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Method for the isolation and liquid chromatographic determination of eight sulfonamides in milk

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SUMMARY

A method for the isolation and liquid chromatographic determination of eight sulfonamides in milk is presented. Fortified or blank milk samples (0.5 ml) were blended with octadecylsilyl ($C_{18}T$) derivatized silica (2 g). A column made from the sample $C_{18}T$ matrix was first washed with hexane (8 ml) following which the sulfonamides were eluted with methylene chloride (8 ml). The eluate contained sulfonamide analytes which were free from interferences when analyzed by high-performance liquid chromatography (HPLC) utilizing UV detection (270 nm, photodiode array). Standard curve correlation coefficients (range, 0.998 ± 0.002 to 0.999 ± 0.001), average percentage recoveries (73.1 ± 7.4 to 93.7 ± 2.7%), and the inter- (3.9–9.6%) and intra-assay (2.2–6.7%) variabilities, were determined for the concentration range examined (62.5–2000 ng/ml) and resulted in a minimal detectable limit of 1.25 ng on column (62.5 ng/ml, 20 μl injection from a final sample volume of 0.5 ml). Savings in terms of time and solvent make this procedure attractive when compared to classical isolation techniques for sulfonamides.

INTRODUCTION

Sulfonamides are used as antibacterial agents and are commonly administered to domestic agricultural species via medicated feeds. In this manner, these compounds prevent and treat disease, increasing the vigor and the general well being of food producing animals. Legal tolerance levels and withdrawal periods prior to slaughter have been established for these drugs. However, these levels are being continuously re-evaluated based on, for example, the recent evidence implicating sulfamethazine as a possible carcinogenic agent[1].

Sulfonamide residue levels have been monitored by various analytical techniques[2–6]. While these colorimetric, thin-layer chromatographic, high-performance liquid chromatographic (HPLC) and gas chromatographic (GC) techniques find application in sulfonamide analyses, a critical factor which governs their usefulness is the sample extraction procedure.

Residue isolation techniques must be such that they isolate the targeted com-

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pound(s) with relatively high percentages of recovery while simultaneously minimizing interferences which may contribute to high background in the analysis. Sulfonamides are generally extracted from solid samples by homogenizing the sample in an extracting solvent. The particulated sample is then repeatedly extracted to increase recovery. Liquid samples are treated similarly by multiple extraction with organic solvents. For example, sulfonamides in milk have been extracted with an acetone–chloroform mixture according to the method of Tishler *et al.*[7]. This method was originally developed for the determination of sulfamethazine in milk but has since found application for meat tissues. In addition to the need for large volumes of extracting solvent the method requires additional clean-up steps in the form of pH adjustments, further extraction and backwashing. Furthermore, emulsion formation during the extraction procedure complicates sulfonamide isolations. Multiresidue sulfonamide isolation techniques which minimize or eliminate problems inherent in these traditional solvent extraction methods are needed.

Recently we have demonstrated[8–11] that by blending biological samples with C_{18} packing material one can prepare a column from which one can selectively elute targeted residues. This method, which we have named matrix solid phase dispersion (MSPD), eliminates many of the difficulties associated with traditional isolation techniques. Based on this fundamental principle, we report here the first use of the MSPD methodology for the rapid extraction, HPLC separation, photodiode array UV detection and quantitation of sulfanilamide, sulfathiazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfisoxazole and sulfadimethoxine as residues in milk.

EXPERIMENTAL

Chemicals and expendable materials

All standard compounds and solvents were obtained at the highest purity available from commercial sources and were used without further purification. Water for HPLC analyses was double distilled water passed through a Modulab Polisher I (Continental Water Systems, San Antonio, TX, U.S.A.) water purification system. Bulk C_{18} (40 μm, 18% load, endcapped from Analytichem, Harbor City, CA, U.S.A.) 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 and methanol. The washed C_{18} was vacuum aspirated until dry. Stock sulfonamide solutions (1 mg/ml) were prepared by dissolving standard compounds with HPLC-grade methanol and diluting to the desired (3.13, 6.25, 12.5, 25.0, 50.0 and 100 μg/ml) levels with methanol. Syringe barrels of 10 ml were thoroughly washed and dried prior to use as columns for sample extraction.

