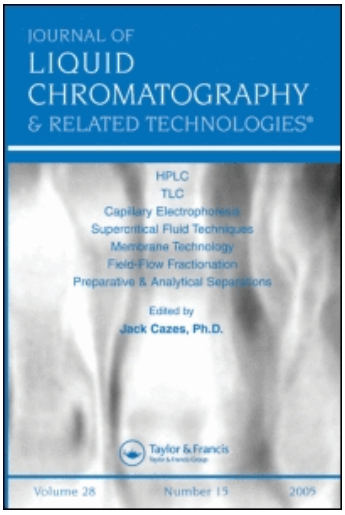

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A MULTIRESIDUE METHOD FOR THE ISOLATION AND LIQUID CHROMATOGRAPHIC DETERMINATION OF SEVEN SULFONAMIDES IN INFANT FORMULA

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ABSTRACT

A multiresidue method for the isolation and high performance liquid chromatographic (photodiode array, UV 270nm) determination of sulfathiazole, sulfisoxazole, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfisoxazole and sulfadimethoxine in a milk-based infant formula is presented. Blank or sulfonamide spiked infant formula samples were blended with octadecylsilyl derivatized silica (C-18) packing material. A column made from the C-18/infant formula matrix was first washed with hexane following which the sulfonamides were eluted with methylene chloride. The eluate contained sulfonamide analates which were free from interfering compounds when analyzed by liquid chromatography. This resulted in correlation coefficients (0.9973 + 0.0016 to 0.9992 + 0.0006), recovery percentages (75.91 + 11.12% to 112.01 + 8.15%) and inter- (5.51 + 1.74% to 15.27 + 8.14%) and intra-assay (1.71

to 8.89%) variabilities for individual sulfonamides, over the concentration range (62.5 to 2000 ng/mL) examined, that are indicative of a useful method for the multiresidue analysis of milk-based infant formula for the presence of sulfonamides.

INTRODUCTION

Nutrient requirements for normal newborn infants are critical, since the rate of growth is most rapid in the first year of life. Thus, infants require a well balanced formula with the proper ratios of fat, protein and carbohydrates and other essential nutrients. These levels have been established by the Infant Formula Act of 1980 (1). The history of infant formulas, outlining nutrient concerns and trends, has been published (2).

The digestion and absorption of food components and elimination of waste products are governed by the development of the gastrointestinal and renal systems. Infants are especially susceptible to conditions which upset the function and/or the normal development of these systems. The introduction of microbial contamination via unsterilized formula can result in diarrhea and subsequent fluid and electrolyte imbalances. Fortunately, due to strict manufacturing guidelines, commercial products are sterile and pose little microbiological threat to infants, if handled properly.

Another, perhaps more important, concern is the introduction of non-therapeutic chemicals which can be harmful to or cause gastrointestinal imbalances in infants. The alteration of normal gastrointestinal microflora as a result of the introduction of antibiotics can result in a disruption of normal digestive processes and lead to diarrhea and associated problems of electrolyte imbalances. Antibiotic compounds can be circuitously introduced to infants from milk based infant formulas and/or infant

meat puree foods. This can occur when the food producing animal has been treated with antibiotics and the resultant milk or meat obtained is then fed to infants via prepared infant foods.

Sulfonamides are broad spectrum antibiotics used widely in the livestock producing industry. Although withdrawal periods and maximal residue limits have been established for animals treated with these drugs, it is unrealistic to assume that no animal derived food products containing residues will reach the market place. Recent evidence implicating sulfamethazine as a possible carcinogen (3) makes this issue even more critical when considering the possibility of sulfonamides being present in infant foods, especially milk-based infant formulas. Thus, the monitoring of sulfonamide residue levels in milk and milk-based infant formulas is needed.

The isolation of sulfonamides from milk involves their extraction utilizing large volumes of extracting solvents, pH adjustments, centrifugations, backwashing and the evaporation of large volumes of solvent (4). Target residues may be lost due to the multiple sample manipulations and/or less than ideal solvent partitioning of residues due to the formation of emulsions. A similar isolation scheme is used for infant formulas. A thorough review of analytical methods for sulfonamides has been published (5).

