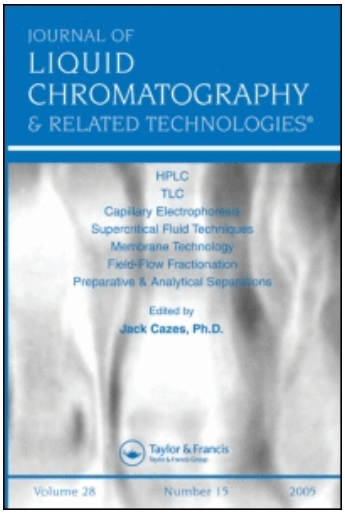

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V. Hormazábal[a]; M. Yndestad[a]
[a] Division of Food Hygiene Norwegian College of Veterinary Medicine, Norway

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DIHYDROSTREPTOMYCIN SULFATE IN KIDNEY AND MEAT USING POST COLUMN DERIVATIZATION

Víctor Hormazábal, Magne Yndestad

Division of Food Hygiene
Norwegian College of Veterinary Medicine
P. O. Box 8146-Dep.
N-0033 Oslo 1, Norway

ABSTRACT

A high performance liquid chromatographic method for the determination of dihydrostreptomycin sulfate in kidney and meat from cow, and swine, has been developed. The sample was treated with trichloroacetic acid, and the supernatant clean-up was performed using a Bond Elut Certify II pretreatment column. The lower limit of quantification was 40ng/g and the limit of detection close to 20ng/g. The recovery of dihydrostreptomycin sulfate varyied from 73.2 to 73.5% and from 80.0 to 82.9% for kidney and muscle, respectively.

INTRODUCTION

Dihydrostreptomycin (DHS) is a semi-synthetic aminoglycoside antibiotic approved for use in food-producing animals in most countries. It is available in various formulations for the treatment of a wide range of Gram-negative and some Gram-positive bacteria.[1-4]

In veterinary medicine, the combination of penicillin and DHS is widely used for the treatment of bacterial infections in cattle, pigs and sheep. This represents a potential hazard to consumers due to residues in the meat.[5-7] DHS is potentially toxic, causing damage in vestibular and auditory function.[8]

Numerous chemical and physical methods have been reported for the analysis of streptomycin (STR)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 β-naphthoquinone-4-sulfonate as the fluorigenic reagent in the mobile phase, has also been developed for the fluorimetric determination of guanidino compounds by HPLC.[10] A method for the determination of both DHS and STR in pork and bovine muscle and kidney using on-line sample enrichment liquid chromatography was published in 1994.[11] More recently, a HPLC method for the determination of both DHS and streptomycin in milk[12] has been published.

The purpose of the present study was to develop a simple, rapid, and sensitive HPLC method for the routine analysis of DHS in kidney and meat.

MATERIALS AND METHODS

Materials and Reagents

Fresh kidney and meat from cows obtained from the local slaughterhouse, were used as control material and for spiking with DHS to conduct recovery experiments. The samples were stored frozen (-20°C).

1-Octanesulfonic acid and 1-heptanenesulfonic acid were obtained from Supelco Inc. (Supelco Park, Bellefonte. USA.). DHS and STR was supplied by Sigma Co. (St. Louis, MO, USA).

All chemicals and solvents were of analytical and HPLC grade. DHS and STR 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. 5.9 with 5 M phosphoric acid and

then to 5.5 with 0.5 M phosphoric acid, and the solution diluted with water to 1 litre and the pH again adjusted to 5.5 with 0.5 M phosphoric acid. The stock solution and working standards were stored in a refrigerator.

1,2-Naphthoquinone-4-sulfonic acid (NQS), potassium salt, tech. 90% was obtained from Aldrich Chemical Co. (Germany), trichloroacetic acid (TCA) by Ferax (Laborat GMBH, Berlin, Germany) and sodium hydroxido p.a. by R.P. Normapur A. R. (France). Bond Elut Certify II cartridges for solid phase extraction, 6 cc/500 mg, were supplied by Varian (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 Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon). The analytical column (operated at 31°C; 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 + Plus (Supelco, Bellefonte, PA, USA). The guard column was connected to an A. 318 precolumn filter with an A-102X frits (Upchurch Scientific , USA).

The mobile phase consisted of a mixture of two solutions, B and C (68 : 32). Solution B consisted of 0.04 M 1-octanesulfonic acid and 0.4 mM NQS at pH c. 3.24, and was prepaired by dissolving 8.65g/L octanesulphonic acid and 110 mg/L NQS in c. 750 mL water. The solution was diluted to 1 litre by addition of water and filtered through a 0.45 μm membrane filter. The pH was adjusted with 1 mL acetic acid. This mobile phase was prepared daily and stored in an amber flask during use. Solution C was acetonitrile. The flow rate was 0.6 mL/min. for 0.5 min., 0.9 mL/min. for 4 min. and finally 0.6 mL/min. for 9 min. The samples were injected at intervals of 14 min. For the determination of DHS aliquots of 25 μL were injected onto the column.

