Modified determination of dihydrostreptomycin in kidney, muscle and milk by HPLC†

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Received 26th June 1998, Accepted 1st October 1998

A method is presented for the determination of dihydrostreptomycin in milk, muscle and kidney by reversed-phase ion-pair high-performance liquid chromatography and post-column derivatisation with β-naphthoquinone-4-sulfonate prior to fluorescence detection. The new sample work-up procedures include acid precipitation of proteins and, in the case of muscle and kidney, removal of fats by solvent extraction followed by solid phase extraction on a cation exchanger. The fluorescence response was linear from 25 to 2000 μg l^{-1} of injected analyte. The detection limits were 10 μg kg^{-1} for milk and 15 μg kg^{-1} for muscle and kidney and the analyte recoveries were on average 93% for milk, 70% for kidney and 75% for muscle.

Introduction

The aminoglycoside antibiotic dihydrostreptomycin (DHS) (Fig. 1) can remain as a slowly removed antibiotic residue in animal products. Several HPLC methods based on fluorogenic post-column derivatisation of the guanidine groups of DHS with β-naphthoquinone-4-sulfonate (NQS) reagent in alkaline media[1–3] have been developed for the determination of DHS residues in meat[4,5] and milk.[6] This paper reports simplified and reliable sample work-up procedures and optimised HPLC and derivatisation conditions that permit detection well below the maximum residue limit (MRL) values.

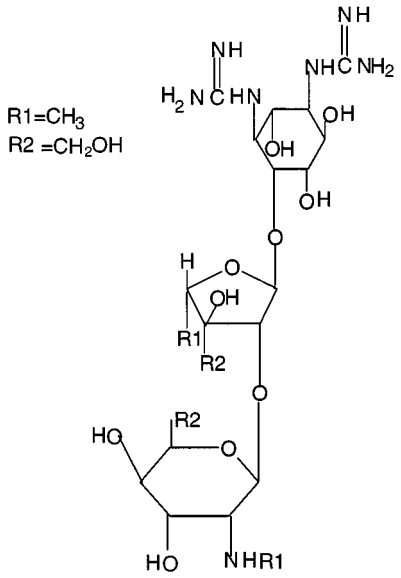

Fig. 1 Structural formula of dihydrostreptomycin ($C_{21}H_{41}N_7O_{12}$ M_n 583.62).

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

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Experimental

Reagents and solutions

(a) Reference standard. Dihydrostreptomycin sulfate (Lot No. 122H04733) was obtained from Sigma (St. Louis, MO, USA). A stock standard solution containing 10 mg l^{-1} of DHS was prepared. The concentrations used for calibration were 50, 100, 250, 500, 1000, 1500 and 2000 μg l^{-1}. The stock standard solution and the solutions for calibration were prepared in a solution of 20 mM sodium 1-heptanesulfonate and 10 mM disodium hydrogenphosphate dihydrate (4.45 g of 1-heptanesulfonate and 1.8 g of disodium hydrogenphosphate per litre of de-ionized water, the pH being adjusted to 5.5 with 5 and 0.5 M phosphoric acid).

(b) Mobile phase. An aqueous solution containing 40 mM sodium 1-octanesulfonate and 0.4 mM NQS was prepared by dissolving 8.65 g of sodium 1-octanesulfonate and 110 mg of NQS in about 900 ml of distilled water. The solution was adjusted to pH 3.2 with 50% m/m acetic acid and diluted to 1 l with distilled water. Finally, the solution and acetonitrile were mixed (50 + 24 v/v) to prepare the mobile phase. The mobile phase was stored in a dark flask and was degassed for 20 min in an ultrasonic bath before use. Acetonitrile (HPLC FAR UV grade) was purchased from Lab-Scan (Dublin, Ireland). 1,2-Naphthhoquinone-4-sulfonate (NQS), potassium salt, technical grade 90%, was obtained from Aldrich (Steinheim, Germany), and sodium 1-octanesulfonate from Sigma. Sodium 1-heptanesulfonate, perchloric acid technical grade 70%, trichloroacetic acid (TCA), potassium dihydrogenphosphate and potassium monohydrogenphosphate were purchased from Merck (Darmstadt, Germany).

