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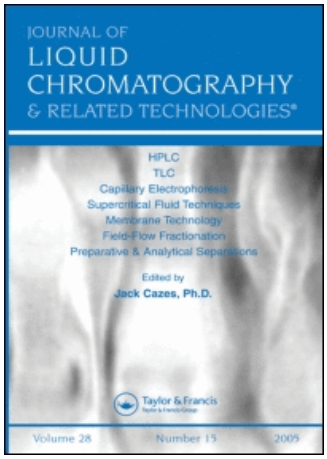

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Online Publication Date: 01 January 2007
To cite this Article: Primus, Thomas M., Jojola, Susan M., Robinson, Stacie J. and Johnston, John J. (2007) 'Determination of Sulfadimethoxine Residues in Skunk Serum by HPLC ', Journal of Liquid Chromatography & Related Technologies, 30:14, 2095 - 2102
To link to this article: DOI: 10.1080/10826070701435095
URL: http://dx.doi.org/10.1080/10826070701435095

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Journal of Liquid Chromatography & Related Technologies®, 30: 2095–2102, 2007
ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701435095

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Abstract: Sulfadimethoxine (SDM) was extracted from skunk serum and isolated by reversed-phase high performance liquid chromatography. SDM was detected by ultra-violet absorbance at 270 nm and quantified by comparison to an external calibration standard. Recovery data were determined by analyzing SDM fortified control serum. The overall mean recovery with relative standard deviations of SDM in fortified skunk serum samples was 99 ± 7%. The recovery for 0.38, 5.2, and 14.2 μg/mL SDM was 96.0 ± 7.5%, 102 ± 6.1%, and 97.3 ± 5.1%, respectively. The method limit of detection for SDM in skunk serum ranged from 0.032 to 0.057 μg/mL SDM with a mean value of 0.040 μg/mL SDM. The method reported is much simpler and equally efficient as previous methods developed for the determination of SDM residues in serum.

Keywords: Sulfadimethoxine, High performance liquid chromatography, Serum, Skunk

INTRODUCTION

The large percentage of rabies cases reported in wildlife occurs in skunks (*Mephitis mephitis*).[1] A functioning deliverable oral bait vaccination of skunks for rabies control is currently in the development stage.[2] While an oral rabies vaccine is being developed, an efficient bait and sachet were

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tested. To test bait ingestion by skunks a biomarker was used in place of a vaccine. Sulfadimethoxine (SDM) dissolved in deionized water was used as a mock vaccine in the sachets. Sulfadimethoxine is a sulfonamide, which is a bacteriostatic and has antibacterial properties when potentiated with trimethoprim. Sulfadimethoxine has been demonstrated clinically or in the laboratory to be effective against a variety of organisms, such as *streptococci*, *klebsiella*, *proteus*, *shigella*, *staphylococci*, *escherichia*, and *salmonella*.[3] Sulfadimethoxine treatment has been shown to reduce the levels of these organisms in respiratory, genitourinary, enteric, and soft tissue infections of dogs and cats. Additionally, sulfadimethoxine has been demonstrated to be a promising biomarker to monitor bait consumption with dogs,[4] raccoons,[5] and badgers.[6]

Most methods for the analysis of sulfadimethoxine have a sample cleanup step following the extraction procedure. Most cleanup steps are accomplished by liquid-liquid extraction, solid-phase extraction columns, or on-line columns prior to the analytical column in the HPLC analysis.[7–10] Others have used liquid chromatography/mass spectrometry (LC/MS) to possibly avoid sample extract cleanup.[11–12] Unfortunately, LC/MS is not a widely available technique for routine analysis. There are also enzyme linked immunosorbent assay (ELISA) test methods to determine sulfadimethoxine in some matrices, which have become commercially available.[13–14]

The ELISA method was providing inconsistent results with calibration standards and skunk serum samples, therefore, the Analytical Chemistry Project (ACP) at the National Wildlife Research Center (NWRC) was asked to develop a simple and efficient method for the determination of sulfadimethoxine in skunk serum. The objective of the Analytical Chemistry Project was to develop a high throughput method without a cleanup step for the analysis of SDM in skunk sera. Bait acceptance and preferences could be determined by applying this method to quantify SDM residues in skunk serum after animals were offered sachets filled with mock vaccine (aqueous SDM solutions).

