.B. (Eds), Utrecht University Faculty of Veterinary Medicine, Utrecht, Netherlands, 601-605.

22) Rushing, L.G. and Bowman, M.C.(1980) *J. Chromatogr. Sci.*, **18**(5), 224-232.

23) Martinez, E.E. and Shimoda, W.(1989) *J. Assoc. Off. Anal, Chem.*, **72**(5), 742-745.

24) Roybal, J.E., Munns, R.K., Holland, D.C., Burkepile, R.G. and Hurlbut, J.A.(1992). *J. AOAC Int.,* **75**(3), 433-437.

25) Sagar, K., Smyth, M.R., Wilson, J.G., McLaughlin, K.(1994) *J. Chromatog. A*, **659**(2), 329-336.

26) Allen, J.L. and Meinertz, J.R.(1991) , *J. Chromatog.*, **536**(1-2) 217-222.

27) Allen, J.L., Meinertz, J.R., Gofus, J.E.(1992) *J. AOAC Int.*, **75**(4), 646-649.

28) Heller, D.N.(1992) *J.AOAC Int.*, **75**(4), 650-654.

29) Allen, J.L., Gofus, J.E. and Meinertz, J.R.(1994). *J. AOAC Int.,* **77**(3), 553-557.

30) Muñoz, P., Reuvers, TH. Martin De Pozuelo, M., Marcos, M[a] V.(1993) *Residues of Veterinary Drugs in Food,* Vol. 2, Haagsma, N.; Ruiter, A.; Czedik-Eysenberg, P.B. (Eds), Utrecht University Faculty of Veterinary Medicine, Utrecht, Netherlands, 504-508.

31) Roybal, J.E., Munns, R.K., Holland, D.C., Hurlbut, J.A., Long, A.R.(1992) in *Analysis of Antibiotic Drug Residues in Food Products of Animal Origin*, Agarwal, V.K. (Ed), Plenum Press, New :York, NY, 197-210.

32) Wilson, R.T., Wong, J., Johnston, J., Epstein, R., Heller, D.N.(1994) *J. AOAC Int.*, **77**(5), 1137-1142.

33) Turnipseed, S.B., Roybal, J.E., Rupp, H.S., Hurlbut, J.A., Long, A.R.(1994) *J. Chrom. B: Biomed. Appl.*, Submitted for publication 11/8/94.

34) Turnipseed, S.B., Roybal, J.E., Hurlbut, J.A., Long, A.R.(1994). *J. AOAC Int.*, Accepted for publication, 1994.

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ch018

Author Index

Anderson, K. L., 58
Beconi-Barker, Maria G., 70
Benedicto, Janet, 144
Berkowitz, David B., 31
Boison, Joe O., 64
Bruhn, Christine M., 18
Carson, Mary C., 108
Charm, S. E., 149
Chu, Pak-Sin, 108
Cooper, J., 5
Crawford, Lester M., 1
Cullor, James S., 44
Dietrich, R., 121
Gatchell, Cathy L., 70
Gilbertson, Terry J., 70
Harik-Khan, Raida, 85,96
Hornish, Rex E., 70
Hurlbut, J. A., 169
Keng, Lily, 64
Korsrud, Gary O., 64
Long, A. R., 169
Lopez-Avila, Viorica, 144
MacNeil, James D., 64
Märtlbauer, E., 121
Medina, Marjorie B., 132
Moats, William A., 58,85,96
O'Carroll, J., 58
O'Rangers, John J., 31
Pfenning, A. P., 169
Quintana, J., 149
Roof, Ryan D., 70
Roybal, J. E., 169
Rushing, J. E., 58
Salisbury, Craig D. C., 64
Saul, S., 149
Scheemaker, J., 149
Shaikh, Badar, 161
Smith, Evan B., 70
Stephany, R. W., 22
Sundlof, S. F., 5
Turnipseed, S. B., 169
Usleber, E., 121
Vidmar, Tom J., 70
van Ginkel, L. A., 22
von Bredow, Jurgen, 108
Zomer, E., 149

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix001

Affiliation Index

Agricultural Research Service, 58,85,96,132
Agriculture and Agri-Food Canada, 64
Association of American Veterinary Medical Colleges, 1
Charm Sciences Inc., 149
Midwest Research Institute, 144
North Carolina State University, 58
Pharmacia and Upjohn, Inc., 70
RIVM/ARO (Netherlands), 22
U.S. Department of Agriculture, 58,85,96,132
U.S. Food and Drug Administration, 5,31,108,161,169
University of California—Davis, 18,44
University of Munich, 121

In Veterinary Drug Residues; Moats, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1996.