(c) Extracting solution for SPE. A phosphate buffer (pH 8.4, 0.22 M) was made by dissolving 37.04 g of potassium monohydrogenphosphate and 1 g of potassium dihydrogenphosphate (anhydrous) in water and diluting to 1 l. The pH was adjusted to 8.4 with 5 M potassium hydroxide.

(d) Ion-pair concentrate for sample preparation. A 0.2 M 1-hexanesulfonic acid solution, used in the sample preparation, was prepared by dissolving 3.76 g of 1-hexanesulfonic acid in water and diluting to 100 ml.

(e) Reaction reagent. A 0.30 M NaOH solution was prepared by dissolving 13.33 g of sodium hydroxide in water and diluting to 1 l.

Apparatus

(a) Liquid chromatograph. Analyses were performed using a liquid chromatograph equipped with two pumps, a Consta-Metric III LDC/Milton Roy pump and a ConstaMetric 3500 LDC Analytical pump, a Gilson Model 231 autoinjector and a Shimadzu fluorescence detector and the detection signal was recorded with a Model C-R 5A Chromatopac integrator (Shimadzu, Kyoto, Japan).

(b) LC column. The analytical column (stainless steel, 150 × 4.6 mm id) and the guard column (stainless steel, 20 × 4.6 mm id) were packed with 5 μm particles of Supelcosil LC-ABZ + Plus (Supelco, Bellefonte, PA, USA).

(c) Filters. A syringe filter (0.45 μm, 25 mm) (Gelman Acrodisc CR PTFE) and a 1 ml Costar Spin X centrifuge filter with a 0.22 μm nylon membrane were used.

(d) SPE column. A benzenesulfonic acid cation-exchange SPE column (3 ml, 500 mg) was obtained from Varian (Palo Alto, CA, USA).

(e) Post-column reaction. The reaction reagent (0.3 M NaOH) pump was coupled *via* a long stainless steel capillary (20 m × 0.25 mm id) functioning as an extra pulse damper to a vortex mixing tee (10 μl internal volume; Visco Mixer from Lee, USA). The analytical column was coupled to the other inlet of the mixing tee and the reactor coil, a long stainless steel capillary (10 m × 0.5 mm id) was connected to the tee outlet. The reactor coil was immersed in a heated water-bath.

(f) Chromatographic conditions. The mobile phase was pumped at a flow rate of 0.7 ml min^{-1} and the post-column reagent solution at 0.3 ml min^{-1}. The analytical column was kept at ambient temperature and the reactor coil temperature was 55 °C. The excitation and emission wavelengths of the detector were set at 270 and 420 nm, respectively.

Before analysis, the HPLC and post-column reaction system was equilibrated by pumping mobile phase and reagent solution for approximately 30 min or until a stable baseline and DHS peak response were obtained. After the analysis, the system was washed by pumping acetonitrile–water (1 + 1) followed by pure methanol, 15 min each, from the analysis pump and at the same time methanol–water (1+ 9) from the post-column pump for 30 min.

Sample extraction procedure

Meat extraction. Muscle or kidney was minced in a food processor and stored at −70 °C until analysis. The frozen homogenate was allowed to come to room temperature and 5 g were placed in a 50 ml polypropylene centrifuge tube. A 10 ml volume of perchloric acid (0.6 M) was added and the tube was placed in an ultrasonic bath (Sonorex RK 100 from Labasco) for about 15 s and then shaken for 5 min on a horizontal shaker. After centrifugation for 5 min at 3500 rpm, the supernatant was transferred to a glass centrifuge tube. Dichloromethane (2 ml) was added and mixed for 15 s in an ultrasonic bath. The resulting mixture was centrifuged for 3 min at 2200 rpm. The upper layer was applied to the SPE cartridge without any pH adjustment.

