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High-performance liquid chromatographic determination of spectinomycin in swine, calf and chicken plasma

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ABSTRACT

A rapid clean-up procedure based on ion-pair solid-phase extraction (SPE) for the high-performance liquid chromatographic (HPLC) determination of spectinomycin in swine, calf and chicken plasma at a limit of detection of 50 ng/ml is described. After dilution with water and adjustment of the pH to approximately 5.6, the plasma is applied to a high-hydrophobic C_{18} SPE column treated with sodium dioctylsulphosuccinate. Spectinomycin is eluted with methanol and derivatized with 2-naphthalene sulphonyl chloride prior to chromatography. The HPLC set-up consists of a dual-column system using two Chromspher silica columns and dichloromethane–acetonitrile–ethyl acetate–acetic acid, in different ratios, as mobile phases. Detection is performed at 250 nm. Quantification is carried out using external standards prepared in blank cleaned plasma. Mean recoveries were 83 ± 3% (n = 5), 93 ± 6% (n = 5) and 92 ± 6% (n = 6) for swine, calf and chicken plasma, respectively, at the 0.1 μg/ml level.

INTRODUCTION

Spectinomycin (Fig. 1) is a broad-spectrum aminocyclitol antibiotic, not belonging to any specified group of antibiotics. In veterinary medicine spectinomycin, mostly in combination with lincomycin, is used for the treatment and prevention of chronic respiratory disease associated with mycoplasmal and coliform infections. It is a dibasic compound, with pK_a values of 6.9 and 8.7. Weakly alkaline solutions of this compound are very unstable, forming actinospectinoic acid [1,2]. It is more stable in moderate acid solutions [1]. Spectinomycin is freely soluble in water and lacks UV-absorbing properties.

Apart from microbiological methods [3–5], only a few chromatographic procedures have been described for the determination of spectinomycin. A gas chromatographic procedure was developed for the determination of spectinomycin in bulk drug samples [2]. Myers and Rindler [6] described an HPLC method for spectinomycin in process samples and fermented beers. This method used ion-pair chromatography followed by fluorimetric detection after post-column oxidation with sodium hypochlorite and subsequent derivatization with *o*-phthaldialdehyde.

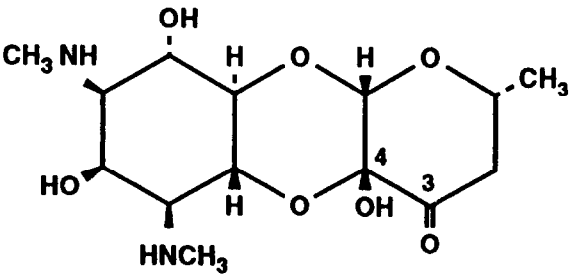

Fig. 1. Structure of spectinomycin.

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In 1985 Tsuji and Jenkins [7] described a normal-phase HPLC method for spectinomycin in veterinary formulations using pre-column secondary amine derivatization by means of 2-naphthalene sulphonyl chloride (NSCl) and detection at 254 nm. The authors reported a detection limit of approximately 4 ng of spectinomycin on the column and a good stability of the derivative. This makes the procedure attractive as a starting point for the determination of spectinomycin in plasma at levels of at least 0.1 μg/ml. Recently an HPLC method, using pre-column derivatization of spectinomycin with 2,4-dinitrophenylhydrazine, for the determination in turkey plasma, was described [8]. This method, however, was developed for monitoring higher levels, *i.e.*, 2–100 μg/ml.

In this paper, a method is presented for the HPLC determination of spectinomycin in plasma at 0.05 μg/ml or higher using the NSCl derivative for chromatography. For this purpose a sample pretreatment procedure was developed based on ion-pair solid-phase extraction (SPE). For chromatography the method of Tsuji and Jenkins [7] had to be modified to obtain lower detection levels. The preliminary results of this method applied in chickens have already been published [9].

EXPERIMENTAL

Reagents and chemicals

Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile, dichloromethane, ethyl acetate, methanol and *n*-butyl chloride (all HPLC grade) were from Rathburn (Walkerburn, UK), sodium dioctylsulphosuccinate was from Sigma (St. Louis, MO, USA), NSCl from Eastman-Kodak (Rochester, NY, USA), acetic acid (99%; HPLC grade) from J. T. Baker (Phillipsburg, NY, USA), citric acid and sodium bicarbonate from Merck (Darmstadt, Germany) and 1-methylpyrrole from Fluka (Buchs, Switzerland). Hydrochloric acid (1 *M*) was prepared from 37% hydrochloric acid (Merck). Spectinomycin · 2HCl · $5H_2O$ (activity 652 μg/mg) was from Upjohn (Kalamazoo, MI, USA).

