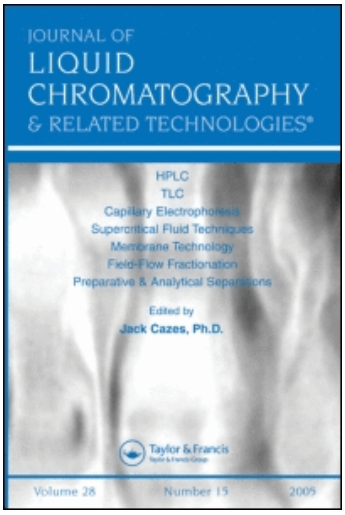

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METHOD FOR THE ISOLATION AND LIQUID CHROMATOGRAPHIC DETERMINATION OF FURAZOLIDONE IN CHICKEN MUSCLE TISSUE

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ABSTRACT

A method for the isolation and quantitation of furazolidone as a residue in poultry muscle tissue is presented. Blank control and furazolidone fortified chicken muscle (7.8-250 ng/g tissue) (0.5g) were blended with 2g of octadecylsilyl derivatized silica (C_{18}). The C_{18}/chicken muscle matrix blend was used to prepare a column that was subsequently washed with 8mL of hexane to remove lipids. The furazolidone was then eluted with 8mL of dichloromethane (DCM). The DCM eluate contained furazolidone analyte that was free from interfering compounds when examined by HPLC utilizing UV detection (365nm, photodiode array). The extracted standard curves (linear regression analysis correlation r=0.9995 $\pm$ 0.0002, n=5), average percentage recovery (99.8 $\pm$ 4.42%,

n=30) inter- (5.9 ± 4.83%, n=30) and intraassay (3.3%, n=5) variabilities for the concentration range examined (7.8 - 250ng/g tissue) were indicative of an acceptable methodology for the analysis of furazolidone in chicken muscle. A minimal detectable limit of 156pg on column was obtained.

INTRODUCTION

Furazolidone (3-[[(5-nitro-2-furfanyl)methylene]amino]]-2-oxazolidone), a nitrofuran, is a yellow crystalline powder that can be mixed with feed to be used for the therapy and prevention of intestinal infections in most species of domestic animals. It has also been used as a feed additive in swine and poultry production. However, furazolidone is mutagenic (1-2) and appears to be carcinogenic. Thus, the U.S. Deptartment of Agriculture/Food Safety and Inspection Service (USDA/FSIS) has included furazolidone in the compound Evaluation and Analytical Capability National Residue Program Plan (3), and Federal law (4) has established a zero tolerance level for furazolidone (FZ) in the muscle tissue of swine. However, because FZ is effective for the treatment of various disorders, the potential for misuse exists, and its illegal or inadvertent use may result in a FZ residue being present in meat, eggs or milk. Furazolidone is widely used in several countries, such as Egypt, for the control of salmonella infection in poultry. It is also used in combination with amprolium as an aid in preventing coccidiosis in chickens. This poses a potential health threat to consumers and necessitates monitoring of animal derived human foods such as chicken meat, eggs and milk for possible furazolidone violations.

Classical spectrophotometric analysis of nitrofurans require the conversion of the nitrofurans into their respective phenylhydrazone (5) derivatives prior to the assay. These methods are time consuming and materials intensive. Therefore a simple, sensitive and rapid quantitative method

for detection of furazolidone residues in chicken meat is needed to help in protection of the consumer.

We report here a method based on the matrix solid phase dispersion (MSPD) techniques (6-11) for the isolation and liquid chromatographic determination of furazolidone in chicken muscle tissue.

EXPERIMENTAL

Reagents: Standard furazolidone [3-[[5-nitro-2-furanyl) methylene] amino]-2-oxazolidone] was obtained from Sigma Chemical Co., St. Louis, MO. Solvents were highly purified analytical grade and were used without further purification. Water for HPLC analysis was double-distilled water passed through a Modulab Polisher I (Continental Water Systems Corp., San Antonio, TX) water purification system. Bulk C_{18} (40 micron, 18% load, endcapped; Analytichem Int., Harbor City, CA) was cleaned by sequentially washing a column (50 mL syringe barrel) of the bulk C_{18} material (22g) with 2 column volumes each of hexane, methylene chloride (DCM) and methanol. The washed C_{18} was vacuum aspirated until dry. A stock furazolidone solution (1000µg/mL) was prepared by dissolving furazolidone with a 1:1 ratio (v/v) of HPLC grade methanol:dichloromethane and diluting to the desired microgram per milliliter levels (0.39, 0.78, 1.56, 3.13, 6.25 and 12.5 µg/mL) with methanol. Syringes barrels (10mL, Becton Dickinson, Ruthertford, N.J.) were thoroughly washed and dried prior to use as columns for sample extraction. Chicken muscle samples were obtained from a local market.