EXPERIMENTAL

Sample Collection

Whole blood samples were collected from skunks 1 to 4 days post exposure to sachets filled with an 80 mg SDM/mL aqueous solution (mock vaccine) of the sodium salt of sulfadimethoxine. Blood samples were collected and approximately 500 μL of whole blood was centrifuged (Eppendorf Micro Centrifuge 5415C) at a relative centrifugal force of 12,600 × g for 10 min. A 200 to 400 μL portion of the serum was transferred to a separate vial and stored in a freezer at −12°C until assayed.

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Reagents

Acetontrile was liquid chromatography grade (Fischer Scientific, Denver, CO). Deionized water was purified using an in-house reversed osmosis water purification system. Technical grade potassium dihydrogen phosphate (Fischer Scientific, Denver, CO) was used to prepare the aqueous 0.025 M phosphate buffer solution. The buffer solution was adjusted to a pH of 5.0 with a 4 N phosphoric acid solution.

Technical grade sulfadimethoxine, sodium salt (99%) was obtained from Sigma-Aldrich (St. Louis, MO). A concentrated stock standard of sulfadimethoxine was prepared by first drying the technical grade compound for 4 hours at 110°C, then dissolving 5.000 mg in 10.0 mL of methanol. Working standards, ranging in concentration from 0.100 to 10.0 μg/mL, were prepared by dilution of stock solutions with mobile phase. All standard solutions were stored in the dark at 23°C.

Response Linearity

Two sets of six sulfamethoxine standard solutions were prepared ranging from 0.100 to 9.93 μg/mL. Data were collected from duplicate injections of each solution and a plot was constructed of analyte peak response (y-axis) vs sulfadimethoxine concentration (x-axis).

Sample Preparation

A 100 μL aliquot of each sample was transferred into a 1.5 mL plastic Eppendorf microcentrifuge tube. To each sample, a 100 μL portion of phosphate buffer was added, followed by 200 μL of acetontrile. The acetonitrile is significant in that most of the blood proteins remaining in the serum are denatured and precipitate out of the sample solution. Each sample vial was vortex mixed and placed in an ultracentrifuge (Eppendorf Micro Centrifuge 5415C) at a relative centrifugal force of 12,600 × g for 10 minutes. The extract was transferred to a sample vial with a 0.350 mL insert (Supelco, Bellefonte, PA), capped, and analyzed by high performance liquid chromatography (HPLC).

High Performance Liquid Chromatography

The HPLC system consisted of a Hewlett-Packard 1090 liquid chromatograph (Palo Alto, CA) equipped with a diode array multiple wavelength detector (Table 1). The mobile phase was prepared by mixing 600 mL of 20 mM dihydrogen phosphate buffer (pH = 5.00) and 400 mL of acetonitrile. The

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Table 1. HPLC parameters for the analyses of skunk serum extracts

Parameter	Conditions
Mobile phase	60:40 Water w/20 mM KH_2PO_4:Acetonitrile
Column cleaner	6:4 Water:Acetonitrile
Flow rate	1.0 mL/min
Injection volume	25 μL
Column	Phenomenex ODS 3 (C18), 5 μm, 250 mm × 4.6 mm i.d. or equivalent (use guard column containing identical HPLC packing)
Column temperature	35°C
Detector	UV @ 270 nm
Run time	15 minutes

mobile phase was degassed by sparging with helium. At the end of each set (28 samples per set) of analyses, the column was washed with a mixture of 6:4 (v/v) water:acetonitrile for 40 minutes.