A spectinomycin standard solution (0.5 mg/ml) was prepared by dissolving 154.3 mg of spectinomycin · 2HCl · $5H_2O$ in 200 ml of water. Working standards were prepared by diluting the standard solution to 0.01 and 0.05 mg/ml in water. The following solutions were prepared: 0.02 *M* citric acid solution, pH 5.6; 0.02 *M* sodium dioctylsulphosuccinate in water (homogenized just before use); and an NSCl solution in acetonitrile (12 mg/ml, prepared just before derivatization). The 0.12 *M* sodium bicarbonate solution was prepared by dissolving 10.0 g of sodium bicarbonate and 100 μl of 1-methylpyrrole in 1 l of water (prepared weekly).

High-hydrophobic C_{18} SPE columns (3 ml) were from J. T. Baker. The columns were pretreated by passing in series, with vacuum off or low vacuum, 3 ml of methanol, 2 ml of 0.02 *M* sodium dioctylsulphosuccinate (slow passage is essential) and 3 ml of citric acid solution. After this pretreatment the column should not be allowed to run dry.

The HPLC columns were a 5-μm Chromspher silica glass cartridge, 100 × 3 mm I.D. (Chrompack, Bergen op Zoom, Netherlands; column C1), with a guard column (10 × 2.1 mm I.D.) filled with pellicular silica (40 μm). For column C2, two coupled 5-μm Chromspher silica glass cartridges, 2 × 100 × 3 mm I.D. (Chrompack), were used.

The mobile phases for HPLC were dichloromethane–acetonitrile–ethyl acetate–acetic acid in the ratios 750:75:18:4.25 (v/v) for eluent 1 and 1000:200:36:8.5 (v/v) for eluent 2.

Apparatus

Evacuated blood collection tubes, containing 150 United States Pharmacopeia units of lithium–heparin, 20-ml stoppered polypropylene tubes (Sarstedt, Nümbrecht, Germany), 7-ml glass reaction vessels with screw cap (Witeg, Mainz, Germany) and 10-ml plastic syringes (Becton Dickinson, Columbus, OH, USA) were used in the procedure.

The instruments were a Hereaus centrifuge (Karlsruhe, Germany), a vortex mixer (Scientific Industries, Bohemia, NY, USA), a Reacti-Vap

Model 18780 evaporation unit, connected to a Model 18790 Reacti-Therm heating module (Pierce, Rockford, IL, USA) and a vacuum manifold (J. T. Baker). For derivatization an oil bath at 100°C was used.

The HPLC apparatus consisted of two HPLC pumps (Pharmacia/LKB, Uppsala, Sweden), an injection valve (Rheodyne, Model 7125, Cotati, CA, USA) equipped with a 50-μl sample loop, a valve-switching unit (multiport streamswitch, Spark Holland, Emmen, Netherlands) containing two six-port switching valves, two variable-wavelength detectors operating at 250 nm (Pharmacia/LKB), a recorder (Kipp, Delft, Netherlands) and an SP 4270 printer–plotter integrator (Spectra Physics, San Jose, CA, USA). The HPLC system is shown schematically in Fig. 2.

Samples

Blood samples were collected in evacuated blood collection tubes and centrifuged at 1000 *g* for 10 min. For recovery studies, blank swine, calf and chicken plasma samples were spiked with spectinomycin at levels of 0.1 and 1.0 μg/ml (blank swine plasma only) at least 15 min before extraction.

Sample preparation

A 2-ml volume of plasma was pipetted into a 20-ml polypropylene tube and 8 ml of water and 0.625 ml of methanol were added. The pH was adjusted to 5.2–5.7 with 1 *M* hydrochloric acid (approximately 60 μl). The solution was vortex-mixed for 30 s and subsequently centrifuged for 10 min at 2500 *g*. The supernatant was collected and the residue was rinsed with 3 ml of citric acid solution. After centrifugation, the citric acid solution was combined with the supernatant. The combined extracts were passed through the pretreated SPE column at a flow-rate of approximately 1–2 ml/min. The column was washed twice with 3 ml of citric acid solution and then allowed to run dry. The column was centrifuged for 15 min at 3000 *g* and further dried in a stream of nitrogen for 20 min. Spectinomycin was eluted with 3 ml of methanol at a rate of about 2 drops per s using the 10-ml syringe. The eluate was collected in the 7-ml reaction vessel and evaporated to dryness in a stream of nitrogen at room temperature.

