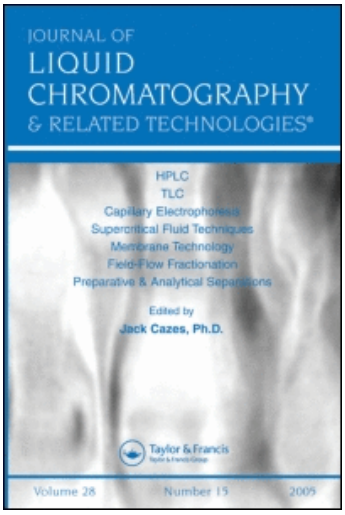

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Determination of Streptomycin and Dihydrostreptomycin in Milk and Meat by Liquid Chromatography – Mass Spectrometry

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Abstract: A liquid chromatographic-mass spectrometry (LC-MS/MS) method for the determination of streptomycin (STR) and dihydrostreptomycin (DHS) sulfate in milk and meat is described. Samples of milk and meat were homogenized with an extraction solution of ammonium acetate/hydrochloric acid/trifluoroacetic acid and centrifuged. The supernatant was filtered, diluted, and injected into the LC-MS/MS. The recovery of STR and DHS from milk varied from 79 to 83%. The recovery of STR and DHS from meat varied from 84 to 90%. The limits of quantification for both milk and meat were 50 ng/mL (g) for STR and 35 ng/mL (g) for DHS.

Keywords: Dihydrostreptomycin, LC-MS/MS, Liquid Chromatography, Meat, Milk, Streptomycin

INTRODUCTION

Streptomycin (STR) and dihydrostreptomycin DHS are aminoglycoside antibiotics with high potency against a wide range of Gram-negative and some Gram-positive bacteria.[1–4] In veterinary medicine, both drugs have been widely used for the treatament of infectious diseases in

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food-producing animals. The combination of penicillin and DHS is 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 and meat.[5–8] DHS is produced by the catalytic hydrogenation of STR.[9]

The use of these classic antibiotics is well studied, and several cases of allergic reactions have been reported.[10] The Maximum Residue Limits (MRLs) for STR and DHS for food-producing animals given by the EU are established in Regulation 2377/90/EEC.[11] The MRL for muscle, skin, fat, and liver is $500\,\mu g/Kg^{-1}$. For kidney and milk, the MRLs are 1000 and $200\,\mu g/Kg^{-1}$, respectively.

Numerous chemical and physical methods have been described for the analysis of STR and DHS, including paper and thin-layer chromatography, spectrophotometry and colorimetry, titrimetry, and polarography.[12] STR and DHS have a guanidino group which reacts with 1,2-naphthoquinone-4-sulfonic acid (NQS) under alkaline conditions and gives highly fluorescent derivatives. These techniques have been proposed for the determination of residues of STR and DHS in food using liquid chromatography (LC) with ion-pairing and fluorescence detection.[13–17]

Mass spectrometry (MS/MS) is a powerful and more sophisticated technique compared to fluorescence detection. It has the advantage that the ratio between different product ions provides additional identification and confirmation. In addition, the risk of false positives is reduced and MS/MS gives results with high sensitivity and quantitative capability. No derivatization is required.

Some papers reported LC-MS/MS methods for the determination of STR and DHS in bovine kidney[18,19] and in milk and honey.[20] The intention of the present study was to develop a time saving, simple, and sensitive LC-MS/MS method for the determination of STR and DHS in meat and milk. The sensitivity should at least meet the requirement of quantitative detection at the MRL level.

EXPERIMENTAL

Materials and Reagents

Fresh drug-free raw milk from cow, obtained from one farm, and cow meat from the local slaughterhouse were used. These samples were used as control material and for spiking with STR and DHS to conduct recovery experiments. The samples were stored frozen ($-20^{\circ}C$).

