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Short communication

High-performance liquid chromatographic determination of spectinomycin in swine, calf and chicken plasma using post-column derivatization

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Abstract

An HPLC method for the determination of spectinomycin in swine, calf and chicken plasma at 0.1 μg/ml or higher is described. The clean-up is based upon ion-pair solid-phase extraction on a High Hydrophobic C_{18} column treated with sodium dioctyl sulfosuccinate. After elution with methanol, spectinomycin is chromatographed on a Spherisorb SCX column using 0.1 *M* sodium sulphate solution (pH 2.6)–acetonitrile (80:20, v/v) as mobile phase. Fluorescence detection is at an excitation wavelength of 340 nm and an emission wavelength of 460 nm after post-column oxidation with sodium hypochlorite followed by derivatization with *o*-phthaldialdehyde. Mean recoveries were 99 ± 2% ($n = 6$), 99 ± 2% ($n = 7$) and 104 ± 2% ($n = 6$) for swine, calf and chicken plasma, respectively, at the 0.1 μg/ml level.

1. Introduction

Spectinomycin is a broad spectrum antibiotic applied in veterinary medicine for mycoplasmal infections or swine dysentery. To obtain information on the behaviour of this compound in the animal, pharmacokinetic data must be available. For this purpose a method for the determination in plasma is required.

As spectinomycin itself does not show any usable UV-absorbing properties, the compound has to be derivatized for HPLC analysis with UV- or fluorescence detection. For this purpose three derivatization procedures have been described in literature: (1) post-column derivatization with *o*-phthaldialdehyde (OPA) after oxidation with sodium hypochlorite [1,2]; (2) pre-column derivatization by means of 2-naphthalene sulphonyl chloride (NSCl) [3]; and (3) pre-column derivatization with 2,4-dinitrophenyl hydrazine [4].

Recently we applied NSCl derivatization to an HPLC determination of spectinomycin in animal plasma [5,6]. However, some practical problems were encountered. First the spectinomycin standard solution had to be prepared in blank cleaned plasma as the yield of the NSCl derivative was enhanced, probably due to the presence of sodium dioctyl sulfosuccinate (NaDOSS) used in the solid-phase extraction (SPE) procedure.

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This reflects more the real situation when analyzing spectinomycin in real samples. Secondly, automation of the HPLC procedure proved to be difficult. The final *n*-butyl chloride solution, in which the NSCl derivative was dissolved, is incompatible with most synthetic materials and therefore with the dispenser-tubing of some type of autosamplers. Moreover, some shift of the NSCl derivative after about ten injections to a shorter retention time was observed, due to NaDOSS, present in the sample solution injected.

For this reason we investigated the application of the post-column derivatization procedure with OPA after oxidation with sodium hypochlorite [1] for the determination of spectinomycin in plasma. The clean-up and concentration procedure is based on ion-pair SPE on a High-Hydrophobic C_{18} column treated with NaDOSS as described earlier [5,6].

2. Experimental

2.1. Reagents and chemicals

Water was purified via Milli-Q (Millipore, Bedford, MA, USA). All chemicals used were analytical grade unless otherwise stated. Acetonitrile (HPLC grade), methanol (HPLC grade), ethanol, boric acid (Suprapur), citric acid, potassium hydroxide, potassium dihydrogen phosphate, sodium hydroxide, sodium sulphate anhydrous, hydrochloric acid (37%), concentrated sulphuric acid, 2-mercaptoethanol, OPA (grade for fluorogenic detection) were all from Merck (Darmstadt, Germany), NaDOSS from Sigma (St. Louis, MO, USA) and sodium hypochlorite (NaOCl, 13% active chlorine) from Janssen Chimica (Beerse, Belgium). Spectinomycin · 2HCl · $5H_2O$ (activity 652 μg/mg) was from Upjohn (Kalamazoo, MI, USA).

A spectinomycin stock solution (1.0 mg/ml) was prepared by dissolving 154.3 mg of spectinomycin · 2HCl · $5H_2O$ in 100 ml of water. Working standards were prepared by diluting the standard solution to 0.004, 0.02 and 0.2 mg/ml in water.