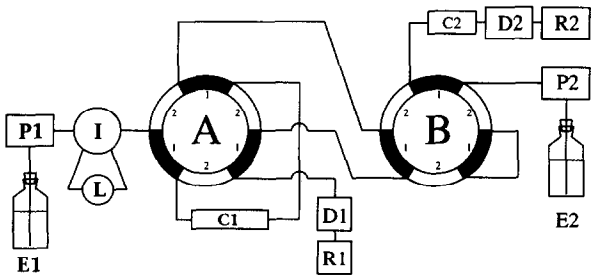

Fig. 2. Set-up of the HPLC system. The figure represents the situation for injection at time 0 (see Table I). P1 and P2, HPLC pumps; E1 and E2, HPLC eluents 1 and 2, respectively; D1 and D2, UV detectors; I and L, injection valve and loop; A and B, switching valves; C1 and C2, HPLC columns; R1, recorder; R2, printer/plotter integrator.

Derivatization

The residue was dissolved in 2.0 ml of 0.12 *M* sodium bicarbonate solution using the vortex mixer for 30 s, and 3.0 ml of the NSCl solution were added. The reaction vessel was closed and vortex-mixed for 30 s. The reaction vessel was placed into the oil bath (100°C) for 15 min and subsequently cooled to room temperature in a water bath of approximately 15°C. A 1.0-ml volume of *n*-butyl chloride was added. The closed vessel was vortex-mixed twice for 20 s and centrifuged for 5 min at 1500 *g* to facilitate phase separation. The total volume of the upper layer is approximately 4 ml (butyl chloride plus acetonitrile). A 2-ml volume of the upper layer was pipetted into a 3-ml glass collection tube and evaporated to dryness in a stream of nitrogen at room temperature. The residue was dissolved in 1.0 ml of *n*-butyl chloride and vortex-mixed for 30 s. The solution was centrifuged for 10 min at 2500 *g* and 50 μl of the supernatant were injected into the HPLC system. When not directly analysed, samples were stored at −20°C. All steps in which *n*-butyl chloride is involved should be performed in glass, as this liquid is not compatible with most plastics.

Standard solutions in blank cleaned plasma

A 2-ml volume of blank plasma was cleaned up as described in the *Sample preparation* section. After the evaporation step 10, 20, 40 and 100 μl of the 0.01 mg/ml and 40, 80, 200 and 400 μl of the 0.05 mg/ml aqueous standard solution were pipetted into the reaction vessels, resulting in a concentration range from 0.1 to 20 μg per 2 ml of plasma. After evaporation, spectinomycin was derivatized as described in the *Derivatization* section.

Chromatography

The system was set up according to the scheme shown in Fig. 2. Pump P1 was used for eluent 1. The flow-rate was set at 0.4 ml/min. Pump P2 was used for eluent 2 and the flow-rate was set at 0.6 ml/min.

Before starting the analysis, the retention time of the NSCl derivative of spectinomycin on column C1 (recorder R1) was determined by injecting a derivatized standard solution in water (no valve switching was used). Then the valve-switching unit was programmed in such a way that valve B (Fig. 2) was switched 30 s before the start of the spectinomycin derivative peak (retention time is approximately 8 min as shown on recorder 1) to place both HPLC columns in series and to transfer the spectinomycin derivative from column C1 to column C2. At the end of the peak (approximately 10 min after injection) valve B was switched back to chromatograph the spectinomycin derivative on column C2, whilst valve A was switched for backflushing column C1. At 25 min after the injection both valves were switched to their initial position and the system was ready for the next injection. Table I shows the schedule of the HPLC system.

A standard curve was obtained by injecting 50-μl aliquots of each of the NSCl-derivatized spectinomycin standard solutions in blank cleaned plasma. Subsequently, 50-μl aliquots of the sample solutions were injected. The content of spectinomycin in test samples was calculated by comparing the peak heights of spectinomycin in the samples with those obtained from standard solutions prepared in blank cleaned plasma.

TABLE I

SCHEDULE OF THE HPLC SYSTEM

Sp = NSCl derivative of spectinomycin; C1 = column 1; C2 = column 2.

Time (min)	Event	Valve position	
		A	B
0–8[a]	Sp on C1	1	1
8–10[a]	Sp from C1 to C2	1	2
10–25	Sp on C2 Backflush C1	2	1
25	End run	1	1

[a] Time depends on retention time of Sp on column 1 (see text).

After ten injections the retention time of NSCl derivative on column C1 should be checked. If the retention time is considerably shifted, column C1 should be flushed with acetonitrile.

