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Review

Chromatographic methods of analysis for penicillins in food-animal tissues and their significance in regulatory programs for residue reduction and avoidance

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ABSTRACT

Chromatographic methods for penicillin analysis in animal tissues play a significant role in the regulation of the use of these drugs in livestock production. Regulatory agencies rely on data generated from these methods to establish withdrawal times and to determine whether presumptive positive tissue samples from slaughtered animals intended for human consumption contain violative levels of penicillins to necessitate regulatory action. The need to develop sensitive, accurate, and reliable methods to support regulatory programs is examined together with emerging techniques that could be taken advantage of to improve the sensitivity and usefulness of current chromatographic methods for tomorrow's regulatory agency.

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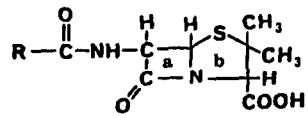
1. INTRODUCTION

Penicillins and cephalosporins belong to the group of antimicrobial drugs commonly referred to as β-lactam antibiotics. In this review, only penicillins will be discussed. Penicillins contain the basic β-lactam ring (a) coupled to a thiazolidine ring (b) (see Fig. 1) to form 6-aminopenicillanic acid, generally referred to as the "penicillin nucleus". To the 6-aminopenicillanic acid moiety are attached side-chains (R), which determine the stability and the spectrum of antimicrobial and pharmacological activities of the different β-lactams. Penicillins are classified as either natural or synthetic; they may also be classified on the basis of their resistance to gastric acidity and penicillinases or as broad spectrum. Penicillin G (benzylpenicillin) and penicillin V (phenoxymethylpenicillin), produced metabolically by molds of *Penicillium notatum* and *Penicillium chrysogenum*, are the natural penicillins. The amino (ampicillin, amoxicillin) and isoxazolyl (oxacillin, cloxacillin, dicloxacillin and methicillin) derivatives of penicillin are semi-synthetic. Phenethicillin and penicillin V are acid-resistant, the isoxazolyl penicillins are penicillinase-resistant while ampicillin, amoxicillin, hetacillin, carbenicillin and ticarcillin belong to the broad spectrum group of penicillins. Fig. 1 shows the basic penicillin structure and the chemical structures of the other penicillins commonly used in veterinary medicine for food-producing animals.

Penicillins are administered by various routes for the control of mastitis in dairy cows and for treating infections of several other tissues in cattle, such as the urinary, gastrointestinal and respiratory tracts. They may also be added to swine feed and to the drinking water of poultry. Generally, penicillin drug formulations are designed to be used according to the approved label dose specifications, which include the dosing rate and frequency (pharmacokinetic values) as well as the route of administration. The veterinary medical profession attends to many species of animals, with highly significant variations in physiology and pathology. Even within the same species, considerable variation might occur in pharmacokinetic values when sick animals are treated [1]. As a result, it is virtually impossible to have an approved drug labelled for every disease condition in animals, and extra-label drug use (*i.e.*, use deviating from the drug label's directions on route of administration, dosage, duration of treatment, species, or indicated disease condition) is therefore a reality of veterinary practice. Penicillins are also administered in lower dosages (prophylactically) to prevent diseases in exposed animals and at subtherapeutic levels in animal feeds to improve feed efficiency and growth of livestock.

The legitimacy of drug use, especially at extra-label dosage rates in food-producing animals, has been questioned by several groups, including those interested in animal welfare and animal rights, those concerned about the safety of the individuals administering the drugs, and those concerned about the perceived potential for human health risks from drug residues in tissues. Health risks usually cited include the development of resistant bacteria by gene transfer to the progeny via the chromosome and gene transfer on R-plasmids in animals treated with penicillin [2], the development of penicillin-resistant pathogenic bacteria in the consumer as a result of increased intake of penicillin residues in animal tissues, and the development of hypersensitivity in some individuals to this drug. Most of the documented cases of allergic responses to the ingestion of penicillin-contaminated food have been linked to milk and milk products [3], but a few cases of penicillin-implicated allergic reactions from meat [3–6] are known (Table 1). In addition, survey



BASIC PENICILLIN NUCLEUS

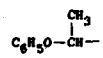
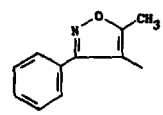
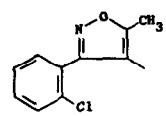
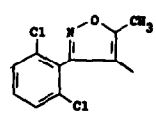
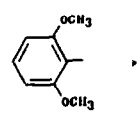
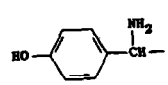
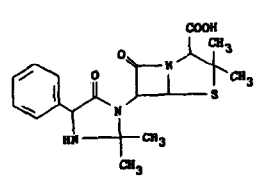
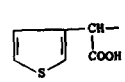
CLASSIFICATION	R	GENERIC NAME
NATURAL	$C_6H_5OCH_2-$	PENICILLIN G
NATURAL, ACID RESISTANT	$C_6H_5CH_2-$	PENICILLIN V
SYNTHETIC, ACID RESISTANT		PHENETHICILLIN
SYNTHETIC, ISOXAZOLYL		OXACILLIN
		CLOXACILLIN
		DICLOXACILLIN
		METHICILLIN
SYNTHETIC, BROAD SPECTRUM		AMPICILLIN
		AMOXICILLIN
		HETACILLIN
		CARBENICILLIN
		TICARCILLIN

Fig. 1. Chemical structures of penicillins commonly used in veterinary medicine for food-producing animals.

TABLE 1

DOCUMENTED CASES OF PENICILLIN-INDUCED ALLERGIC REACTIONS FROM MEAT

Year	Comments	Symptoms
1972	Male victim had previously been treated with penicillin. A pig was slaughtered 3 days after penicillin injection. Victim (a butcher) consumed one bite of meat from the pig and suffered the reaction [3]	Pruritus on face and fingers, generalized rash
1972	A penicillin-allergic patient suffered an acute allergic reaction in West Germany after ingesting freshly processed pork. The meat, which was found to contain 0.3–0.45 U/g penicillin residues, had been eaten from a pig that had been treated with therapeutic levels of penicillin 3 days before slaughter [4]	Angioedema and pruritus
1980	A 45-year-old American female, with prior history of penicillin allergy, suffered an anaphylactic reaction following the ingestion of a frozen meat dinner. Beef was found to contain residues of penicillin [5]	Generalized pruritus, difficulty swallowing, speaking and throat tightness
1981	Two females, aged 37 and 42 years, who had prior history of penicillin allergies, got sick after eating raw pork from a pig that was experimentally injected with 3 million U of penicillin for 3 days and slaughtered. The meat was fed to a group of nine volunteers which included the two female victims [3]	Pruritus on face
1989	A 35-year-old female from Calgary suffered allergic reaction after eating about 4 ounces of roast beef purchased from a local grocery store. The roast was found to contain penicillin G and sulpha residues. Producer did not follow prescribed withdrawal times [6]	Patient broke out in hives and became very sick

studies conducted by regulatory agencies continue to detect penicillin residues in animal tissues at slaughter [7–10]. Why are penicillin residues being detected in tissues of food-producing animals?

Several papers dealing with the reasons for the incidence of antimicrobial residues in meat have been published [3,11,12], including the recent report by Van Dresser and Wilcke [8] on the state of residues in food animals in the USA. Observations made over the years at the Health of Animals Laboratory from the analysis of presumptive positive tissue samples [7] and field operation investigations in Canada report similar findings. According to the US report, penicillin, streptomycin, neomycin, oxytetracycline and sulphamethazine are the most frequently detected residues in animal tissues in the USA. Residues found were predominantly in cows, veal calves and market hogs (barrows and gilts). The reason most frequently cited by field investigators for drug residues was failure to observe withholding time for the drug; in over 80% of the cases for which responsibility was determined, the producer was considered the responsible party.

Pharmacokinetic data developed by manufacturing companies for drug approval are generated for drugs use at the label dose only. For food-producing animals, it is required that, prior to slaughter, an animal be withdrawn from treatment for a recommended period. There are no defined withdrawal periods, however, when penicillin formulations are used in an extra-label manner. It is believed that the unavailability of recommended withholding periods for penicillin use at extra-label doses has largely contributed to the presence of residues in food-producing animals.

These observations and the fact that the livestock industry is a multi-billion dollar industry with substantial global trade ramifications have prompted regulatory agencies in countries throughout the world to take measures not only to safeguard the consumer against any health risks from contaminated meat, but also to ensure that trade restrictions which affect the survival of the industry do not occur.

It is in support of this regulatory process that the analysis of penicillin G residues in animal tissues becomes important. This paper reviews the chromatographic methods of chemical analysis that have so

far been developed for the determination of penicillin residues in animal tissues and examines the role they play in regulatory programs in helping to allay the fears and concerns of the public. Also, anticipated trends in improvements to currently available methods developed for the analysis of penicillin residues in animal tissues are examined.

1.1. Development of methods for the assay of penicillins

Methods are developed for the analysis of penicillins in blood [13–20], urine [15,18,21] and other body fluids [22–25] and tissues primarily to obtain pharmacological and pharmacokinetic data. With biological fluids [26–40] in particular, this information is required in order to establish the adequacy of therapy, establish whether a sick animal fed a β-lactam is, in fact, absorbing the drug from the digestive tract, establish therapeutic levels of drugs in various body compartments, etc. Therefore, the development of analytical methods for the assay of penicillins in biological fluids is very useful to the clinician. For the consumer of food-producing animals, however, it is important that veterinary drug residues are not allowed to occur in the animal tissue intended for consumption. Methods developed for the analysis of penicillin in animal tissues provide the necessary data base required for defining withdrawal times (tissue depletion), and for determining whether presumptive positive tissue samples contain sufficient levels of a defined veterinary drug to necessitate regulatory action. The development of methods for the assay of penicillins in animal tissues is therefore of primary significance to agencies responsible for the regulation of veterinary drugs in food animals.