Extraction Procedure:

Two grams of C_{18} were placed in a glass mortar, and a half gram of chicken muscle was placed onto the C_{18}. Standard

furazolidone (10μL, 0.39, 0.78, 1.56, 3.13, 6.25 and 12.5 μg/mL stock solutions) was randomly injected into the tissue and the fortified sample was allowed to stand for 2 minutes. This fortification level resulted in a final concentration in the tissue of 7.8, 15.6, 31.3, 62.5, 125 and 250 ng/g tissue. Blank control tissues were prepared similarly, except that 10μL of methanol containing no furazolidone was injected into the tissue. The tissues were then gently blended into the C_{18} material with a glass pestle until a homogenous mixture was observed (30 sec). The resultant C_{18}/tissue matrix was transferred into a 10mL syringe barrel containing 2 filter paper disks (Whatman No.1, 1.5 cm diameter). Two filter paper discs were placed on the column head and the column was compressed to a final volume of 4mL with a syringe plunger that had the rubber end and pointed plastic portion removed. A plastic pipet tip (100μL) was placed on the column outlet to increase the residence time of the eluting solvents on the column. The column was first washed with 8mL HPLC-grade hexane by gravity flow under a hood. When flow had ceased, excess hexane was removed by applying positive pressure (pipet bulb) to the column head until any remaining hexane was eluted. The hexane was discarded appropriately. Furazolidone was then eluted with 8mL of DCM as described before for hexane. The DCM extract was dried under a steady stream of dry nitrogen gas. A solution (0.1mL methanol:0.4mL of 0.17M aqueous H_3PO_4, v/v) as added to the dry residue and the sample was sonicated (Ultrasonic T-21B, L&R Manufacturing Company, Kearney, N.J.) to form a suspension. The suspension was then centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburgh, PA) at 13600 x g for 5 minutes. The resultant clear supernatant was filtered through a 0.45μm filter (Biorad, Richmond, CA) and a portion (20μL) was analysed by HPLC.

Liquid Chromatographic Analysis:

Analyses of extracted fortified sample and standard furazolidone was conducted utilizing a Hewlett Packard HP1090 (HP 79994A LC Chemstation) liquid chromatograph equipped with a photodiode array detector (UV 365nm, 30nm bandwidth and a spectrum range of 200-450nm) and liquid chromatograph mobile phase (acetonitrile:0.017M aqueous H_3PO_4, 70:30, v/v) at an isocratic flow rate of 1mL/min. A reversed phase octadecylsilyl (ODS) derivatized silica column (10 micron, 30 cm x 4mm id, Micro Pak, Varian, Sunnyvale, CA) maintained at 40°C was utilized for all determinations.

Data analysis:

Standard curves were generated by plotting peak areas of standard furazolidone and fortified sample extracts for the concentrations examined. A comparison of extracted fortified sample furazolidone areas to areas of pure furazolidone standards run under identical conditions gave percentage recoveries. Inter-assay variability was determined in the following manner: The peak areas for five replicates of each concentration (7.8, 15.6, 31.3, 62.5, 125 and 250ng/mL, 20µL injection volume) were averaged which resulted in a mean ± standard deviation (SD). This SD was divided by its respective mean giving each respective coefficient of variation (CV). The CVs determined for each concentration were then averaged which resulted in a mean ± SD. This was defined as the inter-assay variability. Intra-assay variability was defined as the inter-assay variability percent (5.9 ± 4.83%) were indicative of a suitable method for the determination of furazolidone as a residue in chicken muscle tissue(Table 1).

TABLE 1

Concentrations (conc. ng FZ/g tissue, n=5 replicates each concentration) examined, absolute recoveries and coefficient of variation (CV) between replicates (SD/mean), standard curve correlation coefficients (r) ± standard deviation (SD), inter-(IRV%) and intra-assay variabilities(IAV%) for furazolidone (FZ) isolated from furazolidone fortified chicken muscle tissue.

Conc. (ng FZ/g) FZ added	Conc. (ng FZ/g) FZ found mean ± SD	% Recovery Absolute	CV
7.81	7.18 ± 1.12	91.9	15.59
15.63	15.80 ± 0.73	101.1	4.62
31.25	32.06 ± 1.61	102.6	5.02
62.50	65.25 ± 3.07	104.4	4.70
125.00	122.38 ± 2.85	97.9	2.33
250.00	252.00 ± 8.47	100.8	3.36
Mean % recovery	99.8 ± 4.42%		
r ± SD	0.9995 ± 0.0002		
IRV%	5.9 ± 4.83		
IAV%	3.3		

coefficient of variation for the mean of 5 replicates of the same sample and represents the variability associated with the analytical instrumentation used.

RESULTS AND DISCUSSION

It is necessary to pretreat biological specimens in order to extract drug residues of interest and to remove interferences that may be present. Pretreatment of samples in traditional isolation techniques can include homogenizing or mixing of the sample in the extracting solvent(s), pH adjustments, back-washing of the extract, additional solvent extractions, centrifugations, and the evaporation of large volumes of solvent. Recovery of the target compound may be reduced due to chemical degradations, and less than ideal solvent-solvent extractions due to emulsion formation that may occur during the extraction procedure. In addition to being labor- and material intensive, these multi-step procedures may result in inconsistent assays.

