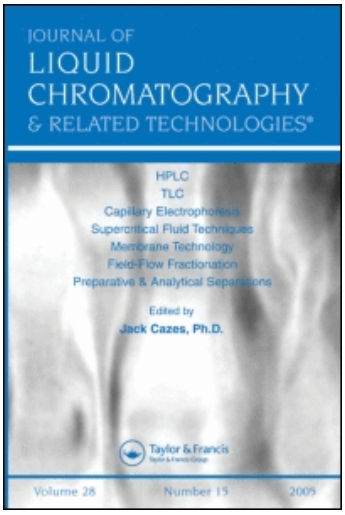

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DETERMINATION OF DIHYDROSTREPTOMYCIN SULFATE IN MILK BY HPLC USING ION-PAIR AND POSTCOLUMN DERIVATIZATION

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ABSTRACT

An isocratic, paired-ion reversed-phase high-performance liquid chromatographic method for the determination of dihydrostreptomycin sulfate in milk by postcolumn derivatization has been developed. The milk was treated with 85% trichloroacetic acid to precipitate proteins, and the supernatant clean-up was performed using a tC18 pretreatment column. The correlation coefficients were 0.999. The lower limit of quantification was 25 ng/ml and the limit of detection close to 15 ng/ml. The recovery of dihydrostreptomycin sulfate varying from 82.6 to 82.8%.

INTRODUCTION

Dihydrostreptomycin (DHS) is a clinically useful aminoglycoside antibiotic with high potency against a wide range of Gram-negative and same Gram-positive bacteria in cattle, pigs, sheep and humans, (1 - 4).

In veterinary medicine the combination of penicillin and DHS is widely used in the treatment of different systemic and local infections e.g. mastitis in dairy cows. This represents a potential hazard to consumers due to persistence of residues in the milk (5-7). DHS is produced by the catalytic hydrogenation of streptomycin (8). Numerous chemical and physical methods have been reported for the analysis of streptomycin and DHS, including paper, thin-layer, and column chromatography, electrophoresis, spectrophotometry and colorimetry, titrimetry, and polarography (9). These methods are time-consuming and have poor specificity, sensivity, and precision.
A post-column derivatization system with ninhydrin as the fluorigenic reagent in the mobile phase, has also been developed for the fluorimetric determination of guanidino compounds by HPLC (10). A procedure for HPLC determination of streptomycin in serum (11), and a method for the determination of streptomycin in meat by HPLC has been published (12). More recently a method for the determination of both streptomycin and DHS in pork and bovine muscle and kidney has also been published(13). However, none of the published methods appeared to be applicable to DHS in milk.

MATERIALS AND METHODS

Materials and Reagens

Whole milk (pasteurized, 3.8% fat) was purchased from local grocery stores, for use as control material and for spiking with DHS to conduct recovery experiments.
1-Heptanesulfonic acid and 1-octanesulfonic acid were obtained from Supelco Inc.Supelco Park, Bellefonte. USA.
DHS and triethylamine were supplied by Sigma Co. (St. Louis, MO, USA).
All chemicals and solvents were of analytical and HPLC grade. DHS stock solution and working standards were prepared by dilution with solution A consisting of 0.02 M 1-heptanesulfonic acid (sodium salt) and 0.01 M di-sodium hydrogenphosphate-2-hydrate,(Ferax, Berlin, Germany), made by dissolving 4.45 g/l heptane sulfonate and 1.8 g/l di-sodium hydrogenphosphate in approx.750 ml of water when making 1 litre of solution. The pH was then adjusted to c. 6.3 with 5 M phosphoric acid and to 6.0 with 1 M phosphoric acid, and the solution made up to volume (1 l) with water and the pH again adjusted to 6.0 with 1 M phosphoric acid. The stock solution and

working standards were stored in a refrigerator.
1,2-ethanedisulfonic acid (disodium salt) was supplied by Tokyo Kasei Kogyo (Tokyo, Japan).
Ninhydrin was obtained from Riedel-de Haën, (Germany), while ortho-phosphoric acid 85% was supplied by Merk (Germany), and trichloroacetic acid (TCA) by Ferax (Laborat GMBH, Berlin, Germany).
Sep-Pak Vac cartridges for solid phase extraction, tC18 Vac (trifunctional) 6cc (1g), were supplied by Waters Division of Millipore (34 Maple Street, Milford, Massachusetts 01757 U.S.A.).