Subject Index

A

Acidification of milk, diuretic drug determination in food-producing animals, 165
Acridines, description, 170
Affinity chromatography, 125
β-Agonists, 23–24
Alar, consumer's perceptions and concerns, 19–20
N-Alkylphenylethanolamines, illegal use in slaughter animals, 23
Allergic reactions, reasons, 6
Amoxicillin, HPLC interface with rapid screening kits for detection in milk, 99–101
Ampicillin, HPLC interface with rapid screening kits for detection in milk, 99–101
Anabolic agents
- growth-promoting purposes in slaughter animals, ban in Netherlands, 24
- hormonal, efficacy for muscle growth promotion, 22–24
- restricted controlled use, 24

Analytical method evaluation within context of use
- concept, 31,108
- screening tests, 32–42

Analytical-scale supercritical fluid extraction, 144
Animal care and management, consumer's perceptions and concerns, 19
Animal-derived foods, human health risks associated with drug residues, 5–14
Animal drug(s), *See* Drugs for animals
Anti-contamination goal procedure, technology, 3–4
Antibiotic(s)
- analysis using immunoaffinity chromatography, 121–131
- consumer's perceptions and concerns, 18–19

Antibiotic(s)—*Continued*
- particle concentration fluorescence assays for rapid detection of trace levels, 132–142
- testing in milk, 45–55,86–94*t*

Arla microtest, performance, 47
Azo derivatives, description, 170

B

Bacterial resistance of drugs, 8–10
Bovine somatotropin
- consumer's perceptions and concerns, 19
- human health risks, 14

Broad spectrum microbial inhibition tests, veterinary drug residue screening, 150
Bromobuterol, illegal use in slaughter animals, 23

C

Calf Antibiotic and Sulfa Test, screening for antimicrobial residues, 64
Capillary zone electrophoresis, diuretic drug determination in food-producing animals, 165–166
Carbonic anhydrase inhibitors, 161
Ceftiofur
- applications, 70
- HPLC interface with rapid screening kits for detection in milk, 101,102*t*
- HPLC–receptorgram for identification, 149–159
- metabolism following intramuscular treatment, 70–71,73

Ceftiofur sodium in tissue
- concentration, 72*t*,73
- excretion, 72*t*,73
- HPLC determination, 74–82
- metabolite profiles, 70,74

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002

Center of Veterinary Medicine–U.S. Food and Drug Administration
guidelines for residue assay evaluation protocols, 45
search for determinative procedure for β-lactam residues in milk, 109
test kit evaluation process, 48–51
Cephapirin, HPLC interface with rapid screening kits for detection in milk, 99–101,104*f*
Charm Farm test
advantages, 69
function, 64
penicillin G detection, 65–68
performance, 46–47
Charm II test
β-lactam detection, 110–111
oxytetracycline detection in milk residues, 58–63
performance, 47
tetracycline detection, 85
Chloramphenicol
HPLC interface with rapid screening kits for detection in milk, 101,103*t*,105
HPLC–receptorgram for veterinary drug identification, 149–159
human health effect, 7–8
Chlorothiazide, determination in food-producing animals, 161–166
Chlortetracycline
HPLC–receptorgram for identification, 149–159
residues in milk, 61–62
CITE Probe, performance, 46–47
Clenbuterol
human health risks, 14
illegal use in slaughter animals, 23
Cloxacillin, HPLC interface with rapid screening kits for detection in milk, 101,102*t*
Confirmatory procedure, description, 109
Consumer's perceptions and concerns, veterinary drug residues, 18–20