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 250 Liquid Chromatograph (Perkin-Elmer) was used, which included an extra pulse-dampened pump (Scientific System inc., USA) and two pump back pression regulators (2 x 250 PSI., Perkin Elmer) with a mobile phase of 0.3 M NaOH at a flow rate of 0.3 mL/min. This was coupled to the vortex mixer, and a reaction coil (Aura Industries, Inc., USA) KRC 15-50 Knitted Reactor Coil, 15 m x 0.50 mm I.D. The reaction temperature was 40°C. The solvent stream was then cooled using a heat exchanger (operated at room temperature), to prevent gas emission in the detector. Flourescence was detected using a LC 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA) with an excitation wavelength of 375 nm and emission wavelength of 420 nm, with a response of 5 and a factor of 512.

Sample Pretreatment

To 8 g kidney or meat, a 5 mL solution A (or standard) was added. The total volume added in this step should amount to 5 mL. One mL 85% TCA in water was then added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, F. R. G.). After centrifugation for approximately 3 min. (5000 rpm), 2 mL dichloromethane was added. The sample was then mixed for 6 s. and centrifuged for 5 min. (5000 rpm). Seven mL of the supernatant (corresponding to 4g kidney/meat) was pipetted into a graduated glass-stoppered centrifuge tube, 0.9 mL 4 M NaOH was added and the mixture then blended. The homogenate was centrifuged for 5 min. (4000 rpm). The upper layer was transferred to a clean tube, 0.9 mL of 0.5 M phosphoric acid added, and the pH adjusted to between 5.5 to 5.8 with 1 M NaOH or 0.5 M phosphoric acid. After 2.5 mL solution D had been added, the sample was mixed and loaded onto a conditioned 500 mg Certify II column. Solution D was made in the same manner as solution A, but the 1-heptanesulfonic acid concentration was 0.06 M (13.35 g/L).

Clean-up on SPE-column

The column was activated with 3mL acetonitrile, followed with 1mL water and 3x1 mL solution D, prior to application of the extract. It is important not to allow the sorbent to dry before applying sample. The aqueous extract was applied to the column and slowly (c. 1mL/min) suctioned through, using a VacMaster system (International Sorbent Technology). The glass stoppered tube was rinsed with 1 mL solution A; this solution was also loaded onto the column. The column was washed and then suctioned to dryness for c. 2 sec.,(with a vacuum of -5 in. Hg.) between every washing, the wash was with

2x5 mL solution A, followed with 3x5 mL NH3 (25%) and 3x1 mL water. The column was suctioned to dryness for 10 sec. (at a vacuum of -10 in. Hg.) and eluted with 2x1 mL 20% formic acid in methanol. The collected eluates were evaporated to dryness under a stream of nitrogen using a Reacti-Therm heating module at 60°C and a Reacti-Vap evaporating unit (Pierce, Rockford. IL, USA). Two hundred μL methanol was added, the sample was mixed for 3-4 s. and evaporated to dryness. The dry residue was dissolved in 400 μl solution A, after which 200 μL chloroform were added. The extract was mixed vigorously for 10 s. followed by centrifugation for approximately 3 min. The water layer was then filtered through a Costar Spin X centrifuge filter unit with 0.22 μm nylon membrane, by centrifugation for 2 min. at 10000 rpm. (5600 g). Aliquots of the aqueous layer (25 μl) were injected onto the column at intervals of 14 min. for the determination of DHS.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for DHS were determined by spiking meat and kidney samples with standard solutions to yield 40, 50, 100, 200, 300, and 400 ng DHS per gram of sample, respectively. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked meat and kidney, with those of standard solutions. The linearity of the standard curves for DHS in meat and kidney were calculated using peak height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean and spiked kidney samples from cow and swine with DHS are shown in Figure 1 and 3. Chromatograms of extract of blank and spiked samples from cow meat with DHS and STR are shown in Figure 2.

The standard curves are linear in the investigated area (40 - 400 ng/g) for DHS in meat and kidney. The corresponding correlation coefficients were r = 0.996 and 0.999 in kidney and meat, respectively. Table 1 shows the recovery and repeatabilities of DHS from kidney and meat from cows:

The recovery of DHS from kidney and meat varied from 73.2 to 73.5% and from 80.0 to 83.0%, respectively. The precision of these recovery studies varied from 1.5 to 3.1% for kidney and from 0.3 to 1.3% for meat.