RESULTS AND DISCUSSION

Sample pretreatment

When developing an SPE procedure, basic (pH > 8.5) as well as strong acid conditions have to be avoided. However, when spectinomycin has to be extracted from an aqueous solution with a pH < 8.5, the compound is positively charged and therefore does not show affinity to a reversed-phase SPE column. For this reason, two possibilities can be considered for SPE, *i.e.*, those based on cation-exchange or on ion-pair extraction.

Ion-exchange SPE (with the use of a weak cation exchanger) indeed provides a good extraction, but suitable elution solvents interfere with the NSCl derivatization, leading to irreproducible results.

Ion-pair extraction proved to be more successful. For this purpose a reversed-phase SPE column was treated with the ion-pair reagent sodium dioctylsulphosuccinate, a common reagent in liquid–liquid extraction procedures of organic bases from acid media into chloroform [10]. The non-polar part of the dioctylsulphosuccinate ion ($DOSS^-$) is attached to the SPE column, allowing spectinomycin to form an ion pair with the

negative DOSS ions. The ion pair $DOSS^-$–$spectinomycin^+$ may be subsequently eluted with an organic solvent such as methanol. Under the conditions described spectinomycin could be nearly quantitatively retained on a SPE column from a solution in citric acid buffer, pH 5.6; at least 50 ml could be applied without loss of recovery. Spectinomycin could be subsequently eluted in a small volume (3 ml) of methanol.

During development of the method, the following observations were made: (i) in testing C_8, C_{18}, phenyl and high-hydrophobic C_{18} SPE columns, recovery from the high-hydrophobic C_{18} type was the best; (ii) the amount of sodium dioctylsulphosuccinate on the column should be at least 0.06 nmol to exclude incomplete elution with methanol, probably due to secondary interaction of spectinomycin with the silanol groups of the column.

Application of this procedure to plasma showed that the sample volume from which spectinomycin could be extracted was more critical. Using 1 ml of plasma (brought to pH 5.5), similarly good recovery for spectinomycin was achieved. However, when extracting the same amount of spectinomycin from 8 ml of plasma, the recovery fell from more than 90% to less than 40%. This phenomenon could be due to the salts naturally occurring in plasma, which might interfere with the ion-pair extraction. This problem could be solved by diluting the plasma five times with water before SPE. The recovery was further improved by the addition of some methanol. In this way it was possible to extract spectinomycin even from 50 ml of 1:5 diluted plasma without loss of recovery. However, aspiration of 50 ml through the columns is not practicable. Therefore, 5- or 2-ml test portions of plasma were chosen.

Derivatization and chromatography

The derivatization of spectinomycin with NSCl deserves some additional attention. Using the procedure of Tsuji and Jenkins [7] it was impossible, in our hands, to reach their detection limit of approximately 4 ng on the column. For this reason various steps of the original procedure were evaluated. First, for obtaining a better limit of detection, we started with a 2-ml volume for derivatization and omitted the dilution step. Next, the optimal derivatization conditions were established. The yield of the NSCl derivative was improved by changing the bicarbonate concentration of 4.2 g/l, as used by Tsuji and Jenkins [7], to 10 g/l, and the content of water in the reaction mixture from 50 to 40%. The concentration of 12 mg/ml NSCl proved to be also optimal under these conditions. The quality of NSCl was found to be very critical. If a product of inferior quality is used, interferences may occur in the HPLC chromatograms. A plateau was found after a reaction time of 10 min. To be on the safe side, a reaction time of 15 min was chosen. For the same reason, 1-methylpyrrole was used, although no catalytic effect was discernible under the optimized reaction conditions.

As for extraction of the NSCl derivative, 1 ml of butyl chloride is sufficient. However, when the organic layer was directly injected onto the HPLC system, a badly shaped peak was observed. For this reason 2 ml of the organic layer were evaporated to dryness, after which the residue was dissolved in 1 ml of butyl chloride, introducing in this way a two-fold concentration factor as well.

The yield of the NSCl derivative increased when spectinomycin was derivatized in the presence of sodium dioctylsulphosuccinate. There was also a slight improvement in the yield when spectinomycin was derivatized in the presence of blank cleaned plasma, probably also due to the presence of sodium dioctylsulphosuccinate. Therefore it is necessary to derivatize the spectinomycin standard solution for HPLC in a blank cleaned plasma.