In this study, we chose the MSPD method for furazolidone isolations because it overcomes many of the complications mentioned above . In the MSPD procedure, the chicken muscle sample (0.5g) is dispersed over a large surface area ($1000 m^2/g$ of C_{18}, theoretical). The C_{18}/chicken muscle matrix column was first washed with hexane following which the furazolidone was eluted with DCM. This column washing sequence resulted in an extract that had minimal interferences as can been seen in LC chromatograms of DCM extracted blank control (Figure 1A) and DCM extracted furazolidone fortified (Figure 1B) tissue. Photodiode array detection (365nm) resulted in a minimal detectable limit of 156pg on column (20 µL injection of a 0.5mL final sample volume). The average absolute percentage recovery (99.8 $\pm$ 4.42%), intra- (3.3%) and

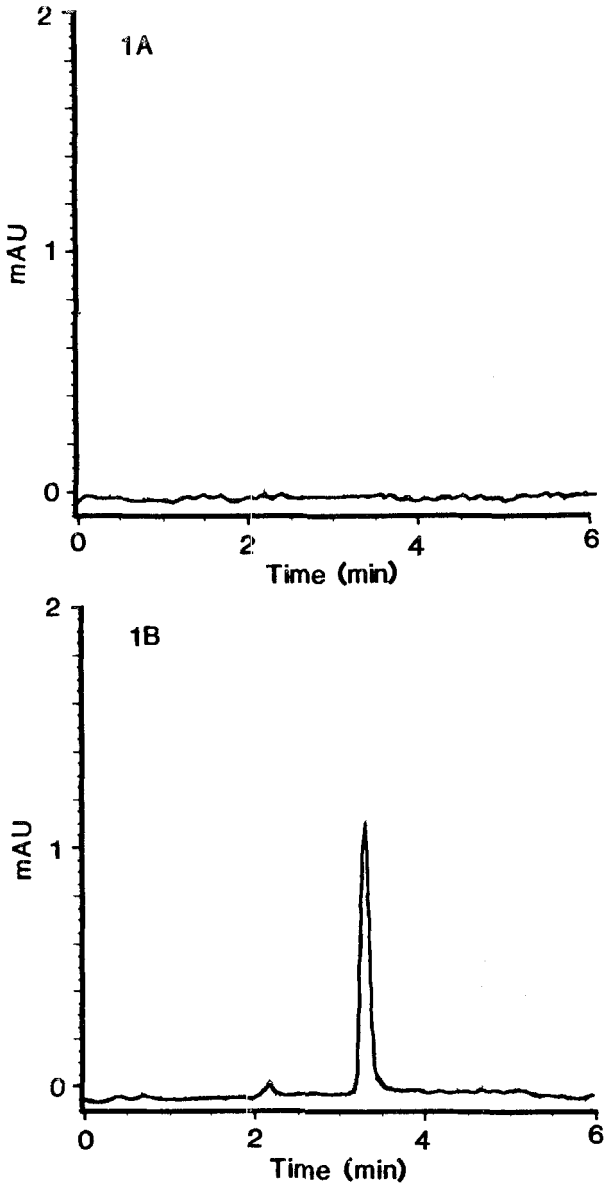

Figure 1. Representative chromatograms obtained from the HPLC/photodiode array (365nm) analysis of the dichloromethane extract of (A) blank control and (B) furazolidone fortified (125 ng/mL, 20 μL injection volume) chicken muscle tissue.

A sequential elution protocol such as outlined above allows one to selectively elute different classes of compounds from the column and therefore remove potentially interfering materials such as lipids and chromophores in hexane, prior to eluting furazolidone in DCM, while other interfering compounds, that are less soluble in DCM, remain on the column. This provides for a clean extract and enhances the detectability of furazolidone. The theoretical aspects of the MSPD technology have been the subject of previous publications(6-11).

Our results are based on fortified samples, such as would be required and obtained for the preparation of standard curves or for conducting recovery studies for the qualitative analysis of drug residues in chicken muscle incurred from the administration of the drug. While the examination of chicken muscle from flocks treated with or fed furazolidone added rations would be ideal such samples were not available to us during this study and is outside the scope of the present methods development research.

The MSPD method outlined here overcomes many of the complications associated with the traditional isolation techniques as it uses small samples, small volumes of solvent, involves few steps and requires no chemical manipulations of the sample. The minimal detectable limit of 156pg (7.8 μg/mL 20 μL injection volume) on column exceeds the level stipulated by the FDA for the limit of quantification (100 μg/g muscle tissue) in approving use of this drug in food producing animals (4).

The savings in terms of time, solvent requirements and disposal costs, and minimal utilization of expendable materials make this method attractive when compared to other classical isolations.

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