1.2. Classification of methods for the analysis of penicillins in animal tissues

Analytical methods for the detection of penicillin residues in animal tissues may be classified into two broad groups: (a) those based on immunological and microbiological techniques which respond to groups of penicillins but do not discriminate among them (screening methods), and (b) those which are compound-specific, involving chemical or physical separation of the specific drug from other components for the detection, identification and discrimination among different penicillins (chromatographic methods).

2. SCREENING METHODS FOR PENICILLIN RESIDUES IN ANIMAL TISSUES

Screening methods for the detection of penicillin residues in animal tissues are usually very sensitive to penicillin G, with detection sensitivities in the μg/kg concentration range, and they provide fairly reliable test results. The procedures for conducting the screening tests are relatively simple and inexpensive, and require very simple equipment. However, because they lack compound specificity and are, at best, semi-quantitative, they have traditionally been used as the method of choice when only a qualitative decision is needed as to whether the tissue sample is contaminated with a microbial inhibitor or not. These screening methods include the classical microbial inhibition tests, such as the Swab test on premises (STOP) [41], the calf antibiotic and sulpha test [42], and the live animal Swab test [43], the inhibition tests which use colorimetric reactions, such as the Brilliant Black reduction test [44] and the Charm farm test [45], and the immunological assays, such as the Charm test II receptor assay [46] and enzyme-linked immunosorbent assays [47].

Until 1977, when the first application of chromatographic analysis for penicillin residues in tissues was published [48], all research that had hitherto been done on penicillin residues in animal tissues had used a screening test. Even today, these methods are still being used. In fact, more than 80% of what we know today about penicillin residues in animal tissues is as a result of the applications of these screening tests to animal studies [49–69]. In 1981, Johnston *et al.* [41] published a screening method, the STOP test, for the detection of antibiotic residues in meat and poultry tissues with a detection sensitivity of 0.0125 μg/g for penicillin G. This test has been used in most abattoirs in Canada and the USA for the past decade. The STOP test requires overnight incubation, so test results are not ready until the following day. With increased consumer demand for more testing and industry demands for quicker turn-around times for sample analysis, it has become prudent to find alternative or more suitable tests to the STOP test for high-volume

TABLE 2

THIN-LAYER CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF PENICILLIN RESIDUES

ACN = Acetonitrile; TZC = 2,3,5-triphenyltetrazolium chloride.

Penicillin formulation and animal experiment conducted; tissue matrix analyzed	Extracting/deproteinizing solvent; assay medium; developing solvent	Detection method and sensitivity of method	Authors and ref.
Procaine penicillin G, sodium penicillin G, spiked into cooked meats, freeze-dried chicken and hamburger; metabolites formed after cooking above spiked tissues were methylated and analyzed	Methanol; silica gel; ethyl acetate–acetic acid–water (8:1:1)	Autoradiochromatography with liquid scintillation counting	DePaolis *et al.* [48]
Ampicillin and penicillin G in imported, casualty and post-mortem animals in the UK; pigs, sheep, beef, veal, goat, kidneys, livers and muscles	ACN–water (9:1); silica gel; toluene–ethyl acetate–diethyl ether–acetic acid (80:10:10:0.75)	Bioautography using *Bacillus cereus* and TZC. Sensitivity for penicillin G (0.01 I.U.)	Smither [65]
Procaine penicillin G (300 000 I.U.) injected intramuscularly to Holstein calves and Yorkshire pigs; ampicillin (200 mg) also injected to similar animals	Methanol; silica gel plates	Bioautography using *Micrococcus luteus* and *Bacillus subtilis*. Developing reagent was TZC	Yoshimura *et al.* [75]
Penicillin B, penicillin V standards spiked into kidney, liver and muscle tissue for method development; diagnostic samples	Methanol; silica gel and cellulose plates; ACN–chloroform–1-propanol–impregnating liquid (16:20:27:16)	Bioautography using *Bacillus subtilis*. Sensitivity to penicillin G (0.08 I.U./g)	Neidert *et al.* [76]
Penicillin G in spiked pork and beef tissue; diagnostic samples: avian, equine, bovine, porcine livers, kidneys and muscles	Methanol; silica gel; chloroform–methanol–acetone–glycerine (49:30:20:1)	Bioautography using *Bacillus subtilis*. Sensitivity to penicillin G (0.03 μg/g)	Salisbury *et al.* [7]

TABLE 3

GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PENICILLIN RESIDUES IN ANIMAL TISSUE

Tissue matrix analyzed; method of analysis, compounds analyzed; analytical sample size	Deproteinizing solvent; extraction method	Detection method and sensitivity	Authors and ref.
Spiked bovine muscle, kidney, liver and adipose tissue; temperature-programmed injector vaporization, DB-1 fused-silica capillary column; penicillins G and V, methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, 25 g homogenized tissue	Acetonitrile (pH 4.0–4.4); extract partitioned into organic aqueous solvents including dichloromethane, water, light petroleum–phosphate. Residue cleaned up on SAX cartridge, dichloromethane–aqueous extract, methylation, diol cartridge clean-up	GC with thermionic (nitrogen–phosphorus) detector, detection limits < 2 ng/g for all β-lactams	Meetschen and Petz [80]

sample screening, especially when other more sensitive screening tests capable of providing test results in a few hours are available today.

3. CHROMATOGRAPHIC METHODS FOR PENICILLIN RESIDUES IN ANIMAL TISSUES

These methods of analysis include thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and electrophoresis. Tables 2–5 catalogue the various chromatographic methods that have so far been developed for the analysis of penicillin residues in food-animal tissues.

3.1. Criteria for chromatographic methods of analysis in regulatory programs

For a chromatographic method developed for the analysis of penicillin in animal tissue to be useful in the regulatory program, it should meet reasonable standards of precision, accuracy, specificity, and, above all, it must be practical to use. Since it is probably intended to be used for the analysis of a large number of diagnostic samples with expected rapid turn-around times, the method should be repeatable and reproducible, and it should not take too long before test results are obtained. Typically, an analytical method for the determination of a veterinary drug is considered to be accurate if the means of the observed recoveries from fortified samples are 80–110% of the added marker residue at levels ≥ 0.1 μg/g or tolerance levels, if the latter are defined. Below 0.1 μg/g or tolerance, mean observed recoveries from fortified samples should be between 60 and 100%. The precision of the method should be such that the intra-laboratory relative standard deviation (R.S.D.) (repeatability) of the assay does not exceed 10% at the tolerance level defined for the residue of interest [70]. It should have a sensitivity well below the tolerance level defined for the veterinary drug of interest and should be capable of confirming the presence of the drug at the violative level. In addition, residues from tissues of food-producing animals that have been experimentally administered with the drug of interest (to produce physiologically incurred or dosed tissue samples) have to be determined to ensure that the initial extraction procedures incorporated into the method, which are usually developed with spiked samples, are adequate to recover residues from true samples.

Determinative chromatographic procedures such as TLC and HPLC, which use UV–VIS, fluorescence, and radioimmunoassay detection, and GC, which relies solely on the use of retention data for identification, are not deemed to be specific enough to support regulatory action. In contrast, chromatographic methods that rely for detection and identification on total molecular configuration, such as infrared spectroscopy (IR) or mass spectrometry (MS), are considered to be specific enough to support regulatory action. The various chromatographic methods, listed in Tables 2–5 and developed for the analysis of penicillin residues in animal tissues, will therefore be reviewed with respect to how useful they are in providing an analytical support service for regulatory programs.

3.2. Isolation techniques for the chromatographic analysis of penicillins in tissues

When procaine penicillin G is administered to a food-producing animal, it is usually absorbed very rapidly. Its distribution is essentially limited to the extracellular fluid space. Protein binding, which is primarily to albumin, is estimated to be 60% for penicillin G, 20% for ampicillin and amoxicillin, and in excess of 90% for the isoxazolyl penicillins. Even at these extensive protein binding rates, significant concentrations of penicillins can still be found in liver, kidney, intestine, bile, lymph and semen [71]. Penicillin is eliminated very rapidly from the body, mainly by the kidney. For example, after injection of an aqueous solution of penicillin G, 60–90% may be recovered within 1 h in the urine. The absorption and elimination characteristics of penicillins in food animals indicate that penicillins would be in a non-covalently bound or unbound state and probably at very low concentration (ng/g) levels in the tissues intended to be analyzed.

The animal tissue samples from which the penicillins are to be determined are typically complex mixtures, composed of a wide variety of carbohydrates, proteins, lipids, etc., as well as penicillins (bound or unbound) if the animal has been so treated. Besides the deleterious effects these materials may have on the chromatographic components,

TABLE 4

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF PENICILLIN RESIDUES IN ANIMAL TISSUES

ACN = Acetonitrile; RP = reversed-phase; K = kidney; L = liver; M = muscle.