Quality Control Samples and Fortification of Controls

Pretreatment samples from two animals were screened and used to prepare quality control samples. Control samples were fortified at 0.38, 5.2, and 14.2 mg/kg sulfadimethoxine with aliquots of fortification standards of sulfadimethoxine in methanol. Each 100 μL portion of control serum was fortified with 5 μL of fortification standards at 8.0, 110, and 300 μg/mL. The aliquot of 5 μL was used to minimize the amount of protein being denatured by methanol. The quality control samples were then assayed with the method described previously.

RESULTS AND DISCUSSION

Response Linearity

A linear regression was performed on the data set and produced an $r^2 = 0.9997$. The plot of log (peak response) vs log (sulfadimethoxine concentration) produced a slope of 0.99140 and an $r^2 = 0.9997$. The average response factor over the range of the calibration curve produced a coefficient of variation of 2.8%. A linear and proportional relationship existed between chromatographic peak response and sulfadimethoxine concentration. Therefore, a single point calibration was used to calculate the concentration of sulfadimethoxine in the sample extracts.

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Method Limit of Detection

The method limit of detection (MLOD) was calculated as the concentration of sulfadimethoxine required in the sample to generate a signal equal to 3 times the baseline noise (peak to peak) observed in the chromatogram of the control extract. The MLOD was estimated from the chromatographic response in height of a control serum extract and an extract from a control serum sample fortified at 0.38 μg/g. The mean MLOD for skunk serum samples was 0.040 μg/mL. For the chromatographic parameters chosen, the retention time of sulfadimethoxine was approximately 6.3 minutes as shown in Figure 1. No significant chromatographic response was noted at the retention time of sulfamethoxine in the chromatogram of the control tissue extract (Figure 2).

Sulfadimethoxine Recoveries from Fortified Control Serum and Residues Detected

Control serum collected from skunks not exposed to sulfadimethoxine was fortified at 0.38 (n = 6), 5.2 (n = 6), or 14.2 μg/mL (n = 3) and produced recoveries of 96.0 ± 7.5%, 102 ± 6.1%, and 97.3 ± 5.1%, respectively. The overall mean recovery of sulfadimethoxine fortified control skunk serum (n = 15) was 99 ± 7%. Sulfadimethoxine residues were calculated as the average of duplicate analyses of samples, when greater than 200 μL of serum was available.

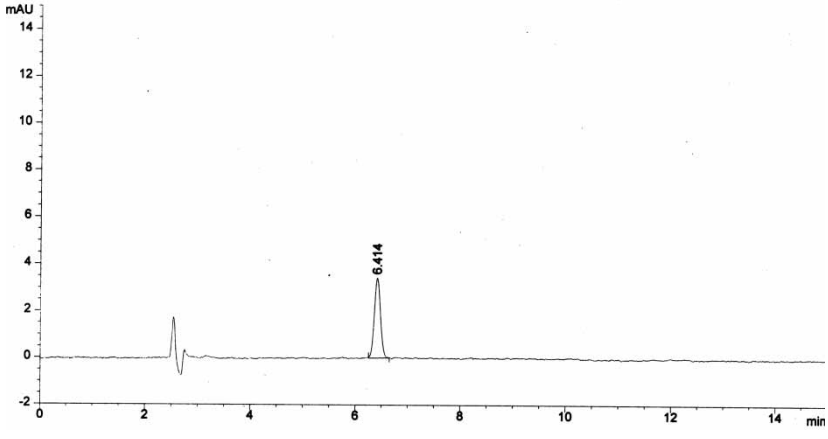

Figure 1. Chromatogram of a 0.60 μg/mL sulfadimethoxine standard detected at 270 nm.

