Method for the Isolation and Liquid Chromatographic Determination of Furazolidone in Milk

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A method for the isolation and liquid chromatographic determination of furazolidone in market milk is presented. Furazolidone-fortified or blank milk samples were blended with octadecylsilyl (C_{18}) derivatized silica. The C_{18}/milk matrix was used to prepare a column that was washed with hexane (8 mL) following which furazolidone was eluted with dichloromethane (8 mL). The eluate contained furazolidone, which was free from interferences when analyzed by high-performance liquid chromatography (HPLC) utilizing UV detection (365 nm, photodiode array). Extracted standard curves were linear (0.998 ± 0.001, n = 5), and the average percent recovery (81.7 ± 8.0%, n = 35) and inter- (9.1 ± 5.5%, n = 35) and intraassay (2.9%, n = 5) variabilities for the concentration range examined (7.8–500 ng/mL of milk, 20-μL injection volume) were indicative of an acceptable methodology for the analysis of furazolidone. A minimal detectable limit of 156 pg (7.8 ng/mL, 20-μL injection volume) on-column was obtained. The method uses small volumes of solvents, has a limited number of sample manipulations, and requires no pH adjustments or back-washing of extracts, making this method attractive when compared to classical isolation procedures for furazolidone.

Furazolidone, a nitrofuran, is an antibacterial drug that is effective as a therapeutic agent for the treatment of bacterial scours, bacterial enteritis, and bloody dysentary in swine (*CFR*, 1988a). It functions as a growth promoter by increasing the general well-being and vigor of treated animals and has been shown to be an effective treatment for bovine mastitis, fowl typhoid, turkey histomoniasis, and infectious hepatitis in chickens (Bryan, 1978). In cattle, its use is limited to the treatment and/or prevention of infectious bovine keratoconjunctivitis (*CFR*, 1988b).

Furazolidone treatment of food-producing animals is restricted due to evidence indicating it to be a mutagenic (Klemencic and Wang, 1978) and carcinogenic agent (Cohen, 1978). In this regard the U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) has included furazolidone in the Compound Evaluation and Analytical Capability National Residue Program Plan (USDA, 1988), and federal law (*CFR*, 1988c) has established a zero tolerance level for furazolidone in the tissue of swine. However, because furazolidone is effective for the treatment of various disorders, the potential for misuse exists, and its illegal or inadvertant use in cattle may result in a furazolidone residue being present in their meat or milk. This poses a potential health threat to consumers and necessitates monitoring of animal-derived human foods such as milk for possible furazolidone violations. Thus, methods for monitoring furazolidone levels should be such that they are rapid, specific, and sensitive enough to allow for furazolidone detection at the minimal levels achievable by present technology.

Sample preparation for furazolidone analysis has traditionally relied on classical isolation procedures that may include solvent–solvent extractions, centrifugations, backwashing, and further extractions in order to isolate furazolidone residue free from interferences (Ernst and Van Der Kaaden, 1980; Nakabeppu and Tatsumi, 1984; Vroomen et al., 1987). The need for isolation methods that minimize sample sizes, time requirements, and expendable materials, especially solvents, exists.

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We have recently developed a multiresidue solid-phase extraction technique for the isolation of other drugs from biological matrices (Barker et al., 1988, 1989; Long et al., 1989a–c) that overcomes many of the limitations of classical techniques. We report here the first use of this methodology, which we have named matrix solid-phase dispersion (MSPD), for the isolation and liquid chromatographic determination of furazolidone in milk.

EXPERIMENTAL SECTION

Chemicals and Expendable Materials. Standard furazolidone [3-[[(5-nitro-2-furanyl)methylene]amino]-2-oxazolidinone (Sigma Chemical Co., St Louis, MO)] and solvents were obtained at the highest purity available from commercial sources and used without further purification. Water for HPLC analyses was double-distilled water passed through a Modulab Polisher I (Continental Water Systems Corp., San Antonio, TX) water purification system. Bulk C_{18} (40 μm, 18% load, end capped; Analytichem Int., Harbor City, CA) was cleaned by making a column (50-mL syringe barrel) of the bulk C_{18} material (22 g) and sequentially washing with two column volumes each of hexane, methylene chloride (DCM), and methanol. The washed C_{18} was vacuum-aspirated until dry. A stock furazolidone solution (1000 μg/mL) was prepared by dissolving with a 1:1 ratio (v/v) of HPLC-grade methanol–dichloromethane and diluting to the desired microgram per milliliter levels with methanol. Syringe barrels (10 mL) were thoroughly washed and dried prior to use as columns for sample extraction.