Recently a method has been developed in this laboratory for multiresidue/multidrug class isolations from biological matrices (6,7) based on the blending of the sample matrix with an octadecylsilyl (ODS) derivatized solid support and appears to overcome many of the complications associated with classical isolations as outlined above. This approach, which we have named matrix solid phase dispersion (MSPD), has also been successfully applied to sulfonamide determinations in milk (8). We report here a further application of this methodology for the isolation and liquid chromatograhic determination of sulfonamides in a milk-based infant formula.

MATERIALS AND METHODS

Chemical and Expendable Materials

All standard compounds and solvents were obtained at the highest purity available from commercial sources and used without further purification. Water for HPLC analyses was triple-distilled water which was passed through a Modulab Polisher I (Continental Water Systems Corp., San Antonio, TX) water purification system. Bulk C-18 (40 micron, 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 gm) and sequentially washing with 2 column volumes each of hexane, methylene chloride (DCM) and methanol. The washed C-18 was vacuum aspirated until dry. Stock sulfonamide solutions (1000 µg/mL) were prepared by dissolving standard compounds with HPLC grade methanol and diluting to the desired ug/mL levels with methanol. Ten mL syringe barrels were thoroughly washed and dried prior to use as columns for sample extraction.

Extraction Procedure

Infant formula (ready to use, milk-based EnFamil Lot-10OCT89ACN00, Mead Johnson Nutritional Division, Evansville, IN.) samples were obtained from a local market. Two grams of C-18 were placed into a glass mortar and an aliquot (0.5 mL) of infant formula (IF) was placed directly onto the C-18. Standard sulfonamides (10 µL,3.125-100 µg/mL stock solutions) and internal standard sulfamerazine (10 µL, 10.0 µg/mL stock solution) were added to the IF and the samples were allowed to stand for 1 min. Blank infant formula (IF) samples were prepared similarly except that 20 µL of methanol containing no sulfonamides were added to

the sample. The samples were then gently blended into the C-18 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 C18/sample matrix was placed into a 10 mL plastic syringe barrel which was plugged 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) 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 (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. To the dry residue were added 0.1 mL of methanol and 0.4 mL of 0.05N H_3PO_4. The sample was sonicated (5-10 min) to disperse the residue, which resulted in a suspension. This was transferred to a micro-centrifuge tube and centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburgh, PA.) at 10,000 x g for 5 min. The resultant clear supernatant was filtered through a 0.45 micron filter (Prep-Disc, Bio-Rad, Richmond, CA.) and an aliquot (20uL) was analyzed by HPLC.

HPLC Analysis

Analysis of sample and standard sulfonamides were conducted utilizing a Hewlett Packard HP1090 HPLC (HP 79994A HPLC Chemstation) equipped with a photodiode array detector set at 270 nm

with a bandwidth of 20 nm and a reference spectrum range of 200-350 nm. The solvent system was a 70:30 ratio (V/V) of 0.05N H_3PO_4 to acetonitrile at an isocratic flow rate of 1 mL/min. A reversed phase, octadecylsilyl (ODS) derivatized silica column (Varian MCH-10, 10 micron, 30 cm x 4 mm id, Varian, Sunnyvale, CA.) maintained at 45C was utilized for all determinations.

Peak area ratio (PAR) 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 spiked sample PARs to PARs of pure standards analyzed under identical conditions gave percent recoveries. Interassay variability was determined as the standard error of the mean of 5-six point sample PAR curves. Intra-assay variability was determined by the standard error of the mean PARs of 5 replicates of the same samples.

RESULTS

Representative chromatograms of extracted infant formula (IF) blank and sulfonamide spiked (250 ng/mL) IF samples are shown in Figures 1A and 1B, respectively. Table 1 gives the compound, concentrations examined, correlation coefficients (+ or - standard deviation), percentage recovery, inter- and intra-assay variability of the seven sulfonamides isolated from spiked infant formula samples.