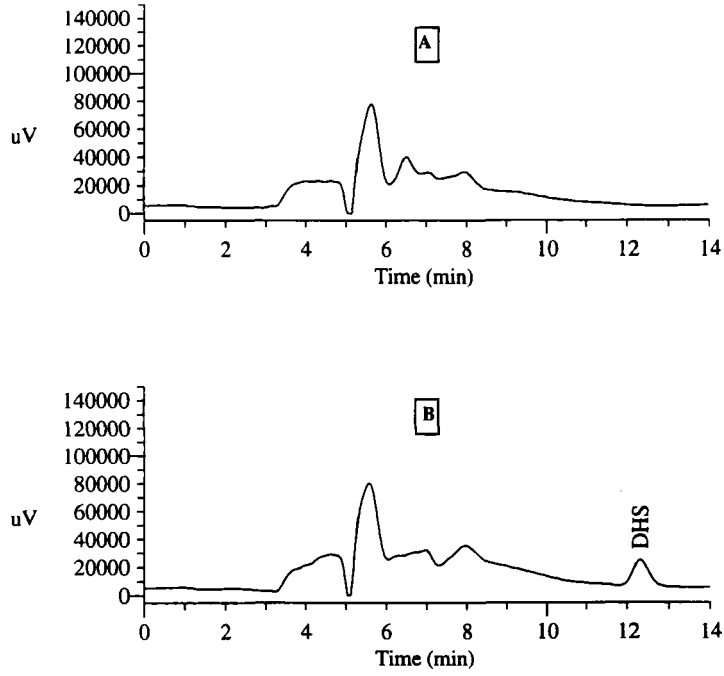

Figure 1. Chromatograms of extracts from cow kidney. **A**: drug-free kidney, **B**: kidney spiked with DHS (400ng/g).

Table 1

Recovery and Repeatability for Dihydrostreptomysin Sulfate from Spiked Samples of Meat and Kidney

Sample	No. of Samples (μg/g)	Amount of DHS in Spiked Samples	Recovery % DHS Mean	SD*
Meat	8	0.1	83.0	1.3
8/g	8	0.4	80.0	0.3
Kidney	8	0.1	73.5	1.5
	8	0.4	73.2	3.1

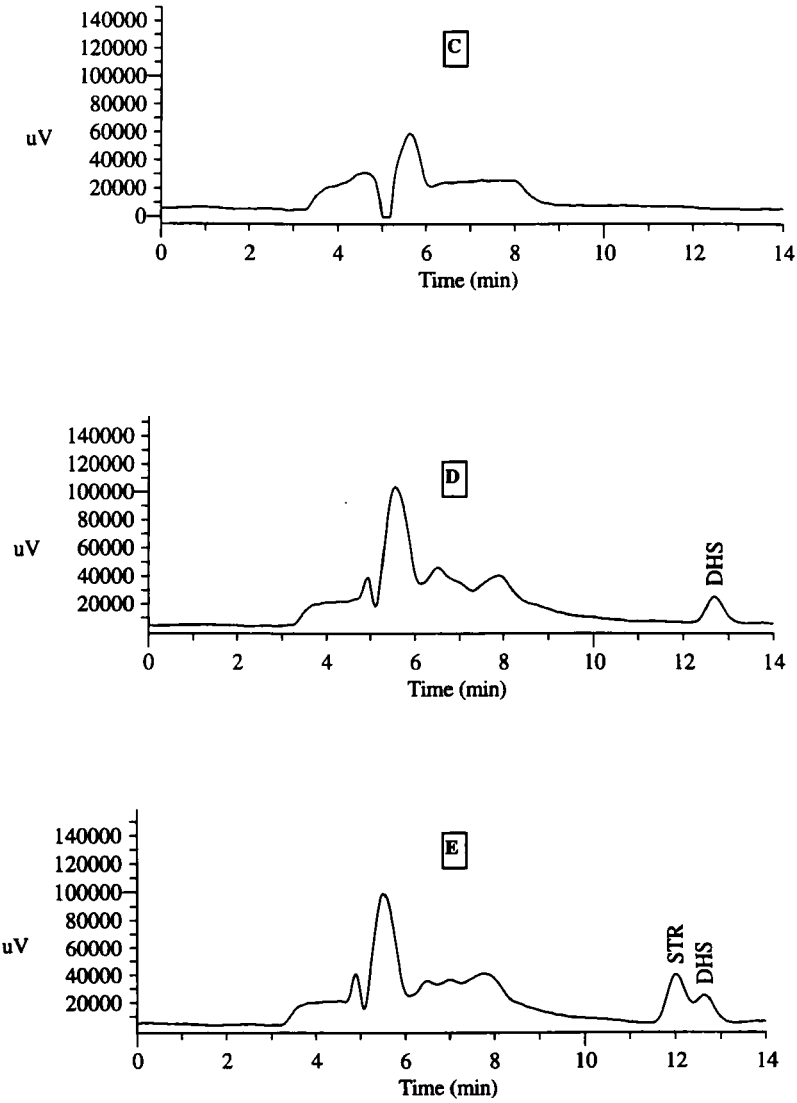

Figure 2. Chromatograms of extract from cow meat. **C**: drug-free meat, **D**: meat spiked with DHS (400 ng/g), **E**: meat spiked with DHS and STR (400 ng/g).