Tissue matrix analyzed; method of analysis; compounds analyzed; analytical sample size	Deproteinizing solvent; extraction method	Detection method and sensitivity	Authors and ref.
Cooked meats and freeze-dried chicken and hamburger; HPLC of methylated penicillins and metabolites; procaine penicillin G, sodium penicillin G, degradation products (metabolites) formed after cooking meat	Methanol	UV detection at 254 nm, MS, NMR	DePaolis *et al.* [48]
Spiked and incurred muscle, kidney and liver tissues from beef and pork; isocratic C_{18} RP chromatography; penicillin G and cloxacillin; 25 g homogenized tissue	ACN; extract was treated with dichloromethane, light petroleum + ACN, phosphate buffer, light petroleum, saturated ammonium phosphate and ACN	UV detection at 220 nm; 50 ng/g penicillin G, 20 ng/g cloxacillin	Moats *et al.* [83]
Spiked muscle, kidney and liver; on-line pre-concentration and manual column switching onto C_{18} RP column under isocratic conditions; 10 g homogenized tissues	Sulphuric acid, sodium tungstate; extract was cleaned up on basic alumina then Sep-Pak C_{18}	UV detection at 220 nm; 50 ng/g	Terada *et al.* [78]
Spiked fish muscle tissue; isocratic C_{18} RP chromatography; ampicillin; 10 g minced tissue	Methanol; extract was treated with propanol, followed by Florisil cartridge clean-up	UV detection at 222 nm; 30 ng/g	Nagata *et al.* [74]
Bovine tissue; initial RP chromatography, fraction collection and rechromatography of fractions by thermospray LC–MS; ampicillin, cloxacillin, penicillin G; 2 g tissues	Sonicate in ACN; methanol, water, ultrafiltration (10 000 rel. mol. mass filter)	Thermospray LC–MS	Tyczkowska *et al.* [77]
Spiked and incurred porcine and bovine (M, L, K) tissues; isocratic C_{18} RP chromatography. Penicillin G with penicillin V as internal standard; 5 g homogenized tissue	Sulphuric acid, tungstate; water extract cleaned up on C_{18} Bond Elut cartridge, eluate derivatized	UV detection at 325 nm; 5 ng/g penicillin G	Boison *et al.* [72]
Spiked and incurred bovine (M, L, K) tissues; C_{18} RP chromatography; penicillin G with penicillin V as internal standard; 5 g homogenized tissues	Sulphuric acid, tungstate; water extract cleaned up on C_{18} Bond Elut cartridge eluate analyzed directly	Thermospray LC–MS; 5 ng/g penicillin G	Boison *et al.* [79]
Cloxacillin standards in typical extraction solvents: methanol, ACN, water, ethanol, 2-propanol and mixtures of these (no tissue matrix involved)		Thermospray LC–MS	Tyczkowska *et al.* [81]
Farmed salmon, penicillin G		Thermospray, ionspray, particle beam, moving belt LC–MS	Pleasance *et al.* [118]

TABLE 5

ELECTROPHORETIC METHODS FOR THE ANALYSIS OF PENICILLIN RESIDUES IN ANIMAL TISSUES

AMP = Ampicillin; AMOX = amoxicillin; ACN = acetonitrile; CLOX = cloxacillin; DCLOX = dicloxacillin; METH = methicillin; PHEN = phenethicillin; K = kidney; L = liver; M = muscle.

β-Lactams analyzed and experimental method; analytical sample size	Extraction method	Detection method and sensitivity	Authors and ref.
AMP, AMOX, penicillin G, CLOX, METH, PHEN, penicillin V spiked into muscle, liver and kidney tissues, potential of 20 V/cm applied to electrodes, examined migration rates in agar and agarose at pH 6 and 8; 5 g tissue samples	Add ACN–deionized water (9:1), centrifuge at 38 000 *g* for 10 min at 4°C, evaporate to dryness. Dissolve in ACN–ethanol–water (0.5 ml)	Bioautography with *Bacillus cereus* and *M. luteus*. AMP (0.002), AMOX (0.002), penicillin G (0.002–0.005), CLOX (0.1–0.2), METH (0.005–0.2), PHEN (0.002–0.01), penicillin V (0.005–0.01)	Smither and Vaughan [84]
AMP, penicillin G in imported, casualty, post-mortem animals in the UK (M, L, K) from goats, sheep, veal, beef and pigs, potential gradient of 20 V/cm applied to electrodes	Add ACN–water to freeze-dried chicken (L) and pig (K) as in ref. 84	Bioautography using *Bacillus cereus* and *Micrococcus luteus*	Smither [65]
UK-produced meat and imported meat; same as reference above	Same procedure above	Bioautography using *Bacillus cereus* and *M. luteus*	Smither *et al.* [85]

such as the injectors, pumps, detectors, column packing material, etc., their presence will frequently interfere with the separation of the analyte of interest. Consequently, once a representative sample size has been defined and the analytical sample to be used for the analysis has been obtained, chromatographic methods of analysis for penicillins in animal tissues require that the penicillins be freed first of all from protein binding, followed by some form of sample preparation and/or purification procedures to isolate the drug of interest prior to chromatographic analysis and detection. These procedures have included solvent extraction/partitioning of the penicillins between aqueous and organic solvents, or the adsorption of penicillins onto suitable adsorbents (C_8, C_{18}, ion-exchange) and eluting them with suitable solvents.

3.3. Sampling animal tissues for penicillin residue analysis

Penicillins are usually distributed fairly homogeneously in non-injection site muscle, liver, kidney, interstitial, and biological fluids. This is, however, not the case with the distribution in injection site muscle mass, where there is a tendency to find localized deposits of penicillin. There is, therefore, a need to define a minimum sample size that must be taken from a bulk tissue in a food-producing animal to provide a representative sample of the bulk sample. When the chromatographic method for the determination of penicillin residues in animal tissues was recently developed [72], the authors had established that, for a detection limit of 5 ng of penicillin G per gram of tissue (*i.e.*, 0.626 ng of penicillin injected onto the column), the minimum tissue mass (analytical sample) fortified with penicillin that had to be extracted was 5 g. With this analytical sample size defined, an experiment was conducted to define the minimum sample size of organ or non-injection site muscle that had to be taken from tissues of experimental steers injected with procaine penicillin G to provide homogeneous representative samples using the criteria that repeatable analytical data must have R.S.D.s of 15% or less. It was found that the R.S.D.s obtained from the analysis of eight individual 5-g samples subsampled from homogenized 40-g incurred muscle, kidney, or liver taken

from various portions of the bulk tissue were $<15\%$. Application of the *t*-test to the calculated means of the eight replicate results from each 40-g lot showed no statistically significant difference ($P = 0.05$) from the means of multiple analysis from other 40-g lots. Similar observations were made when sample sizes of 60–100 g were homogenized and eight 5-g samples were subsampled and analyzed by HPLC. However, when sample sizes $\leqslant 20$ g were sampled from the bulk material and subsampled for analysis, means from quadruplicate analysis were found to be statistically different from lot to lot and R.S.D.s ranging from 20 to 60% were obtained. In the research laboratory at the Health of Animals Laboratory, therefore, sample sizes of approximately 40 g from the bulk material are routinely used for homogenization, before subsampling 5-g amounts for chromatographic analysis for penicillin G. For injection site analysis, it was found that tissue mass in excess of 400 g must be processed prior to subsampling for analysis in order to obtain homogeneously representative samples [73]. In addition, if samples are stored prior to analysis, they have to be sampled into 40-g lots and stored unblended. These stored samples should be blended only on the day of the analysis (to prevent accelerated degradation of penicillins) after they have been allowed to thaw at room temperature.

3.4. Protein precipitation

Protein precipitation to free non-covalently bound penicillins and remove macromolecules from the sample to be chromatographed has been achieved either by direct treatment with methanol [7,48, 74–77], acetonitrile [55,65,77], sodium tungstate and sulphuric acid [72,78,79], acidified acetonitrile (pH 4.0–4.4) [80] or by ultrafiltration [77]. With solvents, deproteinization is usually done after homogenizing the tissue to provide an increased surface area for contact with the deproteinizing reagent to facilitate the process and to provide a favorable extraction medium for the analyte. If the stability of the penicillins in the solvents that have been used for the initial extraction and/or deproteinization step is considered, it becomes apparent that methanol, which is known to degrade penicillins most extensively [81,82] and most rapidly [40] and would be the least appropriate solvent to be used for this purpose, has been used extensively [7,48,74–77]. Ultrafiltration involves the use of molecular mass cut-off filters to remove proteinaceous material. The only drawback with this technique is that not all low-molecular-mass proteinaceous material is removed and other interfering materials may pass through the filter as well. In addition, penicillins may be lost if there is any significant protein binding. The organic extract obtained at this point may either be used directly for chromatographic analysis or subjected to further purification.

3.5. Solvent extraction of penicillin residues from animal tissues

An alternative approach for isolating penicillins from tissues combines deproteinization with a solvent extraction step using either water or an organic solvent under acidic conditions. At the Health of Animals Laboratory, sodium tungstate, sulphuric acid and water are used to deproteinize and extract penicillins from tissues [72]. This technique has been found to work extremely well for isolating penicillins from eggs, plasma, tissue juices, milk, urine, yogourt and cottage cheese. This technique provides a more selective extraction of the sample matrix and offers more scope for the separation of the penicillins from endogenous material, particularly for the polar and acid-labile penicillins, such as ticarcillin and penicillin G. With this approach, proteins in the sample will usually be denatured and left at the liquid–liquid interface. With water as the extracting solvent [72,78], only the water-soluble polar components are partitioned from the tissue matrix into the aqueous phase. In contrast, when an organic solvent which may have a wider distribution of partition constants for tissue components than water is used for extraction [74,80], more endogenous components (both polar and non-polar), in addition to the drug of interest, are extracted. In such cases, further clean-up is usually warranted. The commonest approach to further clean-up of these organic extracts is to back-extract the penicillins into buffered solutions, as was done by Moats [83].

3.6. Further sample clean-up: adsorption and elution techniques

Most tissue extracts treated according to the

methods detailed in sections 3.2.–3.5. would still contain too much, and probably too many, of the other co-extractants (endogenous and exogenous material). The presence of co-extractants in the final extract usually has deleterious effects on the chromatographic components. Co-extractants increase chemical background noise in the detector and make it impossible to determine trace level concentrations of the veterinary drug. They will usually also interfere chromatographically with the drug of interest. Further purification of the penicillins from co-extractives must therefore be conducted.

As a prelude to trace level chromatographic analysis, further purification of the tissue extract, coupled with some form of concentration of the analyte, will have to be conducted at this point. A review of the literature shows that, for the penicillins, this has been achieved by solid-phase extraction and ion-exchange techniques.