DISCUSSION

Classical residue isolation techniques can include the mixing or homogenization of the sample matrix into large volumes of extracting solvents, pH adjustments, backwashing of the resultant

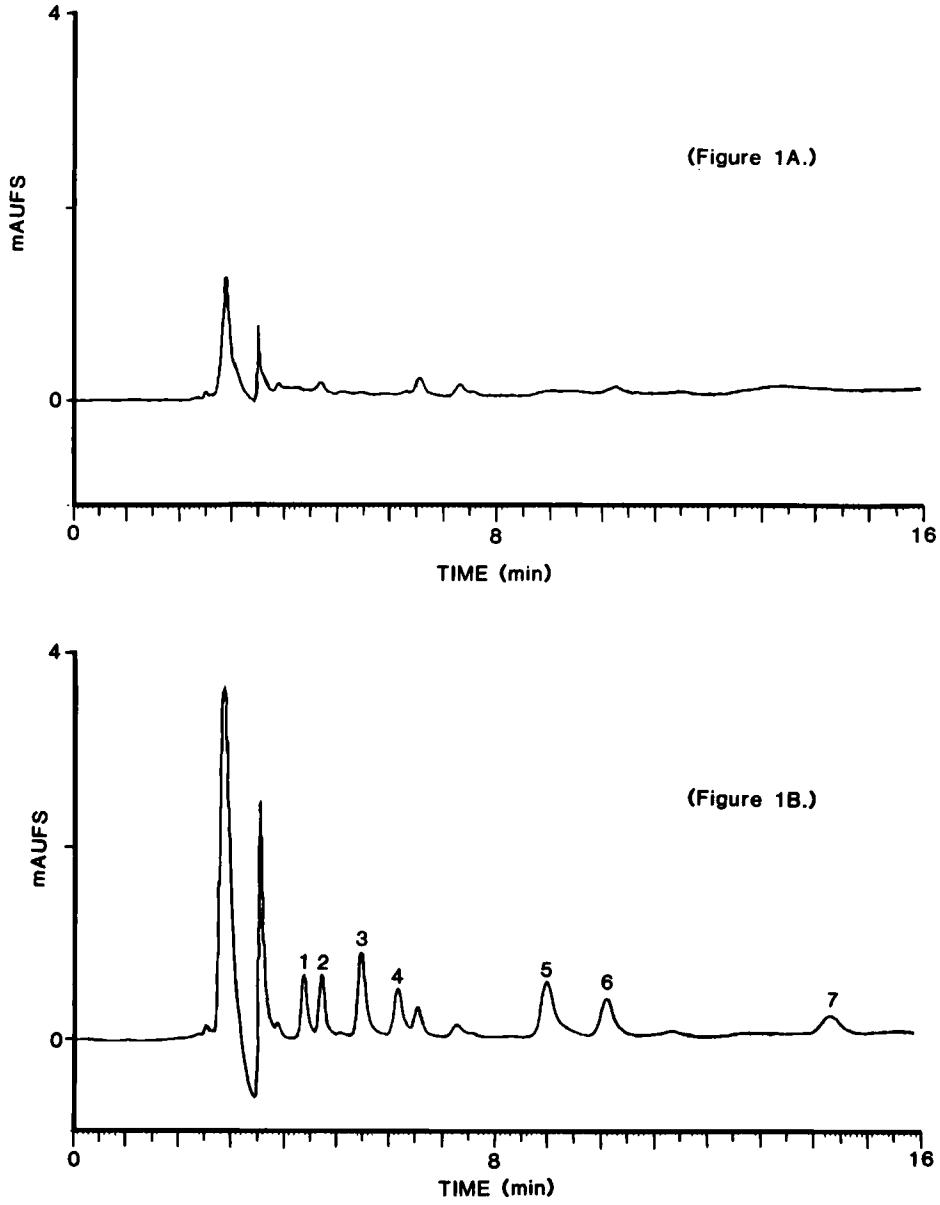

FIGURE 1. Representative chromatograms of the methylene chloride extract of blank infant formula (A) and infant formula spiked with standard sulfonamides (250 ng/mL) and internal standard (200 ng/mL) sulfamerazine (B). Order of elution is sulfathiazole (1), sulfadiazine (2), sulfamerazine (3), sulfamethazine (4), sulfamethoxazole (5), sulfisoxazole (6) and sulfadimethoxine (7).

TABLE 1. Compounds studied, (concentrations of 62.5, 125, 250, 500, 1000 and 2000 ng/ml; 200 ng/ml of sulfamerazine as internal standard; 20 uL injection), correlation coefficients (+ or - standard deviation; SD), percentage recovery, inter- and intra-assay variability of the 7 sulfonamides isolated from spiked infant formula samples. ($\bar{x}$= mean).