Extraction Procedure. Milk samples (vitamin D homogenized, 3.2% butterfat) were obtained from a local market. Two grams of C_{18} was placed in a glass mortar, and a sample (0.5 mL) of milk was placed directly onto the C_{18} and remained as a bead of solution on top of the C_{18}. Standard furazolidone (10 μL, 0.39–25 μg/mL stock solutions) was added to the milk, and the samples were allowed to stand for 1 min. Alternately, furazolidone fortified milk can be placed on top of the C_{18} (2 g) in a mortar with equivalent results. Blank milk samples were prepared similarly except that 10 μL of methanol containing no furazolidone was added to the sample. The samples were then gently blended into the C_{18} with a glass pestle until the mixture was homogeneous. A gentle circular motion with very little pressure was required to obtain a homogeneous mixture. The resultant C_{18}/milk matrix was placed in a 10-mL plastic syringe barrel that was plugged with a filter paper disk (Whatman No. 1). The column head was covered with a filter paper disk, and

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the column contents were compressed to a final volume of 4.5 mL with a syringe plunger that had the rubber end and pointed plastic portion removed. A plastic pipet tip (100 μL) was placed on the column outlet to increase residence time of the eluting solvents on the column. The resulting column was first washed with 8 mL of HPLC-grade hexane. Flow through the column was gravity-controlled in all cases. If the initial flow through the column was hindered, positive pressure was applied to the column head (pipet bulb) to initiate gravity flow. When flow had ceased, excess solvent was removed from the column with positive pressures as described above. The furazolidone was then eluted with 8 mL of dichloromethane (DCM) as described above for hexane. The DCM extract was dried under a steady stream of dry nitrogen gas. To the dry residue were added 0.1 mL of methanol and 0.4 mL of 0.017 M H_3PO_4. The sample was sonicated (5–10 min) to disperse the residue, which resulted in a suspension that was transferred to a microcentrifuge tube and centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburgh, PA) at 13600*g* for 5 min. The resultant clear supernatant was filtered through a 0.45-μm filter (Biorad, Richmond, CA), and a portion (20 μL) was analyzed by HPLC.

HPLC Analysis. Analysis of sample extracts and standard furazolidone was conducted utilizing a Hewlett-Packard HP1090 (HP 79994A HPLC Chemstation) equipped with photodiode array detector set at 365 nm with a bandwidth of 20 nm and a reference spectrum range of 200–550 nm. The solvent system was a 60:40 ratio (v/v) of 0.017 M H_3PO_4 to acetonitrile at a flow rate of 1 mL/min. A reversed-phase octadecylsilyl (ODS) derivatized silica column (Varian MCH-10, 10 μm, 30 cm × 4 mm) maintained at 40 °C was utilized for all determinations.

Standard curves of pure standards and extracted spiked samples were obtained by plotting integration areas of generated peaks for each concentration examined. A direct comparison of spiked sample (n = 35) areas to areas of pure standards chromatographed under identical conditions gave percent recoveries (mean of 35 samples ± standard deviation). The interassay variability was calculated as follows: The mean of the areas for five replicates of each concentration (7.8, 15.6, 31.3, 62.5, 125, 250, 500 ng/mL) was calculated. The standard deviation corresponding to each mean was divided by its respective mean, and this resulted in the coefficient of variation for each concentration. The mean of these coefficients of variation was calculated along with its standard deviation. The mean of the coefficients of variations times 100 was assigned as the interassay variability plus or minus the standard deviation. Intraassay variability was determined as the coefficient of variation (standard deviation of the mean divided by the mean) of the mean area of five replicates of an identical sample.

RESULTS

Representative chromatograms of extracted milk blank and furazolidone-fortified (125 ng/mL) milk samples are shown in Figure 1, parts A and B, respectively. Table I gives the concentrations examined, correlation coefficient (±SD), percentage recoveries, and the inter- and intraassay variabilities of furazolidone isolated from fortified milk samples.

DISCUSSION

The isolation of drug or chemical residues from complex biological matrices can be a time-consuming and material- and labor-intensive task. Ideally, isolation techniques should be simple and time- and labor-efficient while simultaneously limiting expendable materials, especially solvents. The procedure should result in extracts containing the targeted compound, with high recoveries, free from interfering coextractants. 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 solvents. Losses of targeted compounds may result due to chemical degradations, entrainment in pelleted debris, and less than ideal solvent–solvent extractions due to emulsion formations during the extraction procedures. In addition to being labor- and material-intensive, these multistep procedures may result in inconsistent assays.