3.6.1. Solid-phase extraction[a]

Meetschen and Petz [80] used diol cartridges to further clean-up extracts obtained after solvent extraction and ion-exchange techniques. Terada *et al.* [78] used Sep-Pak C_{18} cartridges after the initial extract had been passed through an alumina cartridge by gravity. Boison *et al.* [72] used Bond Elut C_{18} cartridges instead of the Sep-Pak cartridges used by Terada *et al.* [78]. This is because it was discovered during the method development by the former authors that the regular Sep-Pak C_{18} cartridges had $<14\%$ carbon loading whereas the Bond Elut cartridges had a carbon loading of 18%. It was also found that a minimum carbon loading of 17% in the packing bed was required in order to obtain $>70\%$ recovery of penicillin from the reversed-phase solid-phase extraction column. It is therefore important to consider this critical control point in any method development which includes a solid-phase extraction cartridge clean-up step, if penicillin losses are to be kept to a minimum. Table 6 compares the UV detector responses on tissue extracts for penicillins G and V obtained according to the method of Boison *et al.* [72] from muscle tissues fortified with 200 and 300 ng/g penicillins G and V, respectively. Recoveries from cartridges with a carbon loading of $\geqslant 17\%$ were in the order Sep-Pak plus > Bond Elut > SPE* for both penicillin G and V. Recovery from SPE* and the regular Sep-Pak C_{18} cartridges ($\leqslant 14\%$ carbon loading) were significantly lower than for the cartridges with $\geqslant 17\%$ carbon loading. Also, Terada *et al.* [78] used a pre-column to concentrate the dilute penicillin extract from the Sep-Pak cartridge. The concentrated extract was then loaded onto an analytical LC column using a manual switching device, and fed into a UV detector. Unless this column switching stage is automated, this technique would be heavily analyst-dependent and be unattractive for use in a regulatory laboratory.

[a] Any reference to a commercial product in this review does not imply an endorsement or rejection of the product.

4. CHROMATOGRAPHIC ANALYSIS OF PENICILLINS FROM ANIMAL TISSUE

Tables 2–5 show that penicillins have been chromatographically resolved on agarose and agar [65,84,85], silica gel plates [7,12,65,75,76], bonded methylsilicone fused-silica capillary columns [80], and reversed-phase columns [48,72,74,77–79,81,83].

TABLE 6

RECOVERY OF PENICILLINS G AND V FROM DIFFERENT SOLID-PHASE EXTRACTION UNITS

Four replicate analyses were conducted with each type of cartridge. Regular Sep-Pak and Sep-Pak plus are produced by Waters Chromatography (Mississauga, Canada). Bond Elut is produced by Varian (Harbor City, CA, USA). SPE* cartridges are distributed by Scientific Products and Equipment (Concord, Canada).

Solid-phase extraction unit	Detector response (mean ± S.D.) (mm)	
	Penicillin G	Penicillin V
$\geqslant 17\%$ Carbon loading		
t-C_{18} (Sep-Pak plus)	196.5 ± 3.4	92.1 ± 1.1
Bond Elut	171.8 ± 2.6	89.2 ± 1.3
SPE*	167.1 ± 2.8	84.5 ± 1.4
$\leqslant 14\%$ Carbon loading		
C_{18} (regular Sep-Pak)	147.2 ± 3.9	58.8 ± 2.4
SPE*	134.3 ± 2.8	57.5 ± 2.5

4.1. Thin-layer chromatographic method for penicillin residues

Ampicillin [65], procaine penicillin G [48,65,75], sodium penicillin G [7,76] and penicillin V [76] residues in animal tissues have all been analyzed by TLC. The most widely used stationary phase was commercially available silica gel or cellulose plates. Except for DePaolis *et al.* [48] who used autoradiochromatography for detection and a scintillation counter for quantitation, all the other TLC methods have used microbiological inhibition methods (bioautography) for detection and quantitation.

TLC with bioautographic detection has been used as a laboratory technique for the screening of veterinary drugs, including penicillins. The major drawbacks of this technique are that it is labor-intensive, costly, time-consuming, does not distinguish among the various penicillins, and is less sensitive than the screening tests used at the abattoirs for penicillin detection. For a regulatory laboratory without access to sophisticated instrumentation, this technique will generally detect penicillins above a 50-ng/g tolerance level and provide some measure of analytical support for a regulatory program.

4.2. Gas chromatographic methods for penicillin residues

Meetschen and Petz [80] (Table 3) have published the only GC method for the analysis of penicillins (penicillins G and V, methicillin, oxacillin, cloxacillin, dicloxacillin and nafcillin) in animal tissue. The penicillins, after isolation from animal tissue, were methylated, cleaned up further on diol cartridges, evaporated, and dissolved in cyclohexane containing two external standards, 2,4,6-triphenoxy-1,3,5-triazine and 2-(4-biphenyl)-5-(4-*tert.*-butylphenyl)-1,3,4-oxadiazole. This derivatization step was required to form not only the volatile methyl esters of the penicillins, but also to improve their chromatographic properties (thermal stability and decreased polarity) on the fused-silica capillary column. The use of a temperature-programmed vaporization injector instead of a conventional GC (on-column or flash vaporization) injector by the authors results in a markedly improved chromatographic sensitivity and reproducibility for the derivatized penicillins. Since the method involves extensive sample preparation steps with recoveries ranging from 48 to 83%, outside the range specified in section 3.1., it would have been more practical to have included internal standards added to the fortified samples just prior to extraction to correct for sample losses during sample preparation. The method would not be expected to work for the aminopenicillins, unless the amino groups are also derivatized. However, the detection limits of < 2 ng/g claimed for all the penicillins determined by this method make the procedure very attractive for regulatory use.

4.3. Liquid chromatographic methods for penicillin residues

The separation of penicillins from one another and from other endogenous or exogenous components in the tissue extracts has been achieved on octadecylsilyl-bonded reversed-phase HPLC columns [48,72,74,77–79,81,83] and eluted with buffers (at pH values ranging from 2 to 7) containing organic modifiers (methanol, acetonitrile). In the absence of ion-pair reagents, separation on the reversed-phase column occurs as a result of the partition between the non-polar stationary phase and the polar mobile phase; the most polar components elute first and the least polar components elute last.

Most penicillins do not have a specific UV chromophore. Even where absorption bands usually attributable to simple aromatic moieties exist (200 - 235 nm), these are generally not specific or intense. Greater sensitivity is normally achieved at wavelengths below 200 nm. However, at these wavelengths selectivity is poor, resulting in high background interference when trace analyses of tissue extracts are required. Penicillins are therefore chromatographed either in their underivatized (native) forms or as penicillin derivatives with improved UV chromophores.

4.3.1. Detection of native penicillins

4.3.1.1. UV detection. Moats [83], Terada *et al.* [78] and Nagata and Saeki [74] all detected penicillins separated on C_{18} reversed-phase columns by UV detection between 200 and 235 nm. Detection limits for penicillin G [78,83], cloxacillin [83] and

ampicillin [74] residues in animal tissues were determined to be 50, 50 and 30 ng/g, respectively. Under these conditions, the free bases (procaine or benzathine) formed when procaine or benzathine penicillin G salts dissolve in the tissue fluids may be detectable if they are extracted in the initial sample preparation steps. The chromatograms obtained by Terada *et al.* [78] and Nagata and Saeki [74] are less complex than that obtained by Moats [83] (see Figs. 2–4).

4.3.1.2. Mass spectrometry. Tyczkowska and coworkers [77,81] and Boison *et al.* [79] have all used the mass spectrometer as a specific detector for the detection of underivatized penicillins after they have been chromatographed on reversed-phase columns. Tyczkowska *et al.* [77] used thermospray LC–MS to confirm ampicillin, penicillin G and cloxacillin residues in spiked tissues at concentration levels of 10 ng/g, whereas Boison *et al.* [79] were able to confirm penicillin G in tissues at 5 ng/g. Full-scan mass spectra of the components eluting from the LC column into the thermospray ion source of the mass spectrometer, together with retention parameters on the LC column, provide both the qualitative and quantitative information needed to unambiguously identify and determine the penicillin(s) present in the animal tissue. These methods are of significant importance to regulatory agencies because identifications based on these methods can be used in support of regulatory action.

4.3.2. Detection of derivatized penicillins

4.3.2.1. UV detection. Only DePaolis *et al.* [48] and Boison *et al.* [72] have applied a pre-column derivatization method for the analysis of penicillins in animal tissues by HPLC. DePaolis *et al.* [48] subjected penicillin G in animal tissues to different cooking conditions and examined the degradation products [benzylpenicilloic, benzylpenilloic, benzylpenillic acids and α-(1-carboxyethyl)hydrogen benzylpenicilloate], formed in the process by HPLC (as their methyl esters) with UV detection at 254 nm. Boison *et al.* [72] formed the mercury mercaptide derivatives of penicillins G and V and detected them at 325 nm where very few endogenous components would absorb (see Fig. 5). In addition, the derivatization method used by Boison *et al.* [72], is specific only to compounds with an intact β-lactam ring.