Extraction procedure

Milk samples (Vit. D homogenized, 3.2% butterfat) were obtained from a local market. A 2-g amount of C_{18} material was placed into a glass mortar. Standard sulfonamides (10 μl of 3.13–100 μg/ml stock solutions) and internal standard sulfamerazine (10 μl, of 12.5 μg/ml stock solution) were added to the milk and the samples were allowed to stand for 1 min. Blank milk samples were prepared similarly except that 20 μl of methanol containing no sulfonamides were added to the sample. An

aliquot (0.5 ml) of milk was placed on the C_{18} material and the samples were then gently blended into the C_{18} material with a glass pestle until the mixture was homogenous in appearance. A gentle circular motion with very little pressure was required to obtain a homogenous mixture. The resultant C_{18}–milk matrix was placed into a 10-ml plastic syringe barrel which was plugged with a filter paper disc (Whatman No. 1). The column head was covered with a filter paper disc and 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 pipette tip (100 μl, plastic, disposable, Eppendorf) 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 flow through the column was hindered, positive pressure was applied to the column head (pipette bulb) to initiate gravity flow. When flow had ceased, excess hexane was removed from the column with positive pressure as described above. The sulfonamides were then eluted with 8 ml of methylene chloride as described above for hexane. The methylene chloride extract was dried under a steady stream of dry nitrogen gas under a hood. To the dry residue were added 0.1 ml of methanol and 0.4 ml of 0.017 *M* orthophosphoric acid. The sample was sonicated (5–10 min) to disperse the residue, which resulted in a suspension. This was transferred to a microcentrifuge tube and centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburg, PA, U.S.A.) at 13 600 *g* for 5 min. The resultant clear supernatant was filtered through a 0.45-μm filter (Bio-Rad Labs., Richmond, CA, U.S.A.) and an aliquot (20 μl) was analyzed by HPLC.

HPLC analysis

Analyses of extracted samples and standard sulfonamides were conducted utilizing a Hewlett-Packard HP1090 HPLC system (HP 79994A Chemstation) equipped with a photodiode array detector set at 270 nm with a bandwidth of 20 nm, spectrum range of 200–350 nm and a reference spectrum of 450 nm with a bandwidth of 100 nm. The solvent system was 0.017 *M* orthophosphoric acid–acetonitrile (90:10, v/v) at a flow-rate of 1 ml/min, for 5 min increasing the flow-rate to 2 ml/min at 5 min for the remainder of the 16-min run. A reversed-phase octadecylsilyl (ODS) derivatized silica column (Supelcosil LC-18, 3 μm, 7.5 cm × 4 mm I.D., Supelco, Bellefonte, PA, U.S.A.) maintained at 45°C was utilized for all determinations.

Peak area ratio curves of standards and samples were obtained by plotting integration areas of generated peaks as a ratio to the area of the internal standard. A comparison of fortified sample peak area ratios to peak areas of pure standards run under identical conditions gave percentage recoveries (n = 30; 30 samples, 5 replicates of each concentration). The interassay variability was calculated as follows. The mean of the peak area ratios for five replicates of each concentration (62.5, 125, 250, 500, 1000 and 2000 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 and defined as the interassay variability, plus or minus the standard deviation. Intra-assay variability was determined as the coefficient of variation (standard deviation of the mean divided by the mean) of the mean peak area ratio of five replicates of an identical sample.

TABLE I

STATISTICAL DATA OF THE EIGHT SULFONAMIDES ISOLATED FROM FORTIFIED MILK SAMPLES

Concentrations: 62.5, 125, 250, 500, 1000 and 2000 ng/ml. Internal standard: sulfamerazine, 250 ng/ml. S.D. = standard deviation; C.V. = coefficient of variation. Linear regression equation: $y = mx + b$, where m = slope and b = y-intercept.