Clean-up on SPE column. The SCX cartridge extraction column, connected to a 50 ml reservoir *via* an adapter on the Supelco vacuum tank as the eluent resevoir, was previously washed with 5 ml of water purified with a Milli-Q system (Millipore, Bedford, MA, USA) (Milli-Q water). The column was not allowed to dry at this stage. All the sample extract was poured into the SPE cartridge and slowly passed through at a flow rate of about 1 drop per 3 s. When the extract had passed through the column, the column was washed with 3 ml Milli-Q water and vacuum dried for 30 s (at a vacuum of −5 in Hg.) The retained drugs were eluted with 9 ml of phosphate buffer (0.22 M, pH 8.4). The elute was decanted into a 10 ml calibrated flask and 0.5 ml of ion pair solution was added, the pH was adjusted to 5.8–6.0, first with 5 M phosphoric acid (five drops) and then with 0.5 M phosphoric acid, and finally the mixture was diluted to volume with water. A 200 μl volume of the resulting extract was injected on to the HPLC column for the determination of DHS.

Milk. Raw milk was stored frozen at −20 °C or below. The sample was allowed to come to room temperature and 7 g of milk were placed in a 50 ml polypropylene centrifuge tube, 1.5 ml of 85% TCA was added and the mixture was shaken for 3 min on a horizontal shaker. After centrifugation at 3500 rpm for 5 min at 0 °C, the liquid phase of each sample was filtered through a syringe filter (0.45 μm, 22 mm, Gelman). The supernatant was decanted into a 10 ml calibrated flask, 0.5 ml of ion pair solution was added, the pH of the supernatant was adjusted to 3.2–3.5, first with approximately 900 μl of NaOH (25% m/m) and then with 0.3 M NaOH and 0.5 M phosphoric acid, and finally the mixture was diluted to volume with water. About 1 ml of the pH-adjusted supernatant was filtered through a Costar Spin X 0.22 μm nylon membrane by centrifugation for 3 min at 13 000 rpm and 200 μl of the resulting clear supernatant were injected on to the HPLC column for determination of DHS.

Validation study. For the determination of the recovery and between-day precision, the minced tissue or milk was spiked with DHS stock standard solution. The mixture was allowed to equilibrate for 5 min before the extracting solution was added and the sample was extracted and cleaned up as described above. The concentrations spiked were 250, 500, 1000, 1500 and 2000 μg kg^{-1} for kidney and muscle (corresponding to 125, 250, 500, 750 and 1000 μg l^{-1} standard) and 71.4, 142.9, 285.7, 428.6 and 571.4 μg kg^{-1} for milk (corresponding to 50, 100, 200, 300 and 400 μg l^{-1} standard). The experiment was repeated on five different occasions. The detection limits were estimated from the analysis of 20 blank samples as the average signal plus three times the standard deviation.

Results and discussion

Reaction conditions for post-column derivatisation were studied by Kubo *et al.*,[3] who reported maximum sensitivity for DHS at a 0.5 M NaOH reagent concentration, a reaction temperature of 65 °C and a reaction coil length of 15 m. A lower temperature (55 °C) and a reaction coil length of 10 m were chosen in the present work since they produced a better signal-to-noise ratio for the analysis of sample extracts. The NaOH concentration was lowered from 0.5 to 0.3 M after repeated problems with detector cell leakage.

The fluorescence peak area response was linear in the tested concentration range of 25–2000 μg l^{-1}, the correlation coefficient (r^2) being > 0.99. This was also the case for extracts from spiked kidney, muscle and milk over the tested sample concentration ranges of 250–2000, 100–1000 and 50–750 μg kg^{-1}, respectively. Theoretically, a linear response

can only be expected when NQS is present in at least a two-fold excess over analytes, since one DHS molecule contains two derivatizable guanidino groups.