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, and a ISS 100 sampling system equipped with a Lauda RMT6 cooler (14 °C) from Messgueräte Werk Lauda, (Lauda Königshafen, Germany). The integration was carried out using the software programme Omega-2 (Perkin-Elmer), wich was operated on an Olivetti M300 personal computer connected to a Bj-330 printer (Canon). The analytical column (operated at room temperature), (stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 2.0 cm x 4.6 mm I.D.) were packed with 5-µm particles of Supelcosil LC-ABZ (Supelco, Bellefonte, PA, USA).
The mobile phase was a mixture of three solutions B, C, and D (63:19:18). Solution B was 0.04 M 1-octanesulfonic acid Na salt, 0.02 M 1,2-ethanedisulfonic acid disodium salt, and 0.005 M ninhydrin, made by dissolving 8.65 g/l 1-octanesulfonate and 4.68 g/l 1,2-ethanedisulfonic acid in ca. 750 ml of water when making 1 litre of solution. The pH was then adjusted to 3.2 with acetic acid, and 0.891 g/l ninhydrin added and dissolved. The solution made up to volume with water and the pH again adjusted to 3.2 with acetic acid. Solution C was acetonitrile with 0.3% triethylamine (freshly prepared every day), while solution D was methanol. The flow rate was 1.4 ml/min. The column effluent was introduced into a vortex mixer from a heater system for HPLC post column reactions (PCRS 520 - Kratos) equipped with a heat exchanger (vortex mixer is not a mixing tee, but a low volume (1.2 µl) mixing device for two fluid streams). A Series 10 Liquid Chromatograf (Perkin-Elmer), (included pulse-dampened pump) with a mobile phase of 0.3 M NaOH at a flow rate of 0.5 ml/min. was coupled to the vortex mixer, and a reaction coil (Beam Boost

Photochemical Reactor Unit, PTFE 10 m x 0.3 mm I.D.-ITC Handelsgesellschaft m. b. H. Frankfurt, Germany). The reaction temp. was 80 °C. The solvent stream was then cooled using a heat exchanger (operated at room temperature), to prevent gas emission in the detector, which was a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 305 nm and emission wavelength of 500 nm, with a response of 5 and a factor of 256. The samples were injected at intervals of 10 min. Aliquots of 75 µl were injected onto the column for the determination of DHS.

Sample pretreatment

To 6 ml whole milk, were added 0.5 ml solution A (or standard) and 1.5 ml 85% TCA in water. The sample was shaken vigorously for 10 s. followed by centrifugation for approximately 3 min. (4000rpm). After adding 2 ml dichloromethane, the sample was mixed for 5 s. and then centrifuged for 5 min. (4000 rpm).
Six ml of the supernatant (corresponding 4.5 ml milk) was pipetted into a graduated glass-stoppered centrifuge tube, together with 2.5 ml 4 M NaOH. The sample was mixed for 2 s. The homogenate was then centrifuged for 10 min. (4000 rpm). The upper layer were transferred to a clean tube, 2.5 ml of 0.5 M phosphoric acid added, and the pH adjusted to six (+- 0.03) with 1 M NaOH or 0.5 M phosphoric acid. After 1.5 ml solution A had been added, the sample was mixed and the solution loaded onto a conditioned tC18 column.