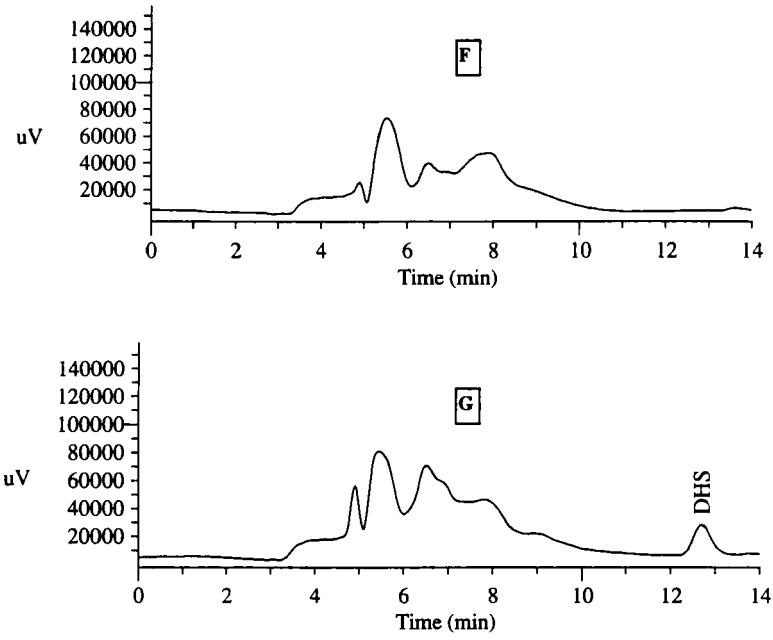

Figure 3. Chromatograms of extracts from swine kidney. **F**: drug-free kidney, **G**: kidney spiked with DHS (400ng/g).

NQS forms high intensity fluorophors with guanidino compounds in alkaline media.[10] DHS, which has two guanidino groups, yields similar fluorophors. In our preliminary studies, non-polar sorbent materials such as tC_{18} SPE-cartridges from Waters, Bond Elut Certify, C_{18} and Bond Elut Certify II from Varian were tested and acceptable recovery were obtained for DHS in meat and kidney pretreated on Bond Elut Certify II. The capacity of the Certify II sorbent material was also tested. If the columns contained 200 mg sorbent material, the recovery of DHS from the kidney and meat homogenates was variable. Increasing the amount of Certify II material in the columns to 500 mg resulted in the analyses of spiked tissue showing good reproducibility.

t-C_{18} from Waters yielded a successful result for determination of DHS in milk,[12] but in meat and kidney the elution already started during washing after 7mL with 30% methanol in water,[12] this is not sufficient to eliminate all interfering peaks near the retention time of DHS in meat and kidney.

Different eluting agents were tested, and the eluting solvents methanol / formic acid (8 : 2, v/v) gave acceptable recovery. The reaction coil and the analytical column must be maintained at a steady temperature and the mobile phases were degassed thorough with helium before use (during analysis we used a solvent system kits from Perkin Elmer), and must be free from fluctuation or an unstable baseline will result.

After all samples and standards had been injected, the analytical column was washed with water-acetonitrile (1:1) in 15 min., thereafter 5 min. gradient to methanol (100%), and 25 min. with methanol. The flow was 0.9 mL/min. The post-column reaction system was washed with water-methanol (9:1) at 0.5 mL/min for 10 min. We used a vortex mixer, but it is not a problem to use a low volume mixing tee instead.

The chromatographic system appeared to be efficient for the determination of DHS in kidney and meat, the limit of quantification being 40 ppb and the limit of detection close to 20 ppb. The detection limits are calculated as 3 times the baseline noise from a drug-free tissue. 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. As shown in Figure 2 (E) the method can also be used for the determination of STR in kidney and meat. The chromatogram of a spiked meat sample with standard solution, to yield 400 ng/g of DHS and STR, shows that baseline resolution of the two drugs is not achieved. Changing the mobile phase composition to water-acetonitrile (70 : 30) produced baseline resolution, but also resulted in retention times of 16.2 and 17.3 min. for DHS and STR, respectively. Because it was considered unlikely that both drugs would be encountered in the same sample, a mobile phase was chosen that allowed identification of each drug while minimizing analysis time. The precision, recovery, and linearity of STR were not validated in this report.

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