COMPOUND	CORRELATION COEFFICIENT r ± SD, n = 5	% RECOVERY $\bar{x}$ ± SD, n = 30	INTERASSAY VARIABILITY %, n = 30	INTRA-ASSAY VARIABILITY %, n = 5
Sulfathiazole	0.9973 ± .0016	75.91 ± 11.12	9.58 ± 3.23	3.15
Sulfadiazine	0.9972 ± .0018	99.62 ± 5.31	9.71 ± 3.47	6.65
Sulfamerazine	Internal Standard	92.67 ± 4.58	5.51 ± 1.74	1.71
Sulfamethazine	0.9992 ± .0006	99.09 ± 8.80	10.91 ± 5.70	3.75
Sulfamethoxazole	0.9989 ± .0008	112.01 ± 8.15	11.35 ± 6.31	4.43
Sulfisoxazole	0.9986 ± .0009	93.08 ± 9.71	10.53 ± 4.74	5.61
Sulfadimethoxine	0.9984 ± .0016	102.99 ± 9.19	15.27 ± 8.14	8.89

extract, additional pH adjustments and/or additional solvent partitioning. These steps may allow one to isolate, with varying degrees of efficiency, targeted residues free from interfering co-extractants. These multi-step procedures, in addition to being time consuming, may result in less than ideal recoveries, accuracy and/or precision. Additionally, multiresidue isolations from a single sample, within a drug class, may be negated due to pH manipulations and differential solvent partitioning characteristics between the residues. These sample preparative steps are essential prior to analysis utilizing sophisticated analytical procedures. Thus, the sample preparative steps may be the most critical aspect of many residue analyses.

Results from the analyses presented here appear to indicate that the present method overcomes some of the limitations of the classical methods. The sample, in this case infant formula (IF), was evenly distributed on a solid (C-18) support, thereby greatly increasing it's surface area and exposing the entire sample to the extraction. Hexane was used to elute lipid material, while the more polar sulfonamides remained on the column. Methylene chloride (DCM) was then used to elute the sulfonamides. The high percentage recoveries and small variabilities (Table 1) 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 IF. The exact distribution of the IF components on the C-18 is not clear but it may involve an association of the lipid component with the lipophilic C-18 polymer and a simultaneous disruption of protein and/or micelle layers which align themselves with hydrophobic regions oriented toward the lipid, while hydrophilic regions extend outward. The hydrophilic regions can then associate with the water and other more polar constituents. This alignment of IF components on the C-18 packing is probably discontinuous in nature as we experienced no difficulty in removing lipid material with the hexane wash. The

theoretical aspects of this phenomena have been thoroughly discussed in a previous report (6,7) and scanning and transmission electron micrographs of uncoated and tissue coated C-18 beads support this hypothesis (unpublished observations).

Chromatograms of a blank IF sample and a spiked IF sample are shown in Figures 1A and 1B, respectively. As can be seen in Figure 1A the blank IF extract was relatively free of interfering compounds. 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 lipids and other compounds, perhaps neutral chromophores, which could otherwise interfere with the analysis. Other more polar chromophores, which are not soluble in methylene chloride, remain on the column. Thus, one can selectively elute the compounds of interest while eliminating potentially interfering compounds.

As a result of the cleanliness of the IF extracts 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 minimal detectable limit for the compounds examined here, utilizing a photodioide array detector, was approximately 1.25 ng on column, which reflects the sensitivity characteristics of the detection system utilized in this study for these compounds. 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 savings in terms of time and solvent, compared to classical extraction techniques, such as the method of Tishler (4), make this procedure attractive. For example, the Tishler method requires 50mL of milk which is extracted several times resulting

in a minimum of 600 mL of extracting solvent that must be evaporated. Additional pH adjustments and washing is necessary before the sample is ready for analysis. The method presented here requires a 0.5 mL sample, 8 mL of hexane and 8 mL of DCM and requires no extensive extract clean-up steps other than drying the DCM, centrifugation and filtering prior to analysis. Furthermore, use of this method as outlined here could result in extracts containing analates relatively free from interfering co-extractants which would aid in their detection by other more sensitive means, such as immunoassay techniques, by eliminating cross-reacting compounds.

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