Compound	*Correlation coefficient ($r \pm$ S.D., $n = 5$)*[a]	*Linear regression equation*	*Recovery (%) ($\bar{x} \pm$ S.D., $n = 30$)*[b]	*Inter-assay variability (%, $n = 30$)*[c]	*Intra-assay variability (%, $n = 5$)*[d]
Sulfanilamide	0.999 ± 0.001	$y = 255.48x - 0.05$	73.1 ± 7.4	5.6 ± 3.3	3.2
Sulfathiazole	0.999 ± 0.001	$y = 407.89x - 0.04$	93.7 ± 2.7	6.6 ± 1.1	2.2
Sulfadiazine	0.999 ± 0.001	$y = 198.95x - 0.02$	81.2 ± 4.8	4.8 ± 2.9	2.7
Sulfamerazine	–	–	81.9 ± 4.6	4.5 ± 2.4	2.7
Sulfamethazine	0.999 ± 0.001	$y = 319.66x - 0.04$	92.7 ± 5.6	4.8 ± 2.7	2.3
Sulfamethoxazole	0.999 ± 0.001	$y = 419.67x - 0.08$	89.4 ± 8.3	4.0 ± 2.0	5.1
Sulfisoxazole	0.999 ± 0.001	$y = 479.48x - 0.04$	88.6 ± 11.2	8.1 ± 3.1	6.7
Sulfadimethoxine	0.998 ± 0.002	$y = 562.73x - 0.09$	89.6 ± 8.1	9.6 ± 3.3	2.3

[a] Mean of five standard curves.

[b] Five replicates at each concentration and $n = 6$ levels (62.5–2000 ng/ml).

[c] Calculate (100 · S.D.)/$\bar{x}$ = C.V. for $n = 5$ replicates at each concentration. Average C.V. values at $n = 6$ levels (62.5–2000 ng/ml) result in $\bar{x}$(C.V.) ± S.D. = inter-assay variability.

[d] Replicates ($n = 5$) of an identical sample, (100 · S.D.)/$\bar{x}$ = C.V. (intra-assay variability).

RESULTS AND DISCUSSION

A critical aspect of drug residue isolations, which dictates the usefulness of any analytical technique, is the sample preparation step. While classical extraction techniques utilizing large volumes of organic solvents isolate compounds of interest they may also result in extracts containing many interferences which necessitates further clean-up steps. As a result of multiple sample manipulations, aside from being time consuming, the accuracy, precision and reproducibility of the technique may be affected. In addition, the multi-step procedures may negate multi-residue isolations from a single sample due to losses of compounds within a class as a result of pH adjustments, reextractions and backwashing.

Results presented here overcome some of these aforementioned limitations and are an extension of previous work conducted in this laboratory[4–7]. The milk was evenly dispersed onto the solid (C_{18}) support which distributed the sample over a large surface area (1000 m^2 per 2 g of C_{18} material) and exposed the entire sample to the extraction process. The initial elution of the column with hexane removed lipid materials and, under these conditions, the sulfonamides remained on the column. Methylene chloride was then used to elute the sulfonamides. The high percentage recoveries and small variabilities (Table I) are a result of what can be envisioned as an exhaustive extraction process whereby a large volume of solvent is passed over an extremely thin layer of milk. The theoretical aspects of he MSPD technique have been the subject of previous publications[8–11] for the isolation of different residues from milk and other matrices.