Clean-up on SPE-column. The column was activated with 2x5 ml methanol, 2x5 ml water, 3x5ml methanol, 3x5 ml water and 2 ml solution A, prior to extract of the milk. The aqueous milk extract was applied onto the column and washed with 1 ml solution A, then with 1 ml 3% methanol in water, and finally with 16 ml 30% methanol in water without sucking, and eluted with 3.5 ml 20% formic acid in methanol with full vacuum. The collected eluates were evaporated to dryness under a stream of nitrogen (60 °C), and dissolved in 300 µl of solution A, after which 200 µl chloroform were added. The extract was mixed vigorously for 10 s. followed by centrifugation for approximately 3 min. Aliquots of the aqueous layer (75 µl) were injected onto the column for the determination of DHS.

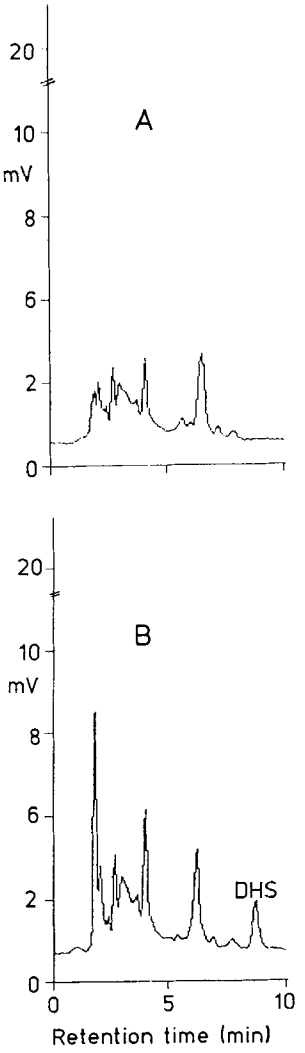

FIGURE 1

Chromatograms of extracts from milk.
A: drug-free milk, **B**: Milk spiked with DHS (400 ng/ml).

TABLE 1

Recovery and Repeatability for Dihydrostreptomysin Sulfate from Spiked Samples of Milk.

Sample	No. of samples	Amount of DHS in spiked samples (µg/ml)	Recovery % DHS Mean	SD*
Wole Milk (6 ml)	8	0.1	82.6	0.7
	8	0.4	82.8	1.2

*SD = relative standard deviation

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for DHS were determined by spiking milk samples with standard solutions to yield 25, 30, 50, 100, 200, 300 and 400 ng DHS per ml of sample, respectively. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked milk, with those of pure standard solutions.
The linearity of the standard curves for DHS in milk was calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean milk samples and spiked samples with DHS are shown in Figure 1.
The standard curves were linear in the investigated area 25 - 400 ng/ml for DHS in milk, while the corresponding correlation coefficients were r=0.999.
Table 1 shows the recovery and repeatabilities of DHS from milk.
The average recovery over the concentration range of the standard curve varied from 82.6 to 82.8%. The precision of these recovery studies varied from 0.7 to 1.2%.
Ninhydrin forms high intensity fluorophors with guanidino compounds in alkaline media (13). DHS, which has two guanidino groups, yields similar fluorophors.
In our preliminary studies, non-polar sorbent materials

such as C18, C18OH, envirelut PAH, C8, C2 from Varian and C18 and tC18 SPE-cartridges from Waters were tested and acceptable recovery for DHS were obtained for DHS in milk pretreated on tC18 from Waters. We recommend however to test each lot of tC18 before use.
The eluting agents were tested, the eluting solvents methanol/formic acid (8:2, v/v) giving 100% recovery. It appeared, however, necessary to reduce the acidity of the eluate before injection onto the analytical column. The eluate therefore had to be evaporated to dryness in a stream of nitrogen gas and reconstituted in the solution A.
The chromatographic system appeared to be efficient for the determination of DHS in milk, the limit of quantification being 25 ppb and the limit of detection close to 15 ppb. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies. The method presented in this paper is selective, robust, sensitive and accurate. This method can also be used for the determination of streptomycin in milk.

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