D

Delaney Amendment of U.S. Food, Drug, and Cosmetic Act, 12
Delvotest P kits
interfacing with HPLC for β-lactam residue measurement, 97–106
performance, 46–47
Desacetylcephapirin, HPLC interface with rapid screening kits for detection in milk, 101,103*t*,104*f*
Desfuroylceftiofur
detection using HPLC, 72–82
metabolism, 70–71,73
microbiologically active metabolite of ceftiofur, 70
Diethylstilbestrol, link to use of hormonal growth-promoting agents, 13
Disc assay, performance, 46–47
Diuretic(s)
determination in food-producing animals
analytical methods, 164–168
characteristics, 163–164
examples approved for use, 162*f*,163
safe milk levels, dose, and withdrawal times, 163,164*t*
function, 161
unapproved uses, 163
Drug(s) for animals
bacterial resistance, 8–10
consumer's concern, 18
diuretic, 161–168
European Union regulatory residue analysis, 22–28
human health risks, 5–14
safety, 1,20
screening tests, 31
Dry milk powder, veterinary drug determination by SCFE–enzyme-linked immunoassays, 144–148
Dye(s)
analysis
feeds, 175
milk, methylene blue, 180–181
tissue, 178–180
water, 175,178
wine, gentian violet, 178
analytical development, 170
categories with medicinal benefit, 170
confirmation, 181–182
metabolism, 173–174
residues in foods, animal origin, 169–182

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002

E

Enzyme-linked immunoassays, 145
Escherichia coli O157:H7, consumer's perceptions and concerns, 20
Estradiol, human health risks, 11–12
Ethacilin, 64–69
European Union regulatory residue analysis of veterinary drugs
 community reference laboratories, 25–27
 performance and competence validation, 28
 residue and contaminant determination–identification, 25,26*f*
 residue control program, 24–25

F

False positive problem, antibiotic residue testing in milk, 44–56
Fluorescence assays for rapid detection of trace levels of antibiotics, *See* Particle concentration fluorescence assays for rapid detection of trace levels of antibiotics
Fluoroquinolone, drug resistance, 10
Food(s)
 animal derived, human health risks associated with drug residues, 5–14
 dye residues from animal origin, 169–182
Food-borne drug residues, effect on human health
 chloramphenicol, 7–8
 β-lactams, 6–7
Food-producing animals, diuretic drug determination, 161–166
Furosemide, determination in food-producing animals, 161–166

G

Gas chromatography–mass spectrometry, determination of dye residues in foods from animal origin, 169–182
Gentian violet, residues in foods from animal origin, 169–182
Government regulation of drugs, consumer's perceptions and concerns, 19
Grade A Pasteurized Milk Ordinance, residue monitoring and farm surveillance, 45–46
Growth hormone, 14

H

Health risks associated with drug residues in animal-derived foods, human, *See* Human health risks associated with drug residues in animal-derived foods
High-performance liquid chromatography
 analysis of oxytetracycline in milk residues, 58–63
 determination of ceftiofur sodium in tissue, 76–82
 determination of dye residues in foods from animal origin, 169–182
 interface with rapid screening kits for β-lactam residue detection in milk, 99–106
 ion pairing, tetracycline antibiotic detection in milk and tissues, 85–94
 receptorgram for veterinary drug identification, 150–159
Hormonal anabolic agents, efficacy for muscle growth promotion, 22–24
Hormonal growth-promoting drugs, human health risks, 10–11
Hormonal no-effect levels, basis for human food safety assessment and residue tolerance level establishment, 12
Hormone ban, increase in black market sale and use of growth-promoting drugs, 14
Hormone residues, consumer's concern, 18
Hormone usage, differences in approach and attitude, 22
Human health risks associated with drug residues in animal-derived foods
 bacterial resistance, 8–10
 food-borne drug residue effect on human health, 6–8

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002

Human health risks associated with drug residues in animal-derived foods—*Continued*
hormonal growth-promoting drugs, 10–11
naturally occurring hormones as growth-promoting agents, 11–12
production drugs, 10–11
somatotropic hormones, 14
synthetic hormonal growth-promoting agents, 12–14
Hydrochlorothiazide, determination in food-producing animals, 161–166