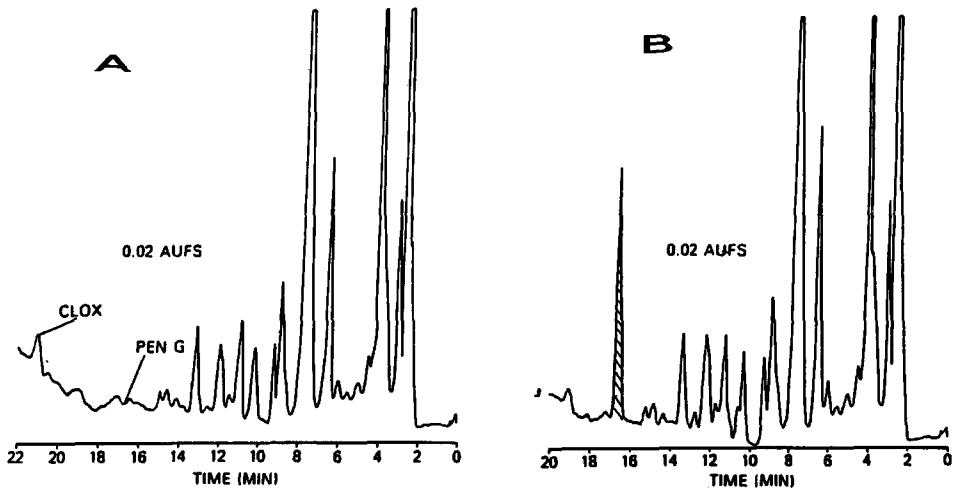


Fig. 2. Chromatograms of (A) a blank beef muscle extract after ammonium sulphate–acetonitrile clean-up, 0.2 g equivalent injected in 200 μl; (B) beef muscle spiked with 1 ppm sodium penicillin G. Varian Model 5000 liquid chromatograph, UV absorbance detector set at 220 nm, 0.02 a.u.f.s., Varian Micropak MCH-10 column, 30 cm × 4.6 mm I.D., solvent gradient 0.01 *M* phosphoric acid–acetonitrile (from 80:20 to 40:60 in 20 min). Flow-rate, 1 ml/min. Arrows indicate retention times of penicillin G and cloxacillin. A small interfering peak has a retention time near that of cloxacillin. The penicillin G peak in B is shaded. *y*-Axis represents the response in absorbance units and the *x*-axis represents the retention time. From ref. 83.

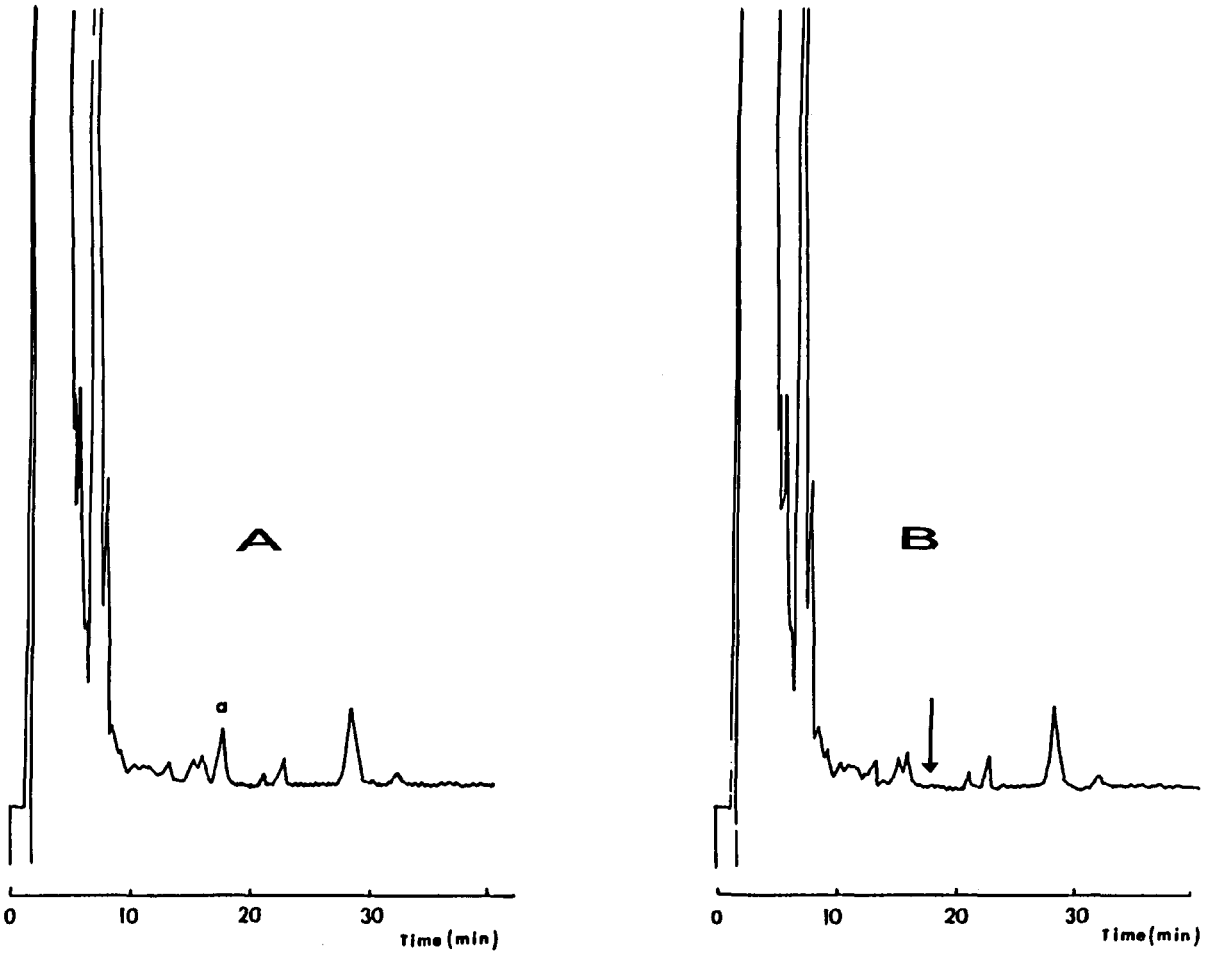


Fig. 3. Typical chromatogram of spiked and unspiked commercial yellow tail fish tissues: (A) 2 μg of ampicillin in 10 g of tissue (peak a = 20 ng ampicillin); (B) unspiked fish tissue (arrow indicates retention time of ampicillin). *y*-Axis represents absorbance and *x*-axis represents the retention time. From ref. 74.

This method is suitable for all the penicillins listed in Fig. 1, including the aminopenicillins, but will not detect free procaine or benzathine bases because they cannot form the mercury mercaptide penicillenic acid complexes. The mechanism for the formation of the mercury mercaptide penicillenic acid derivatives in the presence of 1,2,4-triazole as postulated by Haginaka *et al.* [86] is shown in Figs. 6 and 7.

4.3.2.2. Mass spectrometry. DePaolis *et al.* [48] used the direct insertion probe technique to obtain electron-impact (EI) and chemical ionization (CI) mass spectra in order to characterize some of the degradation products obtained after cooking penicillin in beef and chicken under various cooking conditions. For example, one of the degradation products after treatment with diazomethane was identified from the EI mass spectrum [*m/z* 452 (5.3%), 418 (9%), 393 (1%), 378 (1.8%), 359 (6.2%), 333 (6.7%), 317 (4.8%), 314 (5.7%), 291 (11.4%), 279 (13.3%), 255 (8.6%), 215 (11.4%), 188 (18%), 174 (100%), 118 (30%), 114 (41%), 91 (63%)] and CI mass spectrum (gave an M + 1 ion at *m/z* 453) as α-[(1-methoxycarbonyl)ethyl]-4-methyl

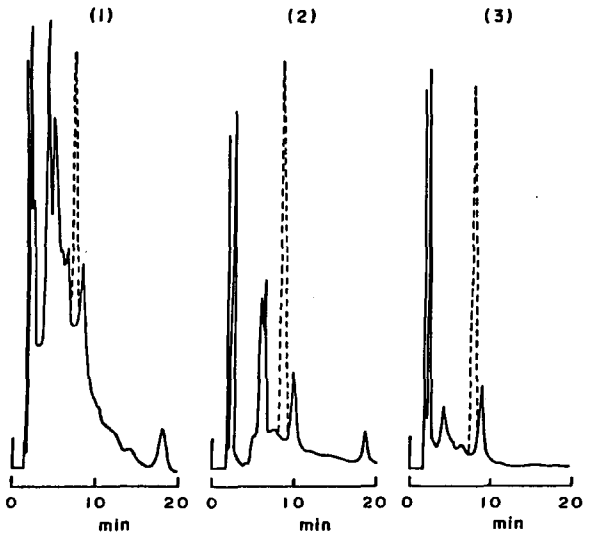


Fig. 4. Typical chromatograms obtained from (1) cattle liver, (2) kidney and (3) muscle by the overall procedure. Broken line: sample spiked with 1.0 μg/g sodium penicillin G. *y*-Axis represents UV response and *x*-axis represents the retention time. From ref. 78.

benzylpenicilloate. This characterization was confirmed by the synthesis of α-(1-carboxyethyl)hydrogen benzylpenicilloate and treatment with diazomethane to form the methylated product which was found to have identical mass spectral characteristics.

4.3.2.3. Nuclear magnetic resonance (NMR) spectroscopy. DePaolis *et al.* [48] also used NMR spectra to support the identification and characterization of some of the isolated degradation products (benzylpenicilloic acid) obtained from penicillin in tissues after cooking. Since then no one has pursued this method of detection primarily because microgram quantities (compared with nanogram amounts) of purified analytes are required for the analysis to be meaningful.

4.4 Electrophoretic methods for penicillin residues in animal tissues

Table 6 shows the pioneering work done by Smither and co-workers [65,84,85] in the application of the slab zone electrophoresis technique for penicillin analysis in animal tissues. With this technique, amoxicillin, ampicillin, penicillin G, penicillin V, methicillin, phenethicillin and cloxacillin in tissue extracts were separated from one another, identified and quantified. In my opinion, the slower slab zone technique has given way to newer refined techniques such as capillary zone electrophoresis and micellar electrokinetic capillary chromatography which appear to offer better sensitivity for the analysis of penicillins and ease of operation. Since higher potential gradients can be achieved with the newer techniques, the time for analysis, which is usually hours for the slab technique, can be substantially reduced to a few minutes [87,88].