The calibration curve obtained by derivatization of various amounts of spectinomycin in blank cleaned plasma shows a good linearity ($r = 0.9992$) for the range 0.05–10 μg/ml. As for the chromatography, the method of Tsuji and Jenkins [7] was modified to some extent. Apart from some modifications in eluent composition and using another type of silica column, a column-switching procedure had to be introduced. Using

a single column, matrix interferences occur, which show retention times around that of spectinomycin. Moreover, it was observed that endogenous components accumulated on the column and eluted at a considerable time after injection. After multiple injections, these compounds may seriously interfere.

Furthermore, it has to be remarked that some shift of the NSCl derivative to a shorter retention time on the first column was observed when sodium dioctylsulphosuccinate was present in the sample solution injected into the HPLC system. Therefore, after every ten injections the retention time of the derivative on column C1 has to be checked. If a shift is observed the column should be rinsed with acetonitrile.

The absorbance of the NSCl derivative of spectinomycin is twice as high at 245 nm as that at 254 nm, used by Tsuji and Jenkins [7]. A wavelength of 250 nm was chosen, as measurement at 245 nm gives baseline instability during column switching. As a result, the detection limit improves by 30% as compared with that obtained at 254 nm.

Spiking studies

Recovery experiments were carried out on swine, calf and chicken plasma at different spiking levels. Each level in plasma was repeated six times. The samples were analysed in duplicate according to the procedure described. The results are presented in Table II.

TABLE II

RECOVERY OF SPECTINOMYCIN FROM SPIKED PLASMA

Plasma	Added (μg/ml)	Mean recovery (%)	Standard deviation	*n*
Swine	0.1	83	3	5[a]
	1.0	91	1.5	6
Calf	0.1	93	6	5[a]
Chicken	0.1	92	6	6

[a] One sample lost.

Typical chromatograms from spiked and blank plasma are shown in Fig. 3. No interference from blank plasma is observed at the retention time of spectinomycin, resulting in an absolute detection limit of 1 ng of spectinomycin (three times the noise) on the column. This means that, starting from a 2-ml test portion and a recovery of approximately 85%, a content of 40 ng/ml of plasma can be quantitated. This content can be reduced to 20 ng/ml by increasing the volume of the test portion to 5 ml. However, in pharmacokinetic studies, for example in chick-

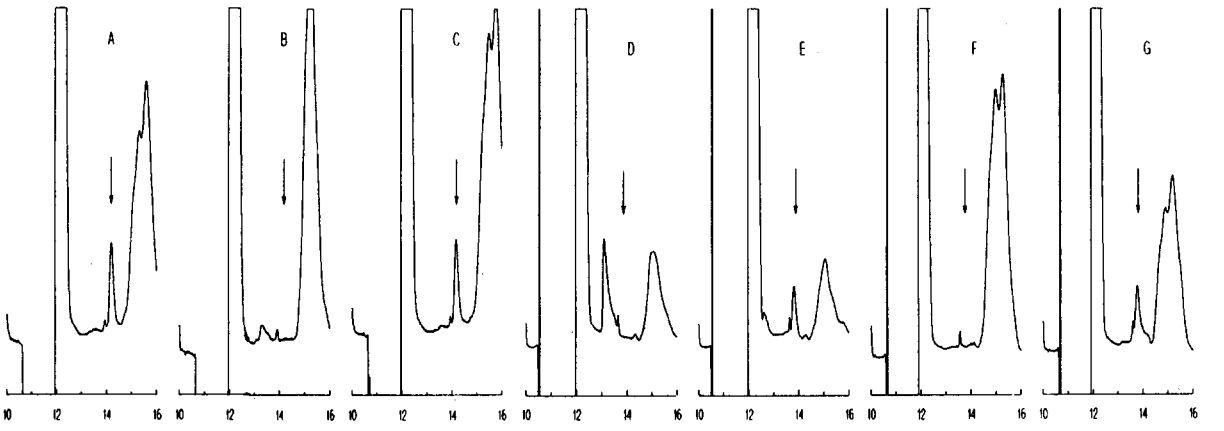

Fig. 3. Chromatograms of (A) a spectinomycin standard solution (0.10 μg/ml), (B) blank and (C) spiked (0.10 μg/ml) swine plasma, (D) blank and (E) spiked (0.10 μg/ml) calf plasma and (F) blank and (G) spiked (0.10 μg/ml) chicken plasma. Absorbance range setting: 0.008 a.u.f.s.

ens, it is difficult to achieve such volumes, particularly as determinations are usually carried out in duplicate.

The procedure has been applied to plasma obtained from chickens treated with Lincospectin 100 soluble powder (Upjohn) via the drinking water [9]. Spectinomycin could be detected only 1 h after the treatment was stopped (mean level 50 ng/ml).

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