To test the purity of the DHS standard, 3 nmol were injected. This gave a single peak that eluted at a retention time of about 13.7 min. Initially the retention time of DHS increased from day-to-day, but this effect could be minimised by daily degassing and storage of the mobile phase in a dark bottle. The retention time was affected by the matrices. Extracts from kidney and milk decreased the retention time by approximately 3% compared with the standard solution, whereas a muscle extract increased it to the same extent.

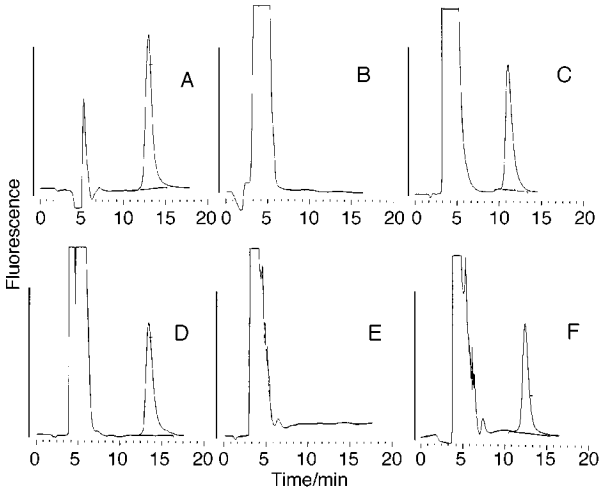

Fig. 2 Isocratic chromatograms of (A) 500 ppb DHS standard, (B) drug free kidney, (C) kidney spiked with 500 ppb of DHS, (D) muscle spiked with 500 ppb of DHS, (E) drug free milk and (**F**) milk spiked with 400 ppb of DHS.

Table 1 Precision and recovery data for the determination of dihydrostreptomycin (DHS) in fortified kidney, muscle and milk

Sample	Concentration spiked/μg kg^{-1}	Concentration found/μg kg^{-1}	RSD (%) (day to day, n = 5)	Recovery (%)
Kidney	250	169	10	68
	500	340	11	68
	1000	708	6	71
	1500	999	5	67
	2000	1521	6	76
				Mean: 70
Muscle	200	166	10	83
	500	355	10	71
	1000	770	7	77
	1500	1038	5	69
	2000	1518	3	76
				Mean: 75
Milk	71.4	65	9	90
	142.9	129	11	90
	285.7	273	9	96
	428.6	416	7	97
	571.4	529	6	93
				Mean: 93

Owing to the matrix and time dependent shifts in retention time and the close retention of streptomycin, there is a risk of mis-identification. To avoid this, it is recommended that two aliquots from sample extracts with a suspected DHS peak are re-chromatographed after addition of DHS and streptomycin, respectively.

The closely related antibiotic streptomycin had about a 10% shorter retention time than DHS, resulting in a double peak when a mixture was injected. The response factor for streptomycin in the present system was about 80% higher than for DHS.

The new work-up procedures produced chromatograms free from interfering or late eluting peaks or baseline shifts in the relevant sensitivity range (Fig. 2). The detection limits were calculated from the analysis of blank samples of diverse origin to be 15 μg kg^{-1} for muscle and kidney and 10 μg kg^{-1} for milk. This is well below the provisional MRL values of 500, 1000 and 200 μg kg^{-1}, respectively.

The recovery of DHS from spiked samples (Table 1) was independent of concentration in the ranges applied. Muscle and kidney gave similar recoveries, averaging 75 and 70%, respectively, whereas milk gave 93%. A higher recovery from milk could be expected from the comparative simplicity of the milk procedure, not including any of the solvent extraction or column clean-up steps needed for the tissues samples. The between-day precision, expressed as RSD, was comparable for the different matrices, ranging between 3 and 11% for the different concentration levels.

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Paper 8/04910F