The following solutions were prepared: water–methanol (80:20, v/v), 0.02 *M* citric acid buffer pH 5.6, 0.02 *M* NaDOSS in water (homogenized just before use), 0.1 *M* potassium phosphate buffer pH 7.0, 1 *M* hydrochloric acid and 0.1 *M* sodium sulphate adjusted to pH 2.6 by dropwise addition of sulphuric acid.

High-hydrophobic C_{18} SPE columns (3 ml) were from J.T. Baker (Phillipsburg, NJ, USA). The SPE columns were pretreated by sequentially passing, with low vacuum or vacuum off, 3 ml of methanol, 2 ml of NaDOSS solution (slow passage is essential) and 4 ml of citric acid buffer. After this pretreatment the SPE columns were not allowed to run dry.

The HPLC column was a 5-μm Spherisorb SCX (250 × 4.6 mm) column (Phase-Sep, Norwalk, CT, USA) with an integral guard column (10 × 4.6 mm) filled with cation-exchange material (Phase-Sep).

The mobile phase for HPLC was 0.1 *M* sodium sulphate pH 2.6–acetonitrile (80:20, v/v) and was degassed before use.

For the post-column reaction system the following solutions were used: (1) a NaOCl solution, prepared by pipetting 10 ml of NaOCl (13% active chlorine) and 10 ml of 0.1 *M* potassium phosphate buffer pH 7.0 to 980 ml of water (post-column reagent A); (2) an 0.4 *M* boric acid buffer pH 10.2, prepared by dissolving 24.4 g of boric acid and 20.0 g of potassium hydroxide in 1 l of water; and (3) an OPA solution prepared by pipetting 10 ml of an 80 mg/ml OPA solution in ethanol and 1 ml of 2-mercaptoethanol to 990 ml of 0.3 *M* boric acid buffer pH 10.2 (post-column reagent B). Reagents were prepared and degassed before use and stored under nitrogen during use. An 0.3 *M* boric acid buffer pH 10.2 was prepared by dissolving 18.55 g of boric acid and 15.0 g of potassium hydroxide in 1.0 l of water.

2.2. Apparatus

Evacuated blood collection tubes containing 150 United States Pharmacopic Units (U.S.P.U.) of lithium heparin, 20-ml stoppered polypropylene tubes (Sarstedt, Nümbrecht, Ger-

many) and 5-ml glass collection tubes (Renes, Zeist, Netherlands) were used.

The instruments used were a table centrifuge (Hereaus, Karlsruhe, Germany), a vortex mixer (Janke and Kunkel, Staufen, Germany) and a Reacti-Vap evaporation unit Model 18780, connected to a Reacti-Therm heating module Model 18790 (Pierce, Rockford, IL, USA). In order to operate several SPE columns simultaneously a vacuum manifold (Baker) was used.

Two reaction coils were used: (1) a reaction coil of 2.2 m × 0.5 mm, made from a reaction coil of 3 m × 0.5 mm (Supelco, Bellefonte, PA, USA) shortened to 2.2 m by cutting 0.8 m, and (2) a reaction coil of 1.7 m × 0.3 mm, knitted from Teflon tubing for HPLC (Chrompack, Bergen op Zoom, Netherlands).

The scheme of the HPLC system is given in Fig. 1. For HPLC pump P1 (Pharmacia/LKB, Uppsala, Sweden) and an autosampler (Spark-Holland, Emmen, Netherlands) was used. For the post-column reaction system (PCRS) two pumps, P2 and P3 (Pharmacia/LKB), were used for post-column reagent A and 0.4 *M* boric acid buffer pH 10.2, respectively. The post-column oxidation was carried out via the reaction coil (2.2m × 0.5 mm) in a waterbath W1 operated at 70°C (temperature constant within 0.2°C). Pump P4 (Pharmacia/LKB) was used for post-column reagent B. The OPA reaction was carried out via the reaction coil (1.7m × 0.3 mm) in waterbath W2 of 25°C (temperature constant within 0.5°C). A fluorescence detector (Millipore/Waters, Milford, MA, USA) operated at an excitation wavelength of 340 nm and an emission wavelength of 460 nm was used for detection of spectinomycin. Peak heights were recorded on an integrator (Spectra Physics, San José, CA, USA).