I

Immunoaffinity chromatography
antibiotic and sulfonamide analysis, 122–131
confirmation of screening tests, 41
Immunoassays, limitations, 132
Immunochemical techniques, confirmation of screening tests, 41–42
Industrial chemicals, sources of violative residues, 2
Industry groups, fueling of consumer's perceptions and concerns, 19
Ion pairing
separation of tetracyclines from interferences in milk, 86,89*t*
technique for changing retention time of analytes, 86
Ion-pairing high-performance liquid chromatography, tetracycline detection in milk and tissues, 85–94

L

β-Lactam(s)
detection and measurement using HPLC interface with rapid screening kits, 96–106
determinative procedures
Charm II test, 110–111
comparison of procedures, 119–120
general experimental design, 109
β-Lactam(s)—*Continued*
determinative procedures—*Continued*
LC analysis
triazole–mercuric chloride derivatives, 113–115*f*
use of automated LC cleanup, 113,116–118
Meetschen and Petz GC procedure, 111–113
HPLC–receptorgram for identification, 149–159
human health effect, 6–7
mandatory screening of milk, 96
methods for screening and quantitation, 133
need for confirmatory methods to check authenticity of screening kits, 97
regulatory method development for detection in milk, 108–120
Lactating cattle, oxytetracycline administration effect on milk residues, 58–63
LacTek Ceftiofur kits, interfacing with HPLC for β-lactam residue measurement, 97–106
LacTek β-lactam kits, interfacing with HPLC for β-lactam residue measurement, 97–106
Lincomycin, HPLC interface with rapid screening kits for detection in milk, 101,103*t*,105
Liquid chromatography
comparison to Charm Farm test of penicillin G detection in hogs, 64–69
diuretic determination in food-producing animals, 165–166
β-lactam determination, 113–118
tetracycline determination, 86
Liquid chromatography–mass spectrometry, determination of dye residues in foods from animal origin, 169–182
Loop diuretic, determination in food-producing animals, 161–166

M

Malachite green, residues in foods from animal origin, 169–182

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002

Mapenterol, illegal use in slaughter animals, 23
Matrix solid-phase dispersion, 42
Maximum residue limits, establishment, 121–122
Meetschen and Petz gas chromatography procedure, β-lactam determinative procedures, 111–113
Metabolism, dye residues in foods from animal origin, 169–182
Methimazole, human health risks, 14
Methylene blue, residues in foods from animal origin, 169–182
Micellar chromatography, diuretic determination in food-producing animals, 166
Microbial inhibition assays, 132
Microorganisms, consumer's perceptions and concerns, 20
Milk
  antibiotic residue testing, 44–56
  HPLC interface with rapid screening kits for β-lactam residue detection, 96–106
  HPLC–receptorgram for veterinary drug identification, 149–159
  oxytetracycline administration effect on residues in lactating cattle, 58–63
  regulatory method development for determination of β-lactams, 108–120
  tetracycline residue detection, 58,85–94
Monoclonal antibodies, analysis of antibiotic and sulfonamide analysis, 121–131
Multi-immunoaffinity chromatography, antibiotic and sulfonamide analysis, 121–131
Multiresidue procedures, development for determination of β-lactam antibiotics in milk, 108–120

N

Nandrolone, levels in slaughter animals, 22–23
Naturally occurring hormones used as growth-promoting agents, human health risks, 11–12
NAXEL–EXCENEL Sterile Powder, 70
Nortestosterone, illegal use in slaughter animals, 23
Novobiocin, HPLC interface with rapid screening kits for detection in milk, 101,103*t*,105

O

Oxytetracycline, effect on milk residues in lactating cattle, 58–63

P

Particle concentration fluorescence assays for rapid detection of trace levels of antibiotics
  advantages, 141,143
  experimental description, 132–134
  penicillin G
    assay procedure, 135,137
    results, 139–142
  sensitivity, 141
  spectinomycin, 134–139
Penicillin G
  evaluation and testing of Charm Farm test with tissues and fluids from hogs, 64–69
  HPLC interface with rapid screening kits for detection in milk, 99–101,103*t*
  particle concentration fluorescence assays for rapid detection, 132–142
  structure, 133,136*f*
Penzyme, performance 47
Phenothiazine congeners, 170
Pig tissues, HPLC–receptorgram for veterinary drug identification, 158*t*,159
Porcine somatotropin, 14
Potassium-sparing diuretics, 161
Preharvest food safety, agreement between consumer and producer, 44
Premature thelarche, link to use of hormonal growth-promoting agents, 13
Procaine penicillin G, evaluation and testing of Charm Farm test with tissues and fluids from hogs, 64–69