5. APPLICATIONS TO TISSUE RESIDUE STUDIES

At a recent meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [89], it was recommended that a maximum residue limit (MRL) for total residues of benzylpenicillin of 0.05 mg/kg for kidney, liver and muscle be established for all food-animal species. This recommendation has been adopted, at least in part, by a number of countries, including USA and Canada. In Canada, for example, the prescribed tolerances are 0.05 mg/kg for penicillin G in cattle, 0.01 mg/kg in turkeys and zero in swine [90]. Similar tolerances have been set in the USA and in other countries. An MRL is the maximum concentration (mg/kg body mass) of residue resulting from the use of a veterinary drug that is recommended by the Codex Alimentarius Commision to be legally permitted as acceptable in food. The JECFA recommendation implies that analytical methods used by regulatory agencies to monitor benzylpenicillin residues in food-animal tissues must be able to detect and/or confirm benzylpenicillin residues at levels of 0.05 mg/kg. It was also noted at this meeting that there was only one published penicillin G tissue residue depletion study [55] based on a chromatographic method of analysis. In that study, tissue residues remaining in kidney, liver and non-injection site muscle of fifteen cross-bred pigs injected intramuscularly with 13 200 I.U. of procaine penicillin G per kg body weight and slaughtered in groups of three at 4 h and 1, 2, 4 and 8 days after the injection were determined by HPLC. In the research laboratory at the Health of Animals Laboratory, extensive tissue depletion studies in feedlot steers have been recently conducted by Korsrud and co-workers [91,92] using the

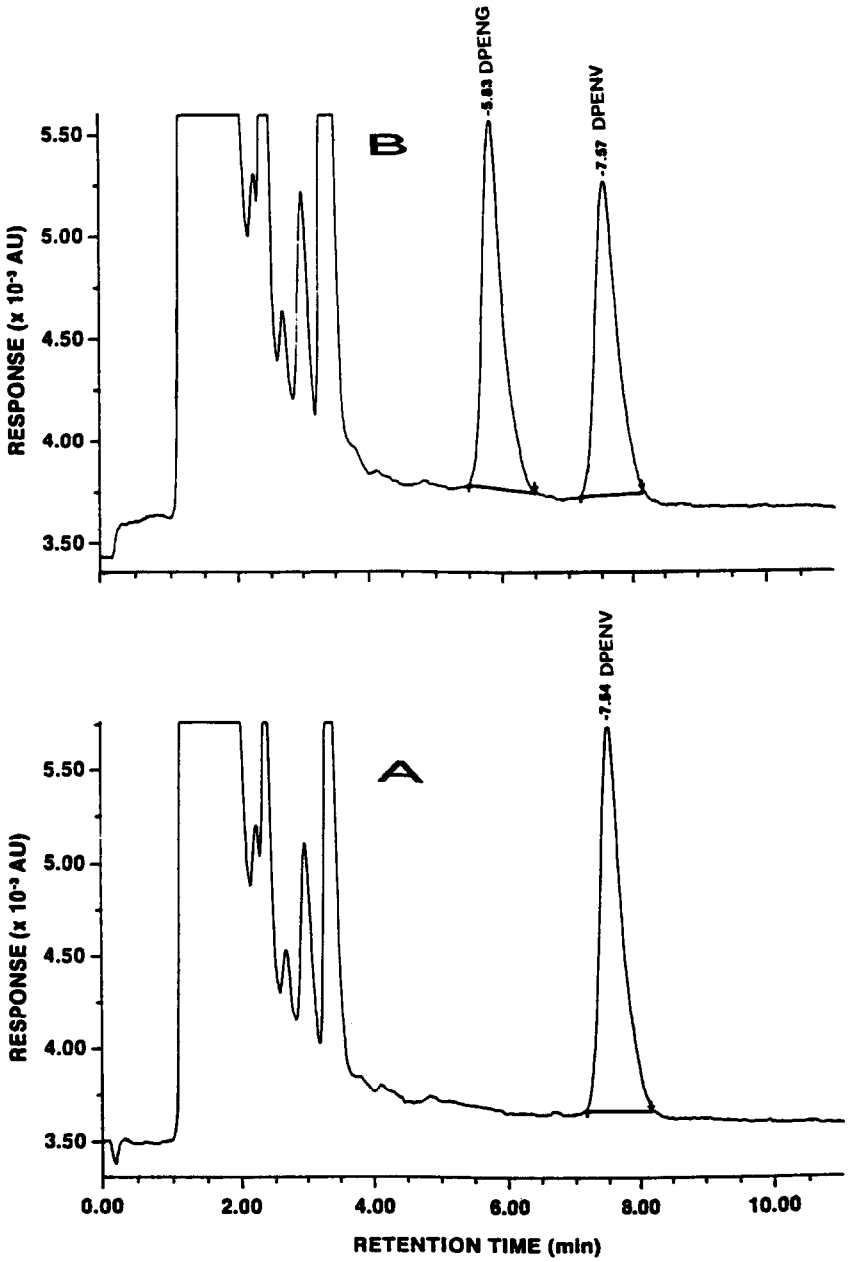


Fig. 5. Chromatogram of a 50-μl injection of a derivatized extract from a control muscle tissue. (A) A control muscle tissue fortified with procaine penicillin G at 200 I.U./kg; (B) a constant amount of penicillin V (300 μg/kg). Peaks represent the mercury mercaptide complexes of penicillin G (DPENG) and penicillin V (DPENV) detected at 325 nm at 0.005 a.u.f.s. From ref. 72.

method developed by Boison *et al.* [72] to measure tissue concentrations of penicillin G when extra-label doses of procaine penicillin G, either alone or in combination with its long-acting analogue, benzathine penicillin G, were injected intramuscularly or subcutaneously to steers. In these studies, the

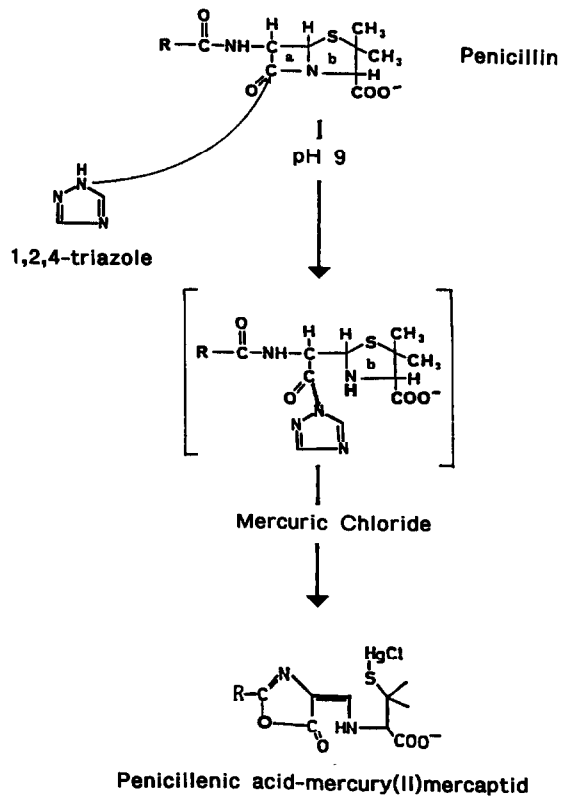


Fig. 6. Postulated mechanism for the 1,2,4-triazole base-catalysed formation of penicillenic acid mercury mercaptides [86].

depletion of benzylpenicillin from non-injection site muscles, kidneys, livers and diaphragm from steers slaughtered 1, 2, 3, 4, 8 and 12 days after the administration of penicillin formulations at dosages ranging from the label dose to 8.2 times the label dose were followed by HPLC. Results from these studies are being used to establish withdrawal periods required when penicillin G formulations are used in an extra-label manner in cattle in Canada [93]. Boison *et al.* [94] have also used the HPLC method to determine the effect of cold-temperature (−20°C and −76°C) storage on the stability of benzylpenicillin residues in plasma and tissues of food-producing animals. The authors found that significant losses of penicillin G residues in gluteal muscle stored at −20°C occurred in as little as 10 days of storage. Such samples lost about 50% of their initial concentration of penicillin G after only 10 days of storage. Losses were found to be less, but still significant, for penicillin-incurred plasma stored at −20°C for more than 2 months before they were analyzed by HPLC. However, penicillin G residues in tissues stored at −76°C were found to be very stable. It was therefore recommended that analytical laboratories receiving samples for penicillin analysis should take note of the extent to which penicillins deplete from tissues and biological fluids and modify their analytical and sample storage protocols ac-

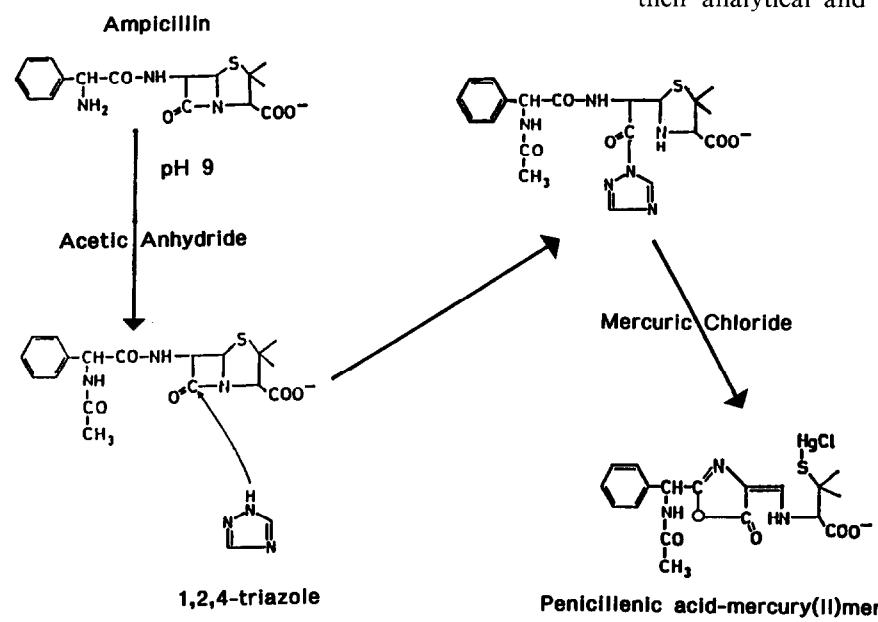


Fig. 7. Postulated mechanism for the 1,2,4-triazole base-catalysed reaction of aminopenicillins with mercuric chloride to form the N-acetylpenicillenic acid mercury mercaptide [86].

cordingly. Otherwise, quantitative analyses conducted on such stored samples will reflect residue concentrations at the time of analysis, but may not adequately reflect concentrations at time of submission.