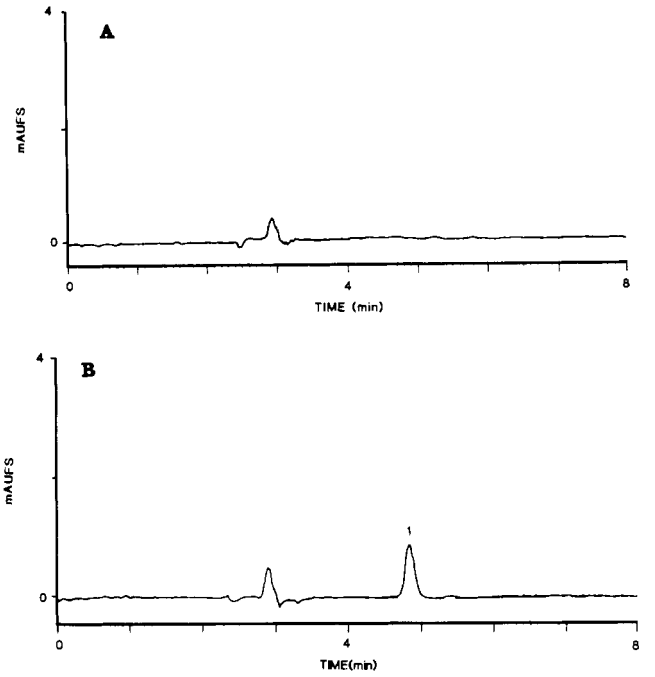

Figure 1. Representative chromatograms obtained from the HPLC/photodiode array (365-nm) analysis of the dichloromethane extract of (A) blank milk and (B) furazolidone (1) fortified (125 ng/mL, 20-μL injection volume) milk.

Table I. Standard Curve Correlation Coefficient, Percentage Recoveries, and Inter- and Intraassay Variabilities (SD = Standard Deviation) Determined for the Dichloromethane Extract of Furazolidone-Fortified Milk Samples (20-μL Injection Volume)

concn, ng/mL	% recovery[a]
7.8	82.8 ± 11.7
15.6	86.9 ± 6.1
31.3	68.2 ± 5.3
62.5	71.6 ± 5.6
125.0	87.9 ± 2.1
250.0	76.9 ± 3.5
500.0	75.4 ± 1.9
interassay var (n = 35), %	9.10 ± 5.5
intraassay var (n = 5), %	2.9
correln coeff (r; mean ± SD, n = 5)	0.998 ± 0.001

[a] n = 35. Five samples at each concentration.

The method presented here overcomes many of the complications outlined above for furazolidone isolations. The dispersion of the milk onto the C_{18} and the subsequent elution of furazolidone from the C_{18}/milk matrix with dichloromethane resulted in extracts containing the furazolidone analyte free of interferences as can be seen in the HPLC chromatogram of blank (Figure 1A) and furazolidone-fortified milk (Figure 1B) samples. The C_{18}/milk matrix was first washed with hexane to remove lipid materials and neutral chromophores that would have interfered with subsequent furazolidone analysis. Furazolidone was then eluted with DCM, which resulted in extracts that had minimal interferences when monitored by photodiode array detection at 365 nm and resulted in a minimal detectable limit of 156 pg on column (20-μL injection of a 0.5-mL final sample volume).

In the MSPD procedure, the sample is dispersed over a large surface area (1000 m^2/2 g of C_{18}). Even though

the washing and extracting solvent volumes are small (8 mL), the mechanism is an exhaustive extraction whereby a large volume of solvent is passed over an extremely thin layer of sample. By using a sequential elution protocol as outlined here, one can selectively elute from the column different classes of compounds and therefore remove potentially interfering materials such as lipids and chromophores prior to eluting furazolidone with DCM. Other more polar chromophores, which are less soluble in DCM, remain on the column. The theoretical aspects of the MSPD methodology have been published previously (Barker et al., 1988, 1989; Long et al., 1989a–c).

Results presented here 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 quantitative analysis of drug residues in milk incurred from the administration of the drug. The purpose of the present study was to examine the application of matrix solid-phase dispersion for the isolation of furazolidone from market milk. While an examination of milk from animals actually administered furazolidone would be ideal, such samples were not available to use and is outside the scope and limits of practicality of the present research. Such studies are currently under way, examining incurred residues of furazolidone in milk obtained from animals so treated, with the assistance of the U.S. Food and Drug Administration.

The method outlined here eliminates many of the problems associated with classical isolation techniques. The method uses small sample sizes and has a minimal number of steps, has no chemical manipulations (such as pH adjustments), and requires a minimal amount of solvent. The minimal detectable limit of 156 pg (7.8 ng/mL, 20-μL injection volume) on column exceeds the level stipulated by the FDA for the limit of quantification (100 ng/mL) in approving uses of this drug in food-producing animals (USDA, 1988). Because the sample has a minimal number of interfering compounds, an increase in sensitivity may be achieved by increasing injection volume and/or dissolving the extract residue in a smaller final volume. The cleanliness of the extract may allow for more sensitive means of detection as well. Use of an internal or external standard may result in a decrease in the reported assay variabilities and, if properly chosen, enhance the recovery of furazolidone. Additionally, other nitrofurans or furazolidone metabolites may be isolated from milk or other food matrices by a similar approach and determined in a similar manner, and the savings in terms of time and solvent requirements make this method attractive when compared to classical isolations.

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