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002

Production drugs, human health risks, 10–11
Progesterone, human health risks, 11–12
Proteins with binding properties to antibiotics, advantages for use in rapid detection methods, 132–133

R

Regulatory method, development for determination of β-lactams in milk, 108–120
Regulatory residue analysis of veterinary drugs, *See* European Union regulatory residue analysis of veterinary drugs

S

Safety of drugs, information sources, 20
Salmonella infections, drug resistance, 9
Screening tests
- advantages, 32
- assessment, 39–42
- criteria, 32–33
- development for particular purpose, 33–35
- for β-lactams, 97
- information needed for use and evaluation, 35–36
- procedures, 108–109
- sensitivity, 36–38
- specificity, 38–39

Solid-phase extraction
- confirmation of screening tests, 42
- diuretic determination in food-producing animals, 166

Somatotropic hormones, human health risks, 14
Spectinomycin
- analytical methods, 133
- particle concentration fluorescence assays for rapid detection, 132–142
- structure, 133,136*f*

Streptomycin, analysis using immunoaffinity chromatography, 121–131
Sulfadiazine, analysis using immunoaffinity chromatography, 121–131
Sulfamethazine
- determination in dry milk powder by SCFE–enzyme-linked immunoassays, 144–148
- HPLC interface with rapid screening kits for detection in milk,101,103*t*,105
- HPLC–receptorgram for identification, 149–159
- immunoaffinity chromatography for analysis, 121–131

Sulfonamides, analysis using immunoaffinity chromatography, 121–131
Supercritical fluid extraction–enzyme-linked immunoassay, veterinary drug determination in dry milk powder, 144–148
Swab Test on Premises, screening for antimicrobial residues, 64
Synthetic hormonal growth-promoting agents, human health risks, 12–14

T

Ten Point Milk and Dairy Beef Quality Program, 96–97
Testosterone, human health risks, 11–12
Tetracycline(s)
- administration routes, 58–59
- applications, 85
- detection
  - ion pairing HPLC, 86–94
  - Charm II test, 85
- HPLC interface with rapid screening kits for detection in milk, 101,103*t*,105
- HPLC–receptorgram for identification, 156,157*f*
- residues in milk, 58

Therapeutic drugs, impact of bacterial resistance, 9–10
Thiazide diuretics, determination in food-producing animals, 161–166

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002

Tissue
- ceftiofur sodium determination, 70–82
- tetracycline detection using ion-pairing HPLC, 85–94

Trichlormethiazide, determination in food-producing animals, 161–166

Triphenylmethane, description, 170

Tylosin, HPLC interface with rapid screening kits for detection in milk, 101,103*t*,105

U

U.S. Department of Agriculture
- Food Safety and Inspection Service responsibility for drugs, 1–2
- STOP test, 33

U.S. Environmental Protection Agency, responsibility for drugs, 2

U.S. Food and Drug Administration, illegal drug residue concerns, 5–6

V

Valio T101 assay, performance, 47

Veterinary drug(s)
- consumer's perceptions and concerns, 18–20
- determination in dry milk powder by SCFE–enzyme-linked immunoassays
  - enzyme-linked immunoassay performance, 146*t*,147–148
  - experimental procedure, 146–147
  - optimization of SCFE conditions, 146*t*,147
  - verification, 148
- European Union regulatory residue analysis, 22–28
- identification using HPLC–receptorgram, 149–159
- monitoring requirements, 150
- occurrence in food, 121
- screening tests, 149

Z

Zeranol, restricted controlled use, 24

September 10, 2012 | http://pubs.acs.org
Publication Date: May 5, 1996 | doi: 10.1021/bk-1996-0636.ix002