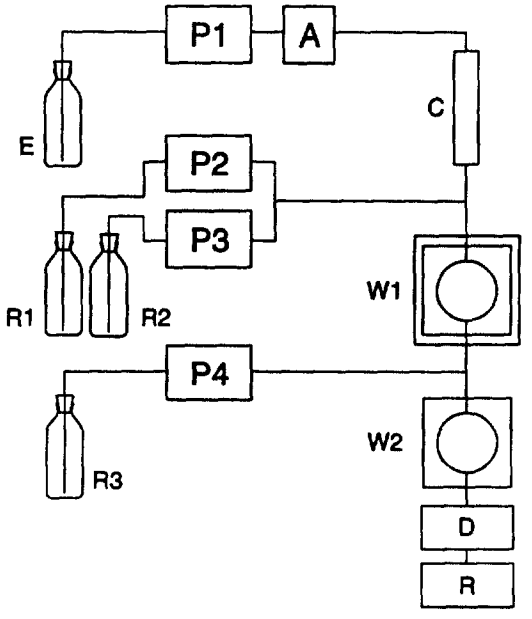

Fig. 1. Set-up of the HPLC system: P1, P2, P3 and P4 = HPLC pumps; E = HPLC eluent; R1 = post-column reagent A (NaOCl); R2 = boric acid buffer; R3 = post-column reagent B (OPA); A = autosampler; C = HPLC column; W1 = water bath (70°C) with coil of 2.2 m × 0.5 mm I.D.; W2 = water bath (25°C) with coil of 1.7 m × 0.3 mm I.D.; D = fluorescence detector; R = integrator.

2.3. 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 at levels of 0.1–1.0 μg/ml at least 15 min before extraction. When samples were not directly analyzed, they were stored at −20°C.

2.4. Sample preparation

The sample preparation procedure was the same as described earlier [6] with some small modifications. A 2-ml volume of plasma was pipetted in the 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 buffer. 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 by means of the manifold. The SPE column was washed two times with 3 ml of citric acid buffer and then allowed to run dry. The SPE

column was centrifuged for 15 min at 3000 *g* and further dried in a stream of nitrogen for 20 min. Spectinomycin was eluted with 4 ml of methanol. The eluate was collected in the 5-ml glass collection tube and evaporated to dryness in a stream of nitrogen at room temperature. The residue was dissolved in 1 ml of water–methanol (80:20, v/v) using a vortex mixer. The solution was centrifuged for 10 min at 2500 *g* and the supernatant was used for HPLC analysis.

NB: If concentrations higher than 50 μg/ml were expected a smaller test portion was taken. However, this test portion must be adjusted to 2 ml with water (which has been shown not to affect the recovery compared to blank plasma) before continuing the procedure.

2.5. Chromatography and post-column derivatization

The system was set-up according to the scheme in Fig 1. The flow-rates for each pump are: P1 = 1.50 ml/min, P2 = 0.25 ml/min, P3 = 0.75 ml/min and P4 = 1.00 ml/min.

Aliquots of 50 μl of the standard and the sample solutions were injected into the HPLC system. The time between two injections was 12 min.

3. Results and discussion

3.1. Sample pretreatment

The background of the development of this sample preparation procedure is discussed in detail elsewhere [6]. With respect to the procedure used earlier some small modifications were introduced. The elution was performed with 4 ml (instead of 3 ml) of methanol, resulting in a nearly 100% recovery of spectinomycin. Moreover, this eluate has to be reconstituted in water–methanol (80:20, v/v) instead of HPLC-eluent, due to the poor solubility of the residue in the latter solvent.

3.2. Chromatography and derivatization

A strong cation-exchange column was used for chromatography. With 0.1 *M* sodium sulphate pH 2.6 in the eluent a reasonable retention for spectinomycin was obtained.

The post-column reaction system consists of two steps. First the secondary amine group of spectinomycin has to be converted to a primary amine. This can be done by treatment with a source of active chlorine such as NaOCl [1,2] at elevated temperature. Thereafter, the primary amine is derivatized with OPA in the presence of 2-mercaptoethanol at high pH resulting in a fluorescent product. The product is rapidly generated at room temperature and the reaction conditions are not critical. Therefore the length of the OPA derivatization coil is not critical. However, the warm effluent after the NaOCl reaction has to be cooled before it reaches the detector to protect the flow cell. The OPA derivatization reagent was found to be unstable under routine conditions. This could be circumvented by storage of the reagent under nitrogen during use. This instability is probably due to oxidation of the thiol group of 2-mercaptoethanol [7].

