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

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A method for the isolation and liquid chromatographic determination of chloramphenicol in milk is presented. Chloramphenicol-fortified or blank milk samples (0.5 mL) were blended with octadecylsilyl (C_{18}) derivatized silica (2 g). The C_{18}/milk matrix was used to prepare a column that was washed with hexane (8 mL) followed by benzene (8 mL). Chloramphenicol was then eluted with ethyl acetate (8 mL). The eluate contained chloramphenicol, which was free from interferences when analyzed by high-performance liquid chromatography (HPLC) utilizing UV detection (278 nm, photodiode array; minimum detectable limit 1.25 ng on-column). Linearity (0.999 ± 0.001), average percentage recovery (68.8 ± 8.3%), and inter- (11.6 ± 6.9%) and intraassay (1.4%) variabilities, for the concentrations examined (62.5, 125, 250, 500, 1000, 2000 ng/mL milk), were indicative of an acceptable method for chloramphenicol. 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 chloramphenicol.

Chloramphenicol (CP) [D-(−)-*threo*-2-(dichloroacetamido)-1-(*p*-nitrophenyl)-1,3-propanediol] is a broad-spectrum antibiotic that is an effective therapeutic agent for the treatment of mastitis in cattle (Schwartz and McDonough, 1984). Its use in the United States is restricted to nonfood-producing animals (U.S. FDA, 1988). This is due, in part, to reported cases of serious hematopoietic disturbances in some human subjects exposed to CP (Oski, 1979; Mercer, 1980).

Because CP is an effective treatment for mastitis in cattle, the potential for its misuse exists, posing a health threat to individuals exposed to CP as a residue in milk. Furthermore, chronic illegal use of CP could lead to the development of resistant bacterial strains, as was evidenced by CP-resistant salmonella apparently resulting from CP use in dairy cattle eventually marketed as hamburger (Spika et al., 1987).

These factors necessitate monitoring of milk supplies for possible violative CP residues. The methods for monitoring CP levels should be such that they are rapid, specific, and sensitive enough to allow for CP detection at the minimal levels achievable by present technology.

Thin-layer chromatographic (Schwartz and McDonough, 1984), spectrophotometric (Devani et al., 1981), liquid chromatographic (Wal et al., 1980) and liquid chromatographic–mass spectrometric (Bories et al., 1983) methods have been used successfully for CP determinations, with recoveries ranging from 68 to 104%. However, a major controlling factor dictating the usefulness of any given analytical technique is the sample preparation or cleanup steps.

Isolations of CP from biological matrices have traditionally relied on classical procedures (USDA, 1979), which may include solvent–solvent extractions, centrifugations, multiple back-washings to remove lipid material, further extractions, and the evaporation of large volumes of solvents in order to isolate CP free from interferences. Such classical isolation methods are labor- and material-intensive. As a result of intensified efforts to monitor drug and chemical residues in the food supply, a need for isolation methods minimizing sample sizes, time requirements, and expendable materials exists.

We have recently developed a multiresidue/multidrug class solid-phase extraction technique for the isolation of drugs from biological matrices (Barker et al., 1988, 1989; Long et al., 1989a–c), which overcomes many of the limitations of classical isolation 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 chloramphenicol in milk.

EXPERIMENTAL SECTION

Chemicals 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 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, endcapped; 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 chloramphenicol solution (1000 μg/mL) was prepared by dissolving with HPLC-grade methanol and diluting to the desired working standard concentration (3.13, 6.25, 12.5, 25, 50, 100 μg/mL) 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 an aliquot (0.5 mL) of milk was placed directly onto the C_{18}. Standard chloramphenicol (10 μL of 3.125–100 μg/mL stock solutions) was added to the milk, and the samples were allowed to stand for 1 min. Alternately, fortified milk can be placed onto the C_{18} with equivalent results. Blank milk samples were prepared similarly except that 10 μL of methanol containing no chloramphenicol was added to the sample. The samples were then gently blended into the C_{18} with a glass pestle until the

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mixture was homogeneous in appearance. 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 circle (Whatman No. 1). The column head was covered with a filter paper circle, 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 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 followed by 8 mL of benzene. (Benzene is a chemical carcinogen and should be handled with adequate caution to limit exposure. Work should be conducted in a hood, and protective clothing should be worn.) 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 pressure as described above. The chloramphenicol was then eluted with 8 mL of ethyl acetate as described above for hexane and benzene. The ethyl acetate 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. This was transferred to a microcentrifuge tube and centrifuged (Fisher Microcentrifuge Model 235, Fisher Scientific, Pittsburg, PA) at 13600*g* for 5 min. The resultant clear supernatant was filtered through a 0.45-μm filter (Micro Prep-Disc, Bio-Rad, Richmond, CA), and an aliquot (20 μL) was analyzed by HPLC.