All chemicals and solvents were of analytical or HPLC grade. STR and DHS were supplied by Sigma Co. (St. Louis, MO, USA). Stock

(1 mg/mL) and working standards (1 μg/mL) were prepared by dilution with Solution A.

Solution A, consisting of 0.1 M ammonium acetate (Merck, Darmstadt, Germany) diluted in 0.1 M hydrochlorid acid (Merck).

Solution B, consisting of 0.5 M ammonium acetate.

Trifluoroacetic acid (TFA) was supplied by Rathburn Chemicals (Walkerburn, Scotland).

Spin-X centrifuge filter units (0.22 μm, nylon type) from Costar (Corning, NY, USA), were used for filtration.

Chromatographic Conditions

The LC-MS/MS instrumentation used for the present method consisted of a Series 200 quaternary pump and autosampler (Perkin Elmer, Norwalk, USA) and an API 2000 MS/MS system (Applied Biosystems, Ontario, Canada) equipped with a Turbo-Ion Spray ion source. The turbo probe vaporizer temperature of the interface was fixed at 450°C. The MS was set to collect ion data in the positive mode. DHS, which differs from STR by the substitution on the middle saccharide ring, generates a virtually similar mass spectrum. The fragments m/z 176, 221, 246, 263, and 407 were found in earlier MS experiments.[21,22] The most abundant transitions of the respective protonated molecular ions (m/z 582,2 for STR and 584,2 for DHS) to m/z 263,2 were used for screening and quantification, while the product ion of m/z 246.2 were used for confirmation of the identity.

A precolumn filter A-138 with an A-102X frits (Upchurch Scientific, USA) was connected to the guard column. The column Allure PFP Propyl 5 μm 150 × 4.6 mm (Restek, Bellefonte, USA, Catalog nr. 9169565–700) were operated at a constant temperature of 23°C. The mobile phase consisted of a mixture of two solutions: Solution C consisted of 985 mL water, 15 mL methanol, and 40 μL TFA; solution D was methanol. The mobile phase operating conditions are shown in Table 1. After separation, the LC effluent was connected to a two position micro electric valve actuator (Vici, Valco Instruments Co. Inc. Texas, USA) programmed in mode two by our provider. Thereafter, the LC fluent was split approximately 1:4 before entering the MS interface.

Sample Pretreatment

Milk

A volume of 200 μL solution A or standard (the corresponding volume of standard solution were diluted to 200 μL with solution A), 20 μL TFA

Table 1. Mobile Operating Conditions

Total time (min)	Flow rate (μL/min)	Solution C (%)	Solution D (%)	TE#1
0.0	900	100		Open
0.5	900	100		Open
3.0	900	75	25	Open
4.0	900	25	75	Close
5.0	800	25	75	Open
6.0	800	25	75	Open
6.1	1200	100		Open
7.5	1200	100		Close
15.0	1200	100		Open

TE#1 = events.

and 500 μL chloroform were added to 400 μL milk sample. The mixture was homogenized for approximately 15 sec with an whirl-mixer. After centrifugation for 5 min at 3600 rpm, the supernatant was filtered through a Spin-X centrifuge filter. To 100 μL of solution B, 100 μL of the filtered supernatant was added. After mixing, 75 μL was injected into the LC-MS/MS system at intervals of 15 min for the determination of STR and DHS.

Meat

A volume of 400 μL solution A or standard (the corresponding volume of standard solution were diluted to 400 μL with solution A), 20 μL TFA and 2000 μL chloroform were added to 400 mg meat sample. The mixture was homogenized for approximately 10 sec with an Ultra-Turrax S 25 N – 10 G dispersing tool (Ika – Warke, Staufen, Germany). After centrifugation for 5 min at 3600 rpm, the supernatant was filtered through a Spin-X centrifuge filter. To 100 μL solution B, 100 μL filtered supernatant was added. After mixing, 75 μL was injected into the LC-MS/MS system for the determination of STR and DHS.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for STR and DHS were determined by spiking drug-free cow milk samples with standard solutions to yield 0, 25, 50, 100, 150, 200, 300, 400, and 500 ng/mL. Drug-free cow meat samples were spiked with standard solutions to yield 0, 25, 50, 100, 150, 200, 300, 400, 500, 600, 800, and 1000 ng/g. For both milk and meat, duplicate samples were used. The recovery was determined by comparing

the analyses of spiked milk and meat with those of satandard solutions of STR and DHS. The linearity of the standard curves for STR and DHS in cow milk and meat were calculated using peak area measurements.