The NaOCl oxidation conditions need to be discussed in more detail. A high response of spectinomycin is obtained even at very low NaOCl concentrations (final concentration of 0.013% corresponding with a flow-rate of the pump of 0.1 ml of NaOCl/min), thereafter a slow decrease is observed, followed by a rapid decrease in spectinomycin response, possibly due to breakdown of spectinomycin. The response of spectinomycin in relation to the final concentration of the NaOCl solution is schematically given in Fig. 2. At a NaOCl concentration of 0.065% (corresponding with a flow-rate of 0.5 ml/min) the response of spectinomycin is approximately 10% of that at a concentration of 0.033% (corresponding with a flow-rate of 0.25 ml/min). A NaOCl concentration of 0.033% instead of a lower concentration was chosen for reasons of better pump performance.

The "Suprapur" grade quality of the boric acid

Table 1
Recovery of spectinomycin from spiked plasma

Plasma	Added (μg/ml)	Mean recovery (%)	Standard deviation (%)	n
Calf	0.1	99	2.0	7
	1.0	91	1.1	3
	10.0	93	0.45	3
	25.0	94	0.06	3
	50.0	98	0.25	3
Swine	0.1	99	1.7	6
	1.0	98	1.3	3
Chicken	0.1	104	2.3	6
	1.0	96	2.1	3

in the post-column reagents is essential for detection at low levels. Boric acid of analytical grade quality gives a higher background fluorescence resulting in a less stable baseline and a higher detection limit.

Some problems were met with respect to the long-term stability of the NaOCl reagent as well. The optimal condition for NaOCl oxidation is pH 10.2. At this pH, however, the reagent possesses a short shelf life. This could be circumvented by preparing the NaOCl reagent in a solution of neutral pH. In the post-column reaction system the right pH is obtained by mixing this solution with 0.4 *M* boric acid buffer pH 10.2. The reason for this instability is that hypohalite ions tend to disproportionate in basic solution to produce halate ions [8].

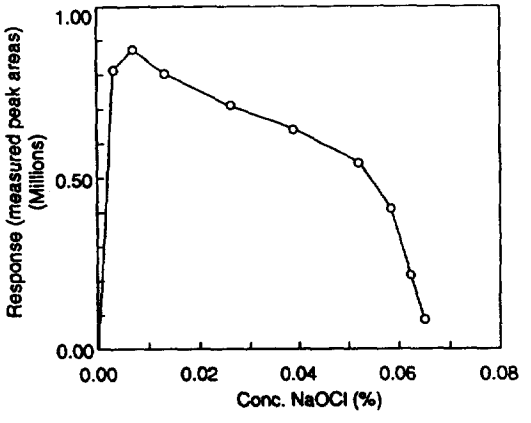

Fig. 2. Response of spectinomycin (in a solution of 0.8 μg/ml) in relation to the final NaOCl concentration in the post-column derivatization reagent. A volume of 50 μl of a spectinomycin solution (0.8 μg/ml) was injected.

3.3. Spiking studies and real samples

Recovery experiments were carried out with replicate samples of blank swine, calf and chicken plasma at different spiking levels. The samples were analyzed in duplicate according to the procedure described. The results are presented in Table 1. A good recovery at all levels investigated, and a low standard deviation for repeatability were attained. To establish the day-to-day variation a spiked calf plasma sample (0.5 μg/ml) was analysed in sixfold on four different days. The results are given in Table 2. Analysis of variance was carried out following a described

Table 2
Day-to-day variation of spectinomycin from spiked calf plasma (0.5 μg/ml)

Day	Mean (μg/ml)	Standard deviation (μg/ml)	n
1	0.433	0.004	6
2	0.438	0.013	6
3	0.437	0.006	6
4	0.445	0.005	6

method [9]. The results showed that there was no significant difference between the within- and between-day precision.

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.

The absolute limit of detection was found to be approximately 5 ng on column (S/N ratio = 3:1). As no interferences of endogenous compounds from plasma of different animals at the retention time of spectinomycin were observed, a spectinomycin level of 0.1 μg/ml plasma can be easily quantitated (limit of quantitation 0.06 μg/ml, taking into consideration a recovery of 90%). The calibration is linear throughout the range 0.2–100 μg/ml ($r = 0.9998$).

