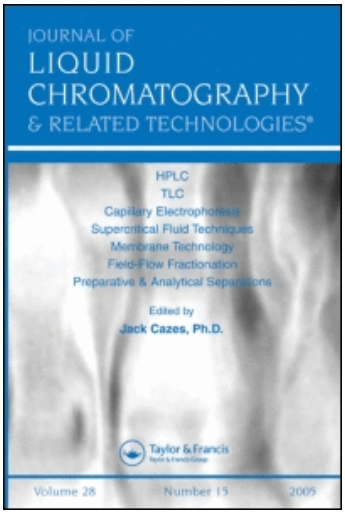

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Isolation and Quantification of Ivermectin in Bovine Milk by Matrix Solid Phase Dispersion (MSPD) Extraction and Liquid Chromatographic Determination

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ISOLATION AND QUANTIFICATION OF IVERMECTIN IN BOVINE MILK BY MATRIX SOLID PHASE DISPERSION (MSPD) EXTRACTION AND LIQUID CHROMATOGRAPHIC DETERMINATION

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ABSTRACT

A technique for the extraction and liquid chromatographic determination of ivermectin residues in bovine milk is described. Avermectin was used as an internal standard. Fortified and blank milk samples (5.0 mL) were blended with 2.0 g C_{18} (octadecylsilyl derivatized silica) in a syringe barrel. After a 2 minute equilibration, the aqueous phase was removed from the column by vacuum aspiration. The ivermectin residues were eluted from the C_{18}/milk matrix with ethyl acetate. After further cleanup by silica solid phase extraction, ivermectin derivatives were formed and then quantified by liquid chromatography with fluorescence detection. The recoveries of fortified ivermectin residues (1.0 - 8.0 ppb) averaged 97.7%. The injected extracts are free from matrix interferences making it easy to calculate the amount of residue present.

INTRODUCTION

The avermectins are macrocyclic lactones derived from the mycelia of the actinomycete *Streptomyces avermitilis* (1). They are active against helminths and arthropods at very low doses (2). Two avermectins have been commercialized to date (3). Abamectin, a mixture consisting of >80% avermectin B_{1a} (B_{1a}) and <20% avermectin B_{1b} (B_{1b}), is widely used as an insecticide and miticide on agricultural crops. Ivermectin, a mixture consisting of >80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and <20% 22,23-dihydroavermectin B_{1b} (H_2B_{1b}) (Figure 1), is widely used as an antiparasitic agent in animals and man.

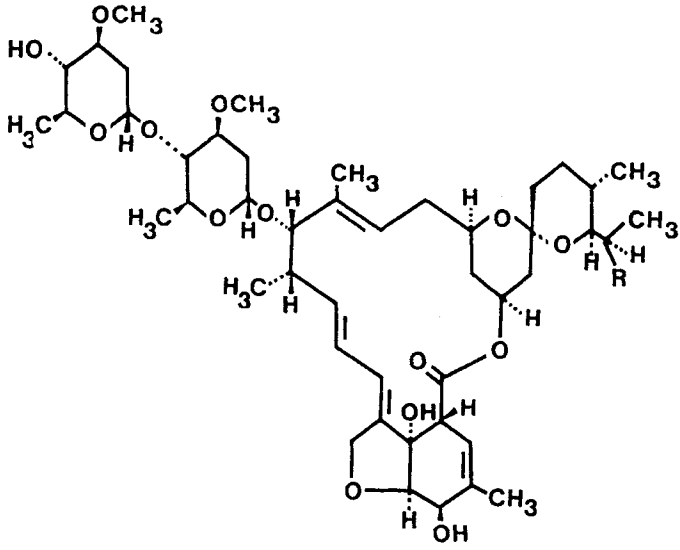

FIGURE 1. The structure of Ivermectin: R= $-C_2H_5$ for H_2B_{1a}, and R= $-CH_3$ for H_2B_{1b}.

Ivermectin residues are depleted slowly from the animal. Tway et al. (4) found the unaltered drug in cattle liver over a period of 28 days post administration. The level of mammary gland excretion of the administered dose is very high (5). Drug depletion studies have shown that dosed ivermectin is secreted through the mammary gland over many weeks (5,6), underscoring concern that residues may be found in retail milk. Ivermectin is currently not approved for use in lactating dairy cows (7). Extrapolation from tissue levels indicates that a regulatory analytical method should be capable of detecting 2 ng/mL (ppb) in milk (Dr. William White, Food and Drug Administration, Center for Veterinary Medicine, personal communication.)