For the determination of recovery rates of STR and DHS, the corresponding doses of standard solutions were diluted with solution A to 620 μL for milk and to 820 μL for meat. To 100 μL standard solution, 100 μL solution B was added and mixed.

RESULTS AND DISCUSSION

The standard curves were linear in the investigated areas from 50 to 500 ng/mL for milk and from 50 to 1000 ng/g for meat. The linear coefficients for STR and DHS in milk and meat were $r = 0.9993$ and $r = 0.9995$, respectively. The recovery and repeatability values for STR and DHS from milk and meat are shown in Table 2. The recovery was calculated directly, without correction for an internal standard. Chromatograms obtained from drug-free milk and meat samples, and from the corresponding samples spiked with STR and DHS are shown in Figures 1 and 2.

The limits of detection for STR and DHS were calculated as three times the peak-to-peak baseline noise ($S/N = 3$) from drug-free cow milk and meat. They were 25 ng/mL(g) for STR and DHS for both milk and meat.

The use of a two position micro electric valve actuator avoids use of unnecessary mobile phase and, thereby, eliminating possible contamination from sample extracts streaming into the MS. It is essential that the

Table 2. Recovery and repeatability for STR and DHS from spiked samples of milk and meat

No. of samples	Amount of drugs (ng/mL/g)	Rec. (%)STR Mean	Rec. (%)STR S.D.	Rec. (%)DHS Mean	Rec. (%)DHS S.D.
Milk					
5	100	80	1.3	81	1.8
5	300	82	2.1	79	0.9
5	500	83	1.5	81	0.8
Meat					
5	100	88	1.9	85	1.1
5	300	87	2.3	84	0.7
5	500	90	1.7	86	1.1

S.D. = Standard deviation.
Rec. = Recovery.

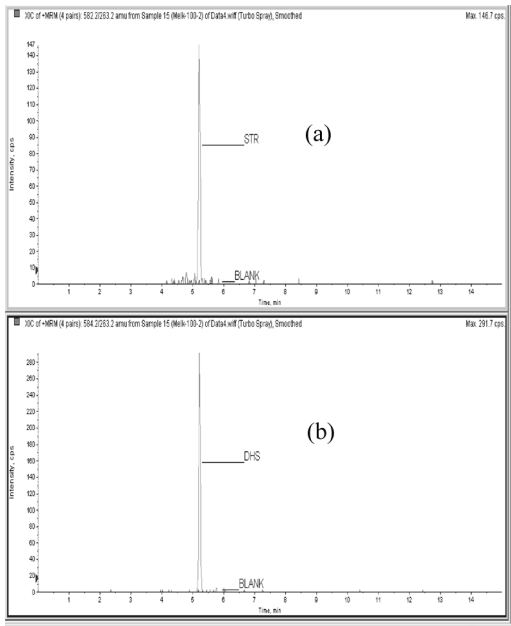

Figure 1. LC-MS/MS chromatograms of extracts from cow milk spiked with 100 ng/mL STR and a blank cow milk sample (a) and spiked with 100 ng/mL DHS and a blank cow milk sample (b).

micro electric valve actuator is programmed in mode two. The micro electric valve actuator appears favorable in all MS analyzing. The actuator was guided from data software under LC pump (events). When the event is open, the mobile phase flows to waste. When the event is closed, the mobile phase flows to the analytical column until a new close event is given; hereafter, the mobile phase flows to the waste. However, the use of a micro electric valve actuator is not an absolute requirement to carry out the described method for STR and DHS.