HPLC Analysis. Analysis of sample extracts and standard chloramphenicol was conducted on a Hewlett-Packard HP1090 HPLC (HP 79994A HPLC Chemstation) equipped with a diode array (UV) detector set at 278 nm with a bandwidth of 20 nm and a reference spectrum range of 200–350 nm. The solvent system was a 65:35 ratio (v/v) of 0.017 M 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 μm, 30 cm × 4 mm) maintained at 35 °C was utilized for all determinations.

Standard curves of pure chloramphenicol standards and fortified samples were obtained by plotting integration areas of generated peaks. A direct comparison of extracted chloramphenicol fortified sample areas to areas of pure chloramphenicol standards run under identical conditions gave percent recoveries. The interassay variability was determined as follows: The mean of the areas for five replicates of each concentration (61.25, 125, 250, 500, 1000, 2000 ng/mL) was calculated. The standard deviation corresponding to each mean was divided by its respective mean, which resulted in the coefficient of variation (CV) for each concentration. The mean of these CV's was calculated along with its SD, multiplied by 100, and defined as the interassay variability plus or minus the SD. 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 blanks and chloramphenicol-fortified (250 ng/mL) milk samples are shown in Figure 1, parts A and B, respectively. Table I gives the concentrations examined, correlation coefficients (±SD), percentage recoveries, and inter- and intraassay variabilities of chloramphenicol isolated from fortified milk samples.

DISCUSSION

The isolation of drug or chemical residues from a complex biological matrix such as milk can be a time-consuming, 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

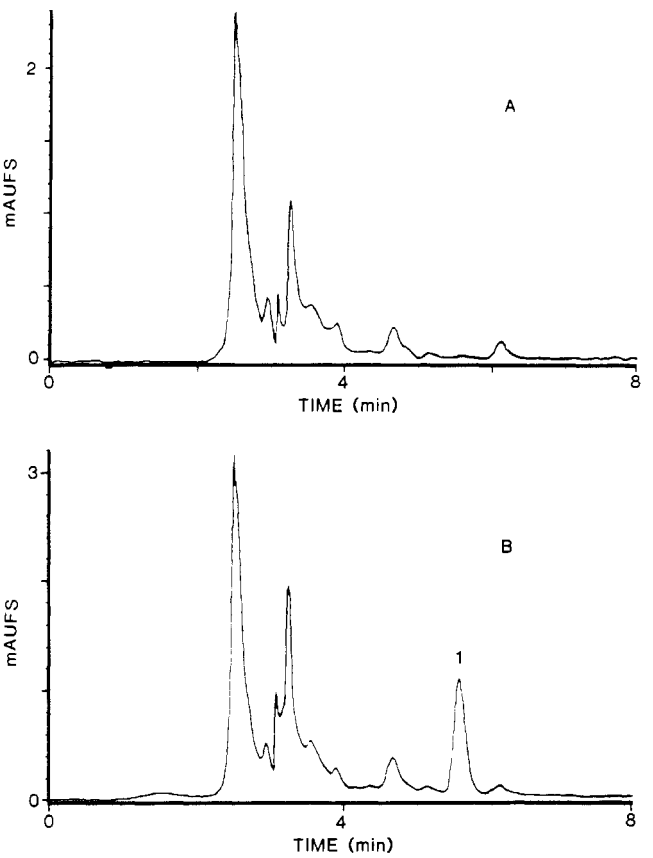

Figure 1. Representative chromatograms obtained from the HPLC photodiode array (278-nm) analysis of the ethyl acetate extract of (A) blank milk and (B) chloramphenicol (peak 1) fortified (250 ng/mL) milk.