As an illustration of the applicability of the described method, plasma of spectinomycin-treated calves was analyzed. To obtain real samples three calves with a mean body weight of 60 kg were injected intramuscularly with a Linco-Spectin solution resulting in 10 mg spectinomycin/kg body weight. The amounts of spectinomycin found in plasma 1 h after injection were 17, 15 and 17 μg/ml, respectively. A chromatogram of a real plasma sample is shown in Fig. 3H.

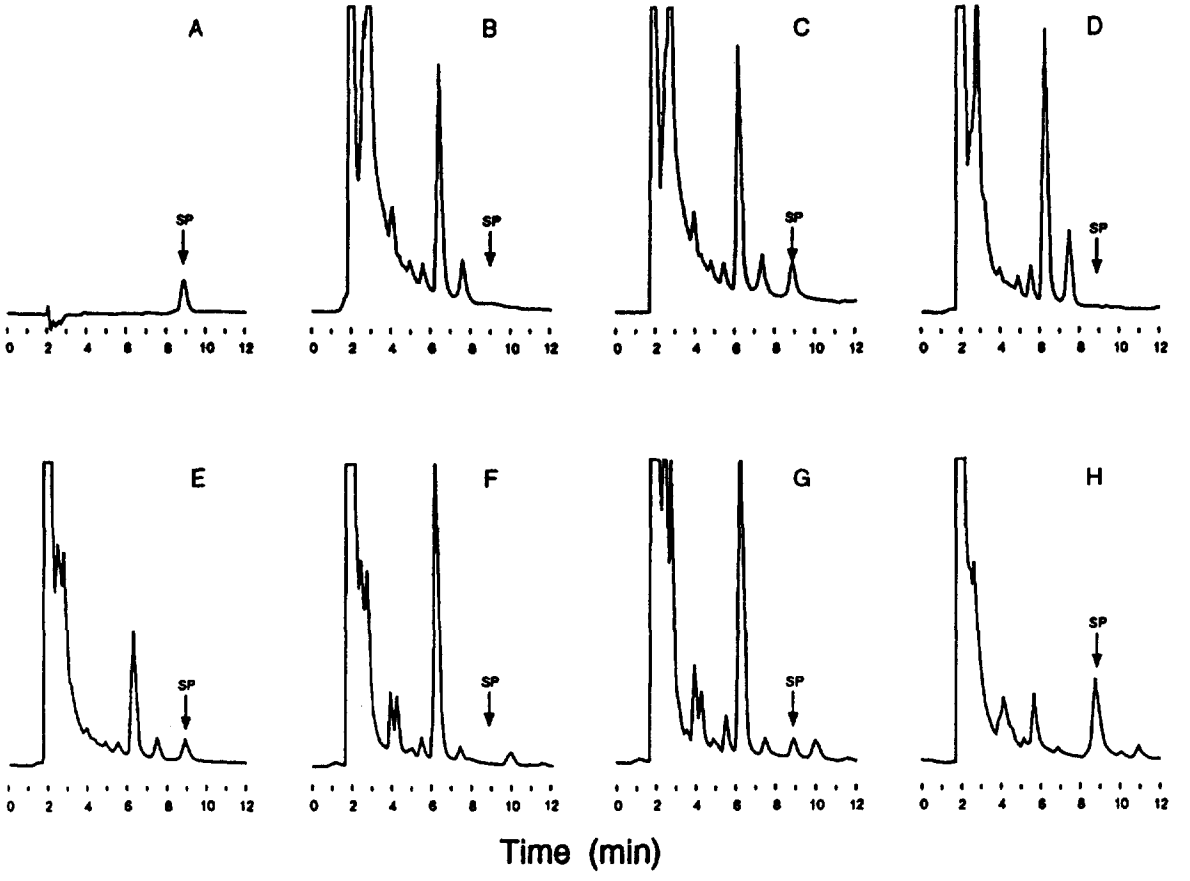

Fig. 3. Chromatograms of (A) a spectinomycin standard solution (0.2 μg/ml), (B) blank and (C) spiked calf plasma (0.1 μg/ml), (D) blank and (E) spiked swine plasma (0.1 μg/ml), (F) blank and (G) spiked chicken plasma (0.1 μg/ml) and (H) real calf plasma (spectinomycin concentration 0.4 μg/ml). Attenuation 16 mV full scale.

Acknowledgement

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