STR and DHS are basic and very hydrophilic compounds. They cannot be retained by the commonly used C_{18} columns. Various methods using ion-pairing reagents have been reported for the determination of aminoglycosides.[13,14] Limited retention on hydrophobic alkyl (ODS) or polar embedded (cyano) HPLC phases makes derivatization or ion-pairing techniques necessary. These modified HPLC techniques are laborious and disrupt reproducibility, and many derivatizing reagents are not LC/MS compatible.

Pentafluorophenyl HPLC phases show greater retention for compounds that have electrophilic properties, like protonated amino groups in basic compounds.[23] A propyl spacer between the functional

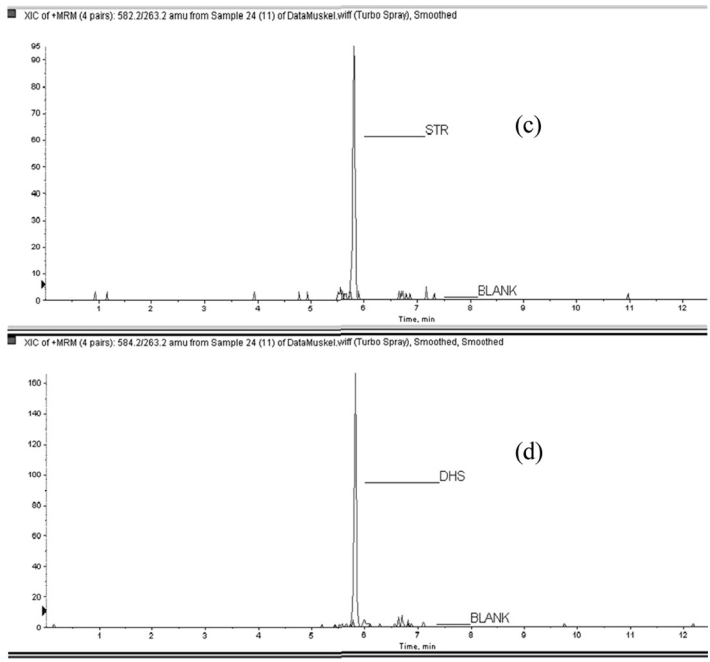

Figure 2. LC-MS/MS chromatograms of extracts from cow meat spiked with 100 ng/g STR and a blank cow meat sample (c), a spiked with 100 ng/g DHS and a blank cow meat sample (d).

group and the silica surface, a penta–fluorophenyl propyl phase, further increases retention. Consequently, when an acidic mobile phase is used to induce protonation of the analytes' amine groups, the Allure PFP Propyl phase makes possible a simple reversed phase HPLC analysis. By regulation of salt concentration in the injected solution, it is possible to regulate the retention time and separation of STR and DHS.

For the determination of STR and DHS by LC-MS/MS, a reversed-phase LC system with an aqueous mobile phase containing TFA and methanol was used. No ion-pairing is necesary. Different solutions were tested for optimum ionization of the analytes and it was found that TFA (40 μL/L) gave the best result. Both higher and lower concentrations of TFA yielded decreased detector response.

The extraction of STR and DHS from milk and meat involved acid to release the protein-bound antibiotics. To the meat sample pretreatment, chloroform was added to ease the absolutely necessary homogenizing step with an Ultra-Turrax. In this way, the contact surface between the extraction solution and the matrix sample is increased. In milk and meat, chloroform eases the separation of water from the solid phase.

CONCLUSIONS

The applications presented here are a good example that LC-MS/MS can offer a number of significant advantages for detection and quantification of STR and DHS in food samples compared to previously published methods. The high selectivity and sensitivity of LC-MS/MS generally requires only a simple clean up procedure and no derivatization. The validation data show that the methods performance is good and can be used for routine analysis. The method presented in this paper is selective, robust, and accurate. The detection limits are below the EU requirement of MRL.

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