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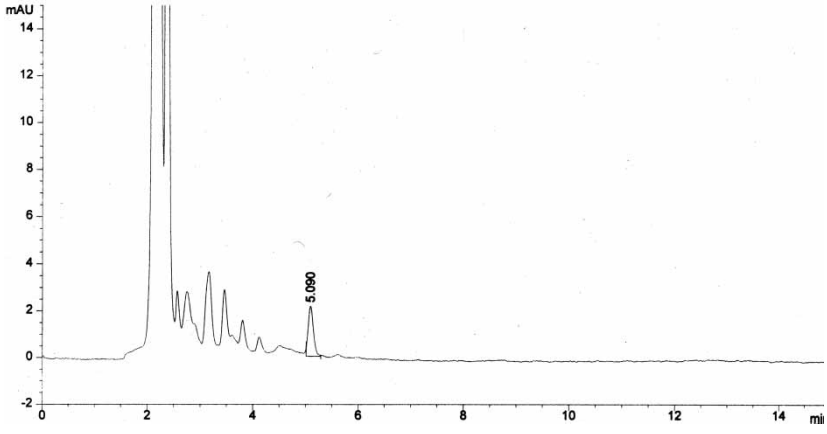

Figure 2. Chromatogram of a control skunk serum extract detected at 270 nm.

Sulfadimethoxine residues in skunk serum of the samples analyzed ranged from <MLOD to 28.7 μg/mL. A chromatogram of a skunk serum extract collected day 1 post exposure is shown in Figure 3. The sulfadimethoxine residues were observed in a few animals 4 days post exposure, as shown in Figure 4, this skunk serum contained 0.050 μg/mL SDM. Very few animals were observed to have detectable levels of sulfadimethoxine after 4 days. The concentration of sulfadimethoxine on day 1 post exposure ranged from 1.1 to 28.7 μg/mL with a mean of 7.4 μg/mL.

The addition of acetonitrile to the serum denatured a large portion of the blood proteins, followed by the centrifuge step which produced a sample free

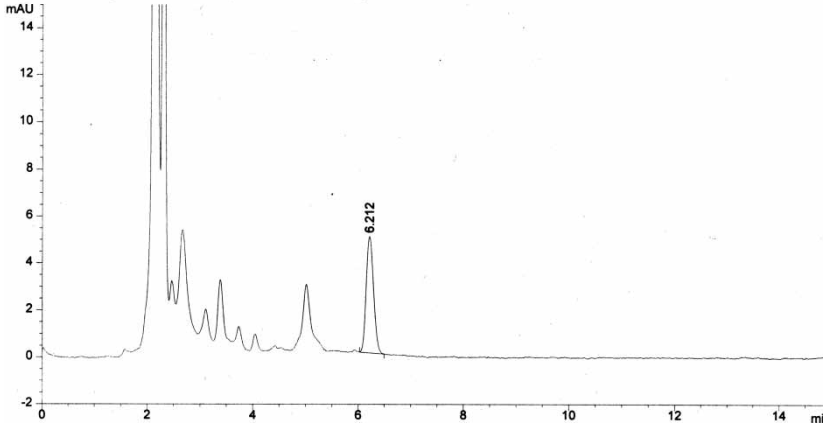

Figure 3. Chromatogram of a skunk serum extract detected at 270 nm, which contains 3.84 μg/mL sulfadimethoxine.

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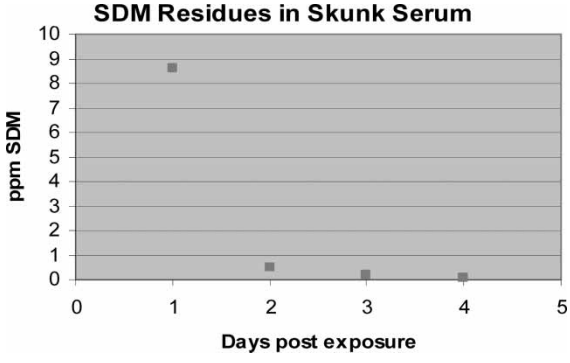

Figure 4. A representative set of sulfadimethoxine residues from a skunk exposed to sachet containing a solution of the biomarker.

of interferences at the retention time of sulfadimethoxine (Figure 2 and Figure 3). Therefore, a cleanup step was not required and, thus, the method proved to be extremely simple and efficient as over 120 samples were analyzed in less than two weeks.

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Received March 7, 2007
Accepted April 17, 2007
Manuscript 6062