Alvinerie et al.(6), Schenck et al.(8) and Kijak (9) have reported methods for the extraction and determination of ivermectin residues in milk. Unfortunately, these methods employ time consuming multiple extraction, centrifugation and liquid-liquid solvent partition cleanup steps. Recently, Barker et al (10) demonstrated that biological matrices can be homogeneously dispersed with C_{18} (40 μm octadecylsilyl derivatized silica), the resulting mixture packed into a column, and various residues selectively eluted from the column. This method, called matrix solid phase dispersion (MSPD), provides a rapid alternative to conventional methods for milk and tissue extractions. Long (11-14) has shown that MSPD can be used for the extraction of a wide variety of drugs from milk.

This paper describes a rapid, reliable method using a modified MSPD extraction with a solid phase extraction (SPE) cleanup for the determination of ivermectin from milk.

EXPERIMENTAL

Apparatus

(a) *Ultrasonic bath*- Branson Model 2200 (Branson Cleaning Equipment, Shelton, CT), or equivalent.

(b) *Micro pipettors*- Electronic Digital Pipette (Rainin Instruments, Woburn, MA) or equivalent.

(c) *Syringe barrels*.- Used as extraction columns; empty reservoirs, 15-mL and 25-mL sizes; 20 μm frits; and Bond Elut Column Adapters (Varian Sample Preparation Products, Harbor City, CA.)

(d) *SPE vacuum manifold*.- equipped with individual flow control valves so that the vacuum to each SPE column can be shut off (Supelco Corp., Bellefonte, PA) or equivalent.

(e) *Spatula*- Stainless steel.

(f) *Polypropylene volumetric flasks*- 50-mL size, for standard solutions (Nalge Co., Rochester, N.Y.) or equivalent.

(g) *Nitrogen evaporator*- N-Evap (Organomation Associates, Berlin, MA) or equivalent.

(h) *Oil bath*- 95-100°C.

(i) *Centrifuge tubes*.- 15 mL, conical, to be silylated once every two months. Fill each tube to the top with Sylon-CT and allow to stand 20 minutes. Rinse thoroughly with toluene followed by methanol, and fill with methanol. Let it stand 20 minutes and then rinse with acetone and allow to dry. These tubes are cleaned by hand immediately after use by soaking in methylene chloride followed by detergent for at least 3 hours, followed by thorough rinsing with hot water, distilled water and acetone.

(j) *Liquid chromatograph*- Model 650-15 fluorescence spectrophotometer (Perkin Elmer Corp., Norwalk CT).

Operating conditions: excitation wavelength 364 nm; emission wavelength 455 nm; time constant 3. Analytical column: Econosil C_{18}, 5 μm, 4.6 x 250 mm (Alltech Associates, Deerfield, IL); guard column, Brownlee Newguard RP-18, or equivalent (Applied Biosystems Inc., San Jose, CA), ambient temperature; series 410 LC pump with ISS-100 autosampler (Perkin Elmer). Injection volume 50 μL; solvent flow rate 1.0 mL/min.

Reagents and Materials

(a) *Solvents.-* *N,N*-Dimethylformamide (DMF), Photrex reagent grade (J.T. Baker, Phillipsburg, NJ); tetrahydrofuran (THF), HPLC grade (Fisher Scientific, Fair Lawn, NJ); all other organic solvents were distilled in glass, suitable for spectrophotometry, and LC grade (EM Science, Gibbstown, NJ).

(b) *Acetic anhydride.-* Analytical reagent grade (Malinckrodt Inc., Paris, KY).

(c) *N-Methylimidazole-* 99% (Aldrich Chemical, Milwaukee, WI)

(d) *Water-* Filtered and deionized, Milli-Q Plus Water Treatment System (Waters Corp., Milford, MA).

(e) *Sodium sulfate-* Anhydrous, granular (12-60 mesh), ACS reagent grade (JT Baker, Phillipsburg, NJ).