Table I. Standard Curve Correlation Coefficient, Percentage Recoveries, and Inter- and Intraassay Variabilities (SD = Standard Deviation) for Chloramphenicol-Spiked Milk Sample

concn, ng/mL	% recovery[a]
62.5	60.8 ± 11.4
125	60.9 ± 13.0
250	65.2 ± 6.7
500	68.1 ± 4.4
1000	78.8 ± 4.2
2000	79.0 ± 5.7
interassay var (n = 30), %	11.6 ± 6.9
intraassay var (n = 5), %	1.4
correln coeff (r; mean ± SD, n = 5)	0.999 ± 0.001

[a] n = 5 (replicates at each concentration).

that contain the targeted residue, with high recoveries free from interferences. Traditional isolation techniques can include homogenizing or mixing of the sample in the extracting solvent(s), pH adjustments, backwashing of the extract, additional solvent extractions, centrifugations, and the evaporation of large volumes of solvents in order to obtain "clean" analytical samples. Unfortunately, losses of targeted compounds may result due to chemical degradations, entrainment in pelleted debris, and less than ideal solvent–solvent extractions as a result of emulsion formations occurring during the extraction procedures and therefore resulting in inconsistent assays. A review of chromatographic methods for chloramphenicol (Allen, 1982) underscores the variability associated with classical isolation techniques for chloramphenicol.

The method presented here overcomes many of the complications outlined above. The blending of the milk

into the C_{18} and the subsequent elution of chloramphenicol from the C_{18}/milk matrix with ethyl acetate resulted in extracts that contained chloramphenicol relatively free from interferences, as can be seen in a comparison of the HPLC chromatograms of blank (Figure 1A) and chloramphenicol-fortified milk (Figure 1B) samples. The C_{18}/milk matrix was first washed with hexane, to remove lipid materials, followed by a benzene wash, which served a similar purpose by removing additional lipid material and interfering chromophores that could have complicated chloramphenicol determinations. Chloramphenicol was then eluted with ethyl acetate, and the resulting extracts showed minimal interferences when monitored by photodioide array detection at 278 nm. The linearity of standards extracted from fortified milk samples, as well as percentage recoveries and inter- and intraassay variabilities, is given in Table I.

In this 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 technique whereby a large volume of solvent is passed over an extremely thin layer of sample. By using a sequential elution protocol, one can selectively remove potentially interfering materials such as lipids and chromophores prior to eluting chloramphenicol with ethyl acetate. Chloramphenicol was not present in either the hexane or benzene extracts as determined by HPLC analysis of an aliquot of each of these fractions, and other more polar chromophores, which were less soluble in ethyl acetate, remained on the column. The theoretical aspects of the MSPD technique have been the subject of previous publications (Barker et al., 1989a,b; Long et al., 1989a–c) for the isolation of different compounds from milk and other biological matrices.

The MSPD method eliminates many of the problems associated with classical isolation techniques. (Wal et al., 1980; Bories et al., 1983). The method uses small samples sizes, has a minimal number of steps, requires no chemical manipulations (such as pH adjustments), and utilizes a minimal amount of solvent. In contrast, classical chloramphenicol isolation (Wal et al., 1980) from biological matrices such as milk require large sample sizes (25 mL of milk), large volumes of extracting solvents (75 mL), multiple extractions, and the evaporation of large volumes of extracting solvents. Because the MSPD sample extract has a minimal number of interferences, an increase in sensitivity may be achieved by increasing injection volume and/or dissolving the extract residue in a smaller final volume. The minimal detectable limit observed was 1.25 ng on-column. The cleanliness of the extract may allow for more sensitive means of detection as well. Use of an internal standard may result in a decrease in the reported assay variabilities and enhance the recovery of chloramphenicol. Thus, the savings in terms of time and solvent requirements make this method attractive when compared to classical isolations and would be useful for market surveys where pasteurized milk is tested for violative chloramphenicol residues.

The results presented here are based on fortified samples. The same 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. The purpose of the pilot study was to examine the application of matrix solid-phase dispersion for the isolation of chloramphenicol from a milk sample, demonstrating the prospect that such methodology may be used to screen for this drug in milk samples. While an examination of milk from animals actually administered chloramphenicol would be ideal, such samples were not available to us and the experiment is outside the scope and limits of practicality of the present research. Such studies are currently under way, examining incurred residues of chloramphenicol in milk obtained from animals used in drug depletion studies, with the assistance of the U.S. Food and Drug Administration.

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