6. FUTURE PROSPECTS

Of the β-lactam antibiotics commonly used for the treatment of food-producing animals, benzylpenicillin (penicillin G) is the drug for which most of the chromatographic methods of analysis have been developed. The emphasis placed on the development of chromatographic methods of analysis for penicillin residues in animal tissues is probably the direct result of the observations made in several survey studies conducted to monitor the prevalence and incidence of veterinary drug residues in animal tissues [7–10]. While the development of methods for penicillin G has now reached a point where penicillin residues in animal tissues can be adequately determined down to concentrations of 5 ng/g, researchers involved in the development of chemical methods for the analysis of other β-lactam antibiotics listed in Fig. 1 should be encouraged to pursue method developments for these other drugs more vigorously, even though these other veterinary drugs appear to be used less frequently in livestock production.

There is no doubt that if producers begin to use these other penicillins more frequently in animal production than benzylpenicillin, the screening tests described in section 2 and used at most abattoirs will detect them. However, it may not be possible, in the absence of sensitive quantitative methods, for the regulatory laboratory to confirm the identity and the concentration of the drug present to support regulatory action. In anticipation of the possible use of these less commonly used penicillins by producers in the future, Boison *et al.* [95], have modified the isocratic LC method [72] developed for penicillin G to a gradient mode to allow the separation and determination of ampicillin, amoxicillin, penicillin V, penicillin G and cloxacillin from tissue extracts (see Fig. 8). This method will soon be evaluated using

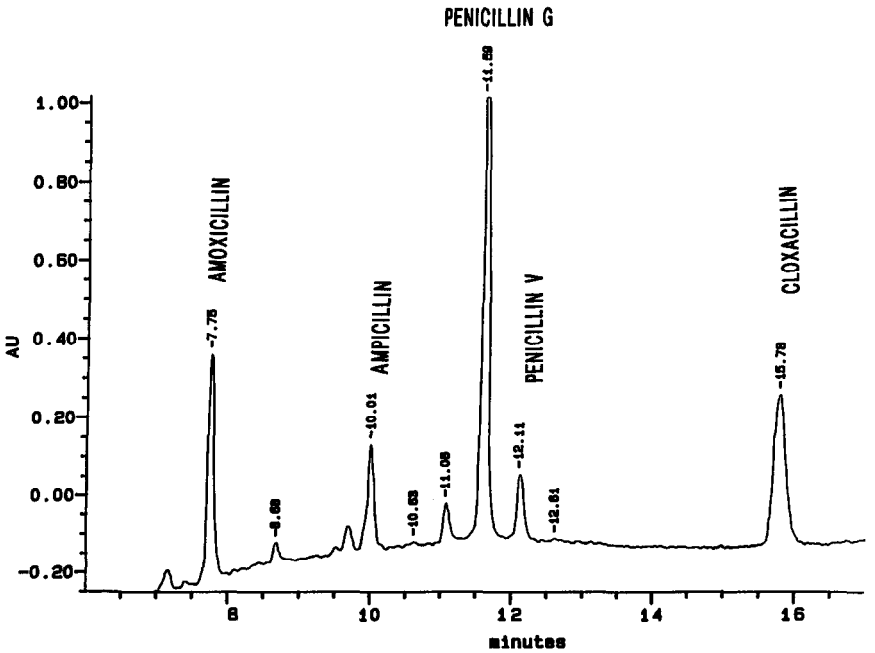


Fig. 8. Gradient elution of five penicillins (mercaptide derivatives of amoxicillin, ampicillin, penicillin G, penicillin V and cloxacillin) extracted from bovine muscle using the method in ref. 72 and separated on a Nova-Pak C_{18} column (15 cm × 4.6 mm I.D.; 5 μm Waters Chromatography) at a flow-rate of 0.8 ml/min with UV detection at 325 nm (0.005 a.u.f.s.). Gradient conditions were as follows: 0–1.4 min, 10:90 A–B; 2–5 min, 20:80 A–B; 5–12 min, 30:70 A–B; 12–14 min, 60:40 A–B; 14–16 min, 60:40 A–B and 16–17 min, 10:90 A–B; A = acetonitrile; B = 0.1 *M* phosphate buffer pH = 6.5 containing 0.0157 *M* sodium thiosulphate.

incurred tissues obtained from animals treated with the other β-lactams, after which it could be incorporated into the laboratory protocol for a multi-residue β-lactam method.

One aspect of the development of methods for detecting penicillin residues in animal tissues which could progress substantially in the near future is in the sample preparation area, where techniques that require little or no sample preparation at all will be developed and used more frequently. These techniques will include capillary electrophoresis and micellar electrokinetic capillary chromatography (MECC). High-performance capillary zone electrophoresis (HPCZE) is performed in a fused-silica capillary column, 20–100 cm × 25–100 μm I.D., filled with an appropriate buffer. Sample (1–30 nl) is introduced into the capillary and the opposite ends of the capillary and electrodes are immersed in small buffer-containing reservoirs. Electric potentials ranging from 200 to 300 V/cm are then applied to the electrodes and charged components in the sample end of the capillary migrate in very narrow bands through the capillary to the electrode of opposite polarity. Separation is based on differences in the electrophoretic mobilities of the charged components. This technique has already been applied to the separation and analysis of several β-lactam antibiotics. For example, Yeo *et al.* [96] used HPCZE to separate penicillin G, ampicillin, amoxicillin, chlortetracycline, nystatin and tylosin tartrate in methanol and ampicillin in a commercial tablet. The major drawback with classical CZE is that it deals with aqueous electrolytic systems, and components can only be separated if they are charged and soluble in water. A variant of this technique, referred to as MECC, combines the advantage of CZE in the separation of charged species with the selectivity required for the separation of uncharged compounds. In MECC, both charged and uncharged compounds, and compounds that are almost insoluble in water, can be separated, because of the hydrophobic nature of the micelles. This method is based on micellar solubilization and electrophoretic migration of the micelle. Solutes are separated by their differential distribution between the micelle and the surrounding aqueous phase and the differential migration of the two phases. For example, Rahn [87] has used MECC to separate the nine β-lactam antibiotics (amoxicillin, ampicillin, 6-aminopenicillanic acid, oxacillin, cloxacillin, ticarcillin, nafcillin and dicloxacillin) in 15 min (see Fig. 9). Nishi *et al.* [88] have applied the same technique to separate seven penicillins (benzylpenicillin, ampicillin, carbenicillin, sulbenicillin, piperacillin, aspoxicillin and amoxicillin) and nine cephalosporin antibiotics in under 20 min (see Figs. 10 and 11). These methods, admittedly, have been applied to standard solutions, but their power to resolve compounds (charged and neutrals) should present obvious advantages in research for developing methods for penicillin residues with a wide range of polarities and solubilities in aqueous and non-aqueous solvents.

Immunoaffinity (antibody-mediated) clean-up methods are becoming popular for the analysis of veterinary drug residues [97–101]. The technique is based on a specific binding between a hapten and an antibody (covalently bound to an activated support) raised against it. In this technique, a sample solution is applied to the immunoaffinity column, and the immobilized antibodies react immunochemically with the specific drug molecules to retain them while the matrix components pass to waste. The column is then washed and the retained components of interest are eluted and analyzed or cleaned up further for analysis. This technique has been used as a clean-up and concentration step prior to chromatographic analysis of chloramphenicol in milk and eggs [97] and tissues [98]. Monoclonal antibodies are highly

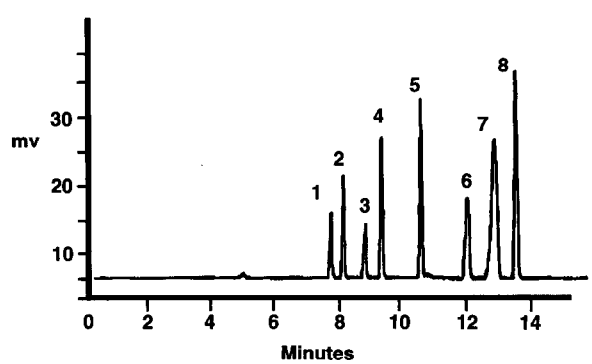


Fig. 9. Micellar electrokinetic capillary chromatography of benzylpenicillin and related antibiotics with UV detection at 214 nm. Peaks: 1 = amoxicillin; 2 = ampicillin; 3 = 6-aminopenicillanic acid; 4 = oxacillin; 5 = cloxacillin; 6 = ticarcillin; 7 = nafcillin; 8 = dicloxacillin. From ref. 87.