Chromatograms of blank (Fig. 1A) and sulfonamide fortified milk (Fig. 1B) extracts show the blank milk extract to be free of compounds which might have interfered with sulfonamide analysis. A method blank contained no interfering compounds. This can be explained by the manner in which the sulfonamides were eluted from the column. The hexane wash removes lipid and other compounds, such as neutral chromophores, which could otherwise interfere with the sulfonamide analysis. Other more polar chromophores, which are less soluble in methylene chloride remain in the column. Thus, one can selectively elute the compounds of interest while eliminating potentially interfering compounds. While this explanation is simplistic, it serves to underscore the basic principle. Observations made during this work suggest the principles involved may be more complex. If one blends sulfonamide standards, which are dissolved in methanol, directly with C_{18} packing material they are not readily eluted with methylene chloride. However, if sulfonamides are fortified into milk, and blended into the C_{18} material they are eluted with high recoveries as indicated by the data presented here. Milk contains proteins, lipids, carbohydrates, salts, etc. which alters the normal elution profile of the C_{18} column. While the elution of sulfonamides from the C_{18} material alone can be considered a reversed-phase mechanism this cannot be said for the C_{18}–milk matrix where the sample constituents contribute a unique chemical characteristic to the column bed. The hydrophobic, ionic and electrostatic qualities contributed by the sample constituents result in unique but reproducible sulfonamide elution profiles. The theoretical aspects concerning the contribution of sample components to separations by MSPD have been discussed[8–11]. Additionally one cannot simply pass milk through a C_{18} cartridge and obtain consistent recoveries of sulfonamides. Passing milk through an SPE C_{18} col-

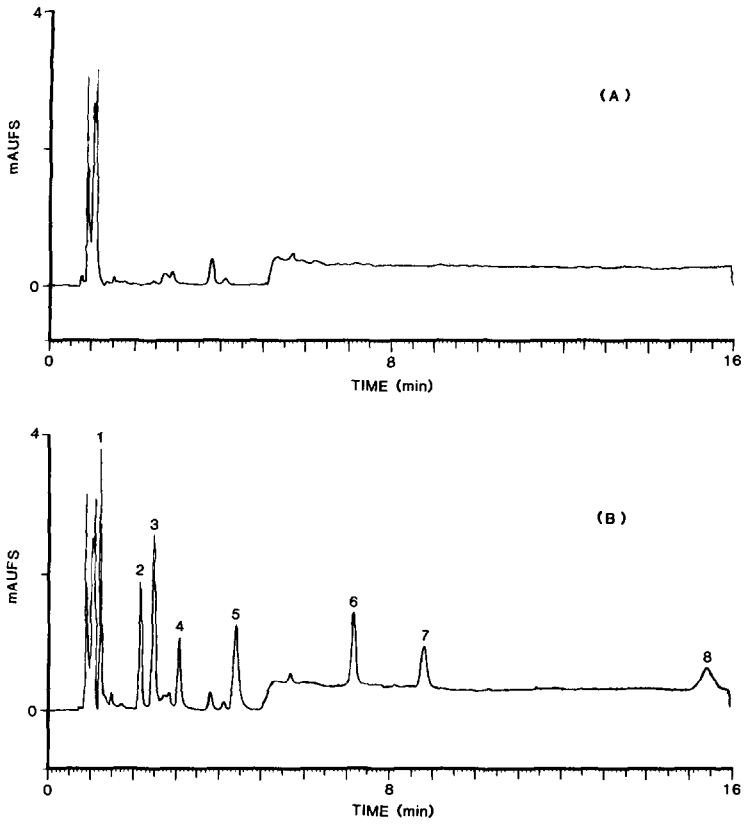

Fig. 1. Representative chromatograms (UV photodiode array, 270 nm) of the methylene chloride extracts of (A) blank milk, and (B) milk fortified with standard sulfonamides (250 ng/ml) and internal standard (250 ng/ml) sulfamerazine. Order of elution is (1) sulfanilamide, (2) sulfathiazole, (3) sulfadiazine, (4) sulfamerazine, (5) sulfamethazine, (6) sulfamethoxazole, (7) sulfisoxazole and (8) sulfadimethoxine.

umn does not appear to sufficiently coat the particles, resulting in inconsistent elution profiles for the sulfonamides, as was experienced in this laboratory. The milk must be mechanically blended into the C_{18} material for consistent results.