(f) *Analytical standard-* Ivermectin, 1.38% (w/w) H_2B_{1a}, 0.21% (w/w) H_2B_{1b}, in glycerol formal was obtained from Merck Sharpe and Dohme Research Laboratories, Rahway, NJ.

(g) *Internal standard-* Abamectin (87% B_{1a} and 9.4% B_{1b}) was obtained from Merck Sharpe and Dohme Research laboratories).

(h) *Stock standard solutions-* Weigh 0.36 g ivermectin standard into a 50 mL volumetric flask, and

dilute to volume with methanol (100 μg/mL H_2B_{1a}). Store at -20°C.

(i) *Fortification standard solutions*- Dilute 0.5 mL ivermectin stock standard solution to volume in a 100 mL volumetric flask with methanol. Store at -20°C.

(j) *Internal standard solutions*- Dissolve 1 mg abamectin standard in 10 mL methanol (100 μg/mL B_{1a}). Dilute 0.5 mL abamectin stock standard solution to volume in a 100 mL volumetric flask with methanol (500 ng/mL B_{1a}). Store at -20°C.

(k) *MSPD column material*- Bulk C_{18}, Bondesil, 40 μm, 18% load endcapped, octadecylsilyl-derivatized silica (Varian).

(l) *Solid phase extraction (SPE) columns*.- Bond Elut LRC silica, 500mg (Varian).

(m) *Sylon CT*- 5% dichlorodimethyl silane in toluene (Supelco, Bellefonte, PA)

(n) *Derivatizing reagent*- Sequentially mix 0.9 mL DMF, 0.3 mL acetic anhydride and 0.2 mL N-methylimidazole just before use.

(o) *LC mobile phase*- Methanol/THF/water (85+15+5, v/v/v)

(p) *Milk samples*- Raw bovine milk was obtained from FDA, Center for Veterinary Medicine, Beltsville, MD, and stored at -80°C.

Sample fortification

An appropriate volume of the 500 ng/mL (H_2B_{1a}) fortification standard (50 - 400 μL) and 200 μL of the B_{1a} internal standard solution were added to a 50 mL graduated cylinder. Control raw milk was added to the 25 mL mark. Standard and milk were mixed by inverting the cylinder 5 times. (Use of a graduated cylinder rather

than a volumetric flask will allow for more efficient mixing of the lipophilic ivermectin with milk lipids.) Five mL aliquots of this milk were taken through the extraction.

Working standards

Working standard solutions suitable for a 4 point standard curve were prepared daily. Appropriate volumes of fortification standard and 100 μL of internal standard solution were added to four 25-mL volumetric flasks. Methanol was added to volume.

Extraction and cleanup

Fit an empty 25-mL syringe barrel (reservoir) with a 20 μm frit. Add 2.0 g of C_{18} to the barrel. Wash the C_{18} sequentially with 5 mL petroleum ether, 5 mL acetone and two 5 mL aliquots of methanol. After the last methanol is eluted, aspirate the column with full vacuum for <5 seconds. (NOTE: Do not wash the C_{18} column with water after last methanol wash.) Close the valve on the vacuum manifold head.

Pipette 5.0 mL milk onto the prepared C_{18} column. Mix the milk with the C_{18} using a stainless steel spatula. Leave the spatula in the column and allow the milk/C_{18} mixture to equilibrate for 2 minutes, occasionally stirring with the spatula.

Carefully remove the spatula from the column, rinsing it with a stream of water from a wash bottle, collecting the water in the column. Open the valve on the vacuum manifold head, and apply sufficient vacuum to elute the milk from the column at a rate of ≤3 drops per

second. When all the milk is eluted from the column, rinse down the sides of the column with ca. 5 mL water from a wash bottle. Elute the water from the column using vacuum. Repeat the water wash. Dry the column by vacuum aspiration for 5 minutes.

Remove the C_{18} MSPD column from the vacuum manifold. Fit a 15-mL size empty syringe barrel with a frit and fill to ca. 5 cm. with sodium sulfate. Attach the sodium sulfate column below the C_{18} MSPD column with an adapter. Place the tandem columns over a 15 mL silylated centrifuge tube. Add 10 mL ethyl acetate to the top (C_{18}) column. Using a rubber pipette bulb, apply sufficient pressure to initiate flow of the solvent. Allow the solvent to drip by gravity flow, collecting the eluate in the silylated tube.