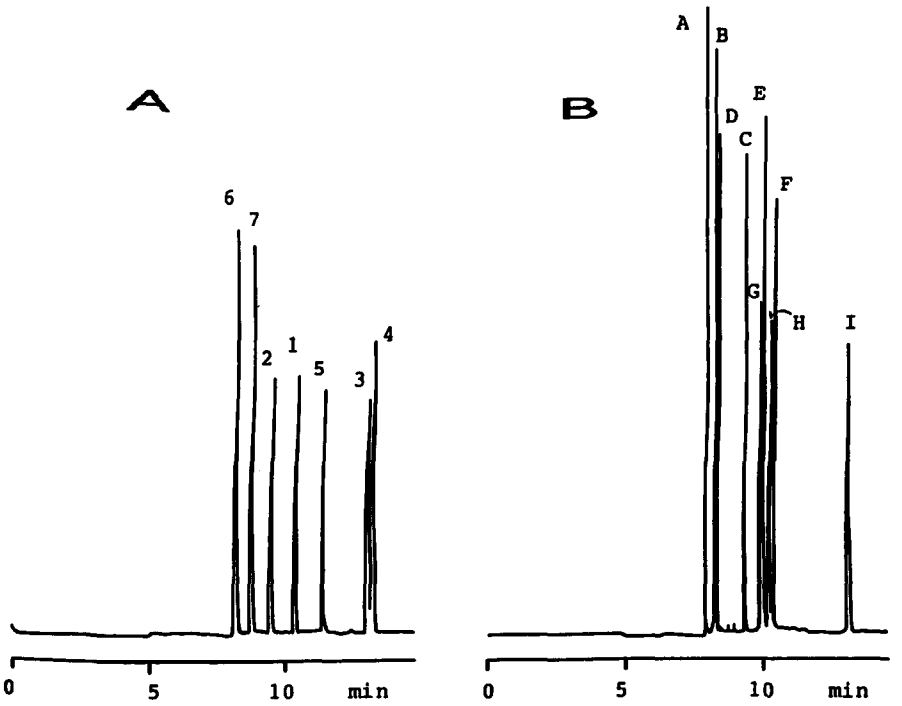


Fig. 10. Micellar electrokinetic capillary chromatography with sodium dodecyl sulphate (SDS) of seven penicillins (A) and nine cephalosporins (B) at 20 kV d.c. voltage with detection at 210 or 220 nm. Peaks: 1 = benzylpenicillin; 2 = ampicillin; 3 = carbenicillin; 4 = sulbenicillin; 5 = piperacillin; 6 = aspoxicillin; 7 = amoxicillin; A = ceftazidime; B = cefotaxime; C = cefoperazone; D = cefmenoxime; E = cefpiramide; F = ceftriaxone; G = cefpimizole; H = cefminox; I = C-TA. *y*-Axis represents UV response and *x*-axis the retention time. From ref. 87.

specific. Therefore, a large amount of sample can be subjected to antibody-mediated clean-up without retention of the matrix components, thus enabling the determination of very low levels of drugs of interest. However, because immunological reactions occur under physiological conditions, only aqueous solutions, and therefore water-soluble compounds, can be submitted to this form of sample clean-up.

The formation of chemical derivatives of penicillins with suitable chromophores to enhance detection sensitivities by HPLC analysis will be another area that will see significant improvement in the future. Several authors have demonstrated that fluoro-labelled penicillin derivatives [16,36,102–106], colored penicillin complexes [107–109] and penicillin derivatives with UV-enhanced chromophores [27,72,78,110–114] can be used to detect low levels of penicillins in pharmaceutical preparations [105–107] and biological fluids, including urine [27,111,112], milk [36], plasma [27,111,112] and fermentation broths [104,109]. Fluoro complexes, including dansylaziridine [104], fluorescamine [105] and *o*-phthaldialdehyde [106] derivatives, have been used in pre- or post-column HPLC methods to determine the concentrations of penicillins in biological fluids and fermentation media down to sub-ng/g levels but this approach has not been used for the analysis of penicillins in animal tissues. Except in the case of post-column reaction techniques where there is increased capital expense because of the cost of commercial post-column reactors, there should be no reason why these techniques cannot be applied to tissue analysis to improve the detection limits of the assays.

Confirmation of penicillins in animal tissues by MS techniques is another area where a lot of activity will take place. A lot of fundamental studies have already been done on the fragmentation and characterization of mass spectra of penicillins [77,79,81,82, 115–124]. While a few of these studies have been

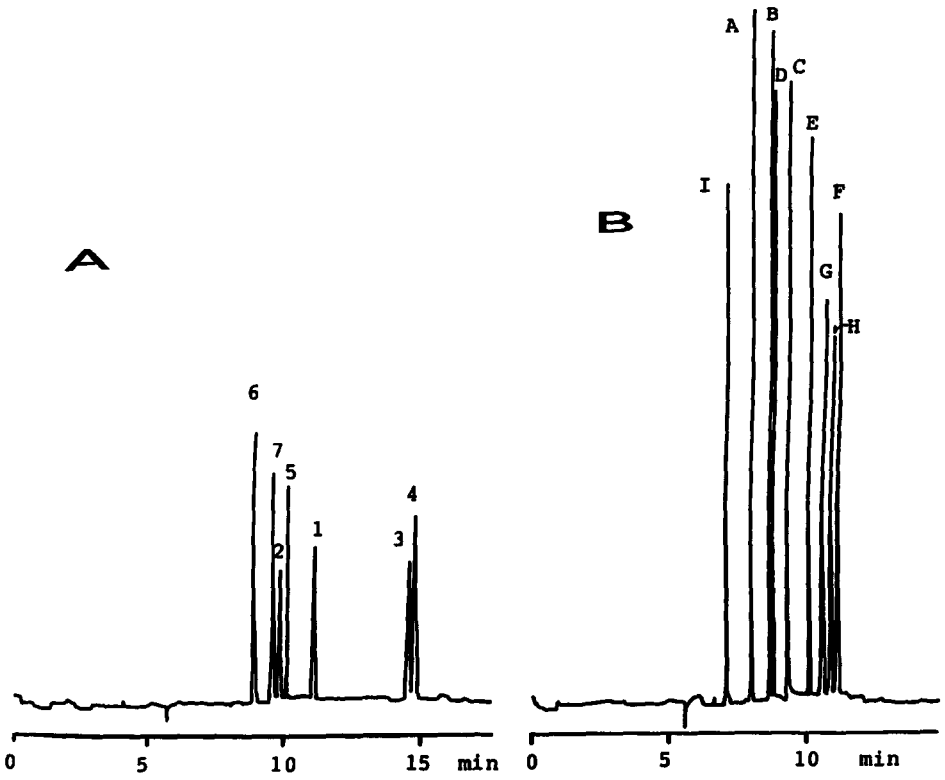


Fig. 11. Micellar electrokinetic capillary chromatography with N-lauroyl-N-methyltaurate of seven penicillins (A) and nine cephalosporins (B) at 20 kV d.c. voltage with detection at 210 or 220 nm. Peak identification as in Fig. 10 [88]. *y*-Axis represents UV detector response and *x*-axis represents the retention time.

conducted on penicillins in tissue matrices [77,79,82, 118], most of the MS work has been limited to standard solutions of the pure compounds [81,115–117,119–123] or biological fluids [124]. New developments in interfacing mass spectrometers [125,126] with LC instruments (plasmaspray, thermospray, ionspray and moving belt) are making it easier to simultaneously determine and provide confirmation of penicillins. Since the cost of a mass spectrometer is prohibitive to most institutions, the new developments in interface technology coupled with improvements in vacuum pump technology will, in the future, enable LC equipment to be coupled directly to simple, inexpensive bench-top mass spectrometers [127]. This will enable affordable mass spectrometers with LC interfaces to be available on the market as we see today with GC–MS instruments.

Finally, one other area in regulatory laboratories where improvement will take place is in the area of laboratory automation for the analysis of penicillin residues in animal tissues. Most methods developed to date require analyst involvement from the initial tissue extraction stages through to the end of the sample preparation stage when the samples are loaded into an HPLC or GC system equipped with an automated sampling unit. These methods become heavily resource-based. As part of a research endeavor to introduce some measure of automated sample analysis into the diagnostic program at the Health of Animals Laboratory, a commercially available sample preparation unit has recently been acquired for evaluation and to determine whether it can be adapted to perform the procedures described in the method published by Boison *et al.* [72]. The sample preparation unit is claimed to be capable of unattended conditioning of solid-phase extraction

cartridges, loading available sample extracts onto the conditioned solid-phase extraction cartridges, eluting them with defined solvents and even adding chemical reagents to the eluent held in controlled-temperature baths for reaction and automatically injecting the sample into an HPLC system. According to the method developed by Boison *et al.* [72] for the determination of penicillin G in animal tissues, all that the analyst has to do is spend 20–30 min per day homogenizing the tissue samples and extracting them for the sample preparation unit to complete the analyses. To make the operation of the automated sample unit practical for diagnostic use, however, the volume of tissue extract obtained according to the method by Boison *et al.* [72] has to be reduced substantially to meet volume constraints imposed by the automated sample preparation unit. This is the subject of current research in the research laboratory at the Health of Animals Laboratory, Saskatoon, Canada.

7. CONCLUSIONS

Very few chromatographic methods (TLC, GC, HPLC and electrophoresis) have been developed and applied to the analysis of penicillin residues in animal tissues. There is, however, a need to develop such methods for the determination of penicillin residues because screening methods used in the field as a prelude to laboratory analysis have better sensitivities than most of the laboratory methods. When sensitive laboratory methods become available for all the penicillins, regulatory agencies can use the data generated from depletion studies of these veterinary drugs in animal tissues to define withdrawal periods and establish tolerances and action levels for the use of all the β-lactam antibiotics in animal tissues. The definition of withdrawal periods using sensitive, quantitative laboratory methods when these drugs are used in an extra-label manner will be extremely useful, because it will provide the much needed guidelines required to encourage responsible agricultural practice among livestock producers and thereby reduce the incidence of β-lactam antibiotic residues in food-producing animals and improve the global livestock trade. Emerging techniques, such as CZE, MECC and affinity chromatography will, in the future, be applied extensively to the analysis of penicillin residues in food animal tissues. As well, laboratory automation of analytical procedures for the analysis of penicillin residues in animal tissues will be incorporated into diagnostic laboratory protocols and LC–MS confimatory methods will continue to play an important role in regulatory analysis.

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