As a result of the cleanliness of the milk extracts so obtained a scale-up of this procedure could allow for the determination of sulfonamide levels in the low-ppb range and is presently being pursued. The minimum detectable limit utilizing a photodiode array detector was between 31.25 and 62.5 ng/ml (20 μl injection from 0.5 ml sample volume, 1.25 ng on-column) which reflects the sensitivity characteristics of the detection system utilized and the compounds analyzed in this study. The consistency of recovery at each concentration is reflected in the standard deviation of the average recoveries shown in Table I. The recoveries for eight different sulfonamides underscores the utility of the MSPD approach in terms of multiresidue isolations. Because of the cleanliness of the extract, an increase in sensitivity could be achieved by increasing injection volume and/or dissolving the extract residue in a smaller final volume. Additionally, by extending the theoretical aspects of this method, it is likely

that multi-residue sulfonamide determinations in other milk based products or liquids, as well as tissues or blood components, could be achieved with similar results.

The results presented here are based on fortified samples, such as would be required and obtained for the preparation of standard curves and for conducting recovery studies for the quantitative analysis of drug residues in milk incurred from the administration of a drug. The purpose of the present study was to examine the application of matrix solid phase dispersion for the simultaneous isolation of the eight sulfonamides from a single milk sample, demonstrating the prospect that such methodology may be used to screen for a wide range of drugs in a single sample with the understanding that it is unlikely that more than two compounds may be present in an actual sample. While an examination of milk from animals actually administered these eight sulfonamides would be ideal, such samples were not available to us and is outside the scope and limits of practicality of the present research. Such studies are currently underway, examining incurred residues of individual sulfonamides in milk obtained from animals used in drug depletion studies, with the assistance of the United States Food and Drug Administration.

CONCLUSIONS

The savings in terms of time and solvent requirements, compared to classical extraction techniques[3], make this procedure attractive. For example, the Tishler method[7] requires 50 ml of milk which is extracted several times resulting in a minimum of 600 ml of extracting solvent which must be evaporated. Additional pH adjustments and washing are necessary before the sample is ready for analysis. The method presented here isolates eight sulfonamides simultaneously, requires a 0.5-ml sample, 8 ml of hexane and 8 ml of methylene chloride and requires no extensive extract clean-up steps other than drying the methylene chloride, centrifugation and filtering prior to analysis. Furthermore, use of the MSPD method as outlined here results in extracts containing several sulfonamides which are relatively free from interfering coextractants which could aid in their detection by other more sensitive means, such as immunoassay techniques, by eliminating cross-reacting compounds.

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REFERENCES

1 N. Littlefield, *Technical Report, Chronic toxicity and Carcinogenicity Studies of Sulfamethazine in B6CF$_1$ Mice*, National Center for Toxicological Research, Jefferson, AR, 1988.
2 W. Horwitz, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 104–130.
3 M. Petz, *Z. Lebensm.-Unters.-Forsch.*, 180 (1984) 267–279.
4 R. M. Simpson, F. B. Suhre and J. W. Shafer, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 23–26.
5 R. Malisch, *Z. Lebensm.-Unters.-Forsch.*, 182 (1986) 385–399.
6 P. A. Ristuccia, *J. Liq. Chromatogr.*, 10 (1987) 241–276.
7 F. Tishler, J. L. Sutter, S. N. Bathish and H. F. Hagman, *J. Agric. Food Chem.*, 16 (1968) 50–53.
8 S. A. Barker, A. R. Long and C. R. Short, *J. Chromatogr.*, 475 (1989) 353–361.

9 S. A. Barker, A. R. Long and C. R. Short, in W. Huber (Editor), *Proceedings of the Sixth Biennial Symposium of the American Academy of Veterinary Pharmacology and Therapeutics, Blacksburg, VA, June 13–16, 1988,* American Academy of Veterinary Pharmacology and Therapeutics, Blacksburg, VA, 1988, pp. 55–56.
10 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 739–741.
11 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 813–815.