Remove and discard the columns and evaporate the ethyl acetate eluate to dryness at ≤50°C with the aid of a stream of nitrogen. A small amount of oily residue will remain. Add 2 mL of 40% ethyl acetate in hexane to the centrifuge tube, vortex mix and place in ultrasonic bath for 1 minute.

Place a silica SPE column on the vacuum manifold and wash with 3 mL of 40% ethyl acetate in hexane. Transfer the contents of the silylated centrifuge tube to the silica SPE column and elute at a rate of 1 drop per second. Rinse the silylated centrifuge tube two times with 1 mL 40% ethyl acetate in hexane, transferring the rinsings to the silica SPE column. Save the silylated tube for later collection of SPE column eluate. Wash the silica SPE column with an additional 5 mL 40% ethyl acetate in hexane. Discard all the 40% ethyl acetate in hexane washes.

Elute the silica SPE column with 5 mL of 50% ethyl acetate in methanol, collecting the eluate in the silylated centrifuge tube. Evaporate the eluate to

dryness at ≤60°C with the aid of a stream of nitrogen. Less than 0.05 mL of residue should remain in the silylated tube. Excess moisture, methanol or residue can lead to incomplete derivatization.

Derivatization of Ivermectin

For preparation of standards, add 5.0 mL of each of the working standard solutions to a silylated centrifuge tube. Evaporate to dryness at ≤60°C with the aid of a stream of nitrogen. Add 0.1 mL of freshly prepared derivatizing agent to the sample and standard silylated tubes. Stopper tubes, vortex briefly and centrifuge for a few seconds at low speed. Place the tubes in a 95°C oil bath. The solution should turn black.

After 1 h remove tubes, allow to cool. Add ca 1 mL chloroform to each tube and vortex mix. Elute chloroform on a silica SPE column which was prewashed with 4 mL chloroform. Wash the silylated centrifuge tube with three 1 mL portions of chloroform and elute each through the silica SPE column. Wash the silica SPE column with 2 mL chloroform, collecting all the eluates. Evaporate to dryness at ≤60°C with the aid of a stream of nitrogen. Dissolve residue in 0.5 mL methanol, vortex mix and centrifuge briefly. Inject 50 μL of each standard and each sample into the LC system.

Determine the peak heights of the H_2B_{1a} and B_{1a} fluorescent derivatives. Calculate the peak height ratios for each of the sample and standard injections as follows:

Peak height ratio =

(Peak Height H_2B_{1a}/Peak height B_{1a})

Construct a linear regression curve of the peak height ratio vs ng/mL H_2B_{1a} in the standards. Using the peak height ratios of the milk sample injections, calculate concentration (ng/mL) of H_2B_{1a} injected from the standard curve.

Calculate concentration of H_2B_{1a} in the milk sample as follows:

$$H_2B_{1a} \text{ ng/mL (sample)} = H_2B_{1a} \text{ ng/mL (injected)}/5$$

where 5 is the number of mL milk taken through the extraction.

RESULTS AND DISCUSSION

To test the performance of the method, raw milk samples were fortified initially with ivermectin only. Later samples were fortified with ivermectin and abamectin (internal standard). Milk samples were extracted, cleaned up, and derivatized. Derivatized residues were detected by liquid chromatography with fluorescence detection (Figure 2A). Representative chromatograms of control milk samples show no interfering matrix background peaks (Figure 2B). The recoveries of fortified ivermectin residues from the milk (1.0-8.0 ppb) were calculated both with and without the internal standard. The results are shown in table 1.

Ivermectin was rapidly extracted from milk using MSPD. When the milk was mixed with the C_{18}, ivermectin and the milk lipids were adsorbed in a thin layer onto the surface of the C_{18} particles. The polar milk matrix components and most of the milk proteins were eluted with the aqueous phase. Ivermectin was eluted from the column with ethyl acetate.

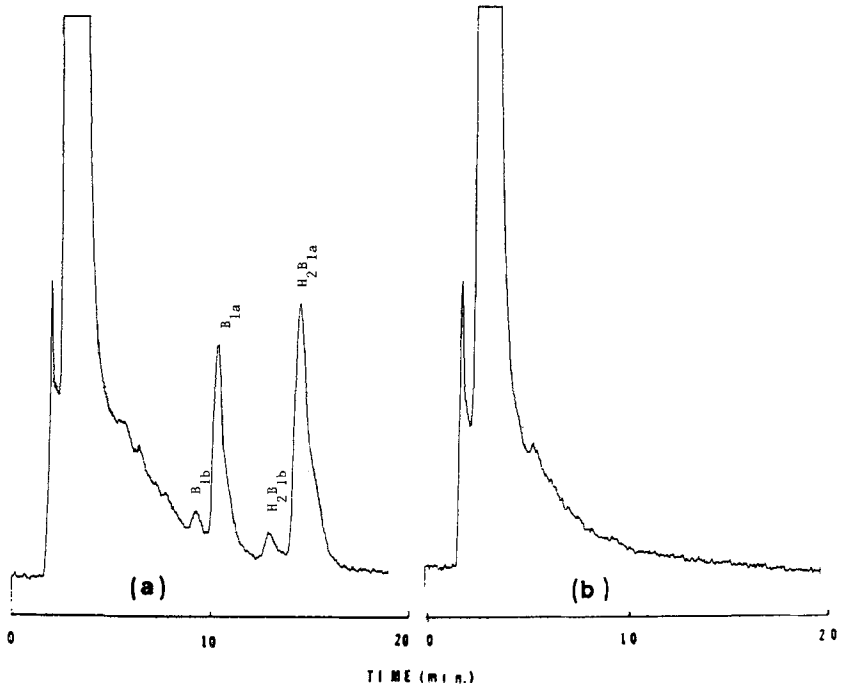

FIGURE 2. Typical chromatograms of (a) milk fortified with 4.0 ppb ivermectin (H_2B_1) and abamectin internal standard (B_1), and (b) control milk.

TABLE 1.

Recoveries of Ivermectin from Bovine Milk.

Fortification level (ppb)	% Recovery (% RSD)[a] calculated using internal standard	% Recovery (% RSD)[b] calculated without internal standard
1.0	97.0 (2.0)	85.7 (8.4)
2.0	104.1 (0.7)	83.5 (4.6)
4.0	97.7 (0.9)	91.5 (10.3)
8.0	99.9 (1.7)	81.0 (8.5)

[a] n=3
[b] n=7

The ethyl acetate MSPD eluate contained some milk co-extractants, which interfered with the derivatization of ivermectin. Further cleanup using commercially available SPE columns was explored. The ethyl acetate MSPD extracts from milk spiked with 50 ppb ivermectin were evaporated and the residue was cleaned up using an alumina, Florisil or silica SPE column, as per published procedures (15-18). The efficiency of the SPE column cleanup was evaluated using LC with UV detection (17). The silica SPE cleanup developed by Vuik (18), for the determination of avermectin in cucumbers, was far superior to both the alumina and Florisil SPE cleanups for these particular extracts. The silica extracts exhibited a minimal number of interfering coextractants when analyzed by LC-UV, and a minimal amount of residue remaining after the solvent was evaporated.

MSPD has been widely used for the extraction of drugs from milk. Typically, 2.0 g C_{18} is mixed with 0.5 g milk in a mortar and pestle. The resulting homogenate is transferred into a syringe barrel. Van Pouck et al. (19) have developed a modified MSPD method for the determination of sulfonamides in milk. Five g milk is mixed directly with 2.0 g C-18 in a syringe barrel. This eliminates the need to transfer the homogenate and allows larger volumes of milk to be extracted, thereby increasing the sensitivity of the method.

The MSPD method eliminates many of the problems associated with traditional isolation techniques. Traditional methods for the isolation of ivermectin from milk (6,8-9) employ multiple solvent extractions and centrifugation steps which extract the ivermectin along with the milk fat. Ivermectin is then separated from the fat by multiple liquid-liquid partition steps. Using the MSPD method, ivermectin is rapidly separated from the milk matrix coextractants without having to use tedious

liquid-liquid extraction and centrifugation steps. The savings in time make the MSPD method attractive when compared to traditional techniques.

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