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High-performance liquid chromatographic method for the determination of spectinomycin in turkey plasma

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ABSTRACT

A selective high-performance liquid chromatographic (HPLC) method with ultraviolet–visible (UV–VIS) detection was developed to measure therapeutic concentrations of spectinomycin in turkey plasma. Treatment of plasma samples with 3% trifluoroacetic acid in acetonitrile facilitated spectinomycin extraction and protein precipitation. After centrifugation, the stable derivatization reagent, 2,4-dinitrophenylhydrazine, was added to an aliquot of the supernatant, and the mixture was incubated for 30 min at 70°C. Excess reagent was quenched with acetone and additional heating. The resulting derivative, a proposed spectinomycin-hydrazone, was separated from other compounds by reversed-phase HPLC during a short gradient run. The absorbance of the effluent was monitored spectrophotometrically with the UV–VIS detector set at 205 nm. The detector response was linear through the range of interest, 2–100 μg/ml.

INTRODUCTION

Spectinomycin, a broad-spectrum aminocyclitol antibiotic, is a labile, non-chromophoric compound [1–4]. Avian plasma represents one of the most complex biological matrices; consequently, the development of a high-performance liquid chromatographic (HPLC) assay for spectinomycin in turkey plasma presents a challenge for the analytical chemist. To date no procedure for forming ultraviolet–visible (UV–VIS) or fluorescent derivatives of spectinomycin has been successfully applied to the HPLC analysis of this drug in a biological matrix. Tsuji and Jenkins [2] after trials involving 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD chloride), 1-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF), dansyl chloride, fluorescamine and 2-diphenylacetyl-1,3-indandione-1-hydrozone developed a lengthy and complex procedure for derivatization of spectinomycin using 2-naphthalenesulfonyl chloride (NSCl). Unfortunately, the best results they [2] could obtain were limited to the screening of bulk drug preparations. A similar attempt was performed in our laboratory to derivatize the three available hydroxyls in spectinomycin (Fig. 1) using benzoyl chloride. While derivatization did occur, the method was not applicable to blood plasma analysis due to the overwhelming masking effect of the non-selective derivatization of amino and hydroxy metabolites which formed major peaks after the procedure. Attempts to

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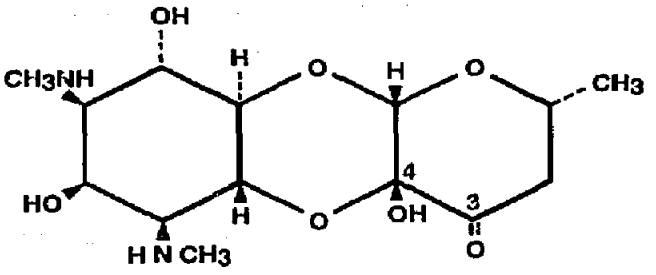

Fig. 1. Structure of spectinomycin.

improve the resolution by using longer columns and pre-HPLC cartridge clean-up procedures also failed.

Transformation of seconday amino groups to primary amines as an approach to derivatize spectinomycin with *o*-phthalaldehyde [1], besides being a complex procedure, is still applicable only to fairly clean preparations of the drug. In our laboratory we tried to directly derivatize spectinomycin's secondary amino groups (two available) with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD fluoride). And again, even though derivatization of standard spectinomycin solution was possible, the massive response of the fluorescence detector to easily derivatized amino acids, amines and immines in blood plasma made it impossible to resolve the analyte from the matrix.

Finally, we directed our attention to the carbonyl group of carbon 3 (see Fig. 1). The first attempt was made using dansylhydrazine, but was only partially successful due to poor solubility of spectinomycin in the largely organic reaction mixture, which resulted in poor recovery of the derivative.

We now describe a derivatization procedure for spectinomycin which is suitable for HPLC analysis of this drug in a biological matrix. The method uses 2,4-dinitrophenylhydrazine (2,4-DNPH) as a reagent to derivatize the carbonyl group of spectinomycin. The methodology meets the requirements for monitoring therapeutical levels (50–100 μg/ml) of this antibiotic in blood plasma using reversed-phase chromatography and selective, sensitive detection at 405 nm.

EXPERIMENTAL

Materials

Spectinomycin dihydrochloride pentahydrate, 2,4-DNPH containing 30% water and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and acetone were supplied by Fisher Scientific (Raleigh, NC, USA). Water was distilled and deionized with a Dracor water system (Durham, NC, USA). Dulbecco's phosphate-buffered salt solution (1X PBS buffer, 0.15 *M* NaCl) was obtained from Cellgro Mediatech (Washington, DC, USA).

Stock solution of 10 mg/ml spectinomycin was prepared in 1X PBS buffer (pH

7.0). Working concentrations of 2, 10, 25, 50 and 100 μg/ml were prepared daily with 1X PBS buffer. Plasma was spiked with stock solution and diluted with blank plasma to obtain the desired concentrations. All plasma samples were kept on ice until derivatization.

For sample clarification Ultrafree MC filter units (30 000 nominal molecular weight limit) and a Millipore personal centrifuge (6400 rpm fixed speed, 2000 *g*) were used (Millipore, Bedford, MA, USA). Derivatization vials, screw-cap septum vials, 1.5 ml with PTFE-laminated discs, were supplied by Pierce (Rockford, IL, USA).

Sample preparation and derivatization

A 100-μl aliquot of plasma was vortex-mixed with 400 μl of 3% TFA in acetonitrile and centrifuged at 2000 *g* for 3 min. A 250-μl aliquot of clear supernatant was pipetted into the derivatization vial and 200 μl of 5 mg/ml 2,4-DNPH in acetonitrile (derivatization reagent) were added; the contents were mixed and then heated at 70°C for 60 min. After heating, the sample was cooled on ice for 2 min and 30 μl acetone were added. The sample was mixed and then heated for an additional 10 min. After cooling on ice, the sample was clarified by passage through a 30 KDa molecular mass cut off filter and kept at room temperature until injected to the HPLC system.

High-performance liquid chromatography

A 20-μl aliquot of the sample was introduced into the HPLC system through a Rheodyne Model 8125 injector. Solvent delivery was accomplished with a Perkin Elmer Series 410 gradient pump. Separation was achieved using a 3.3 cm × 0.46 cm I.D. stainless-steel cartridge packed with 3 μm Pecosphere C_{18} CR (Perkin Elmer, Norwalk, CT, USA). Mobile phase A consisted of water–acetonitrile (1:1.5 v/v) and mobile phase B of acetonitrile–methanol (19:1, v/v). Gradient chromatography was performed as follows. The column was equilibrated 10 min in 100% A prior to sample injection. Following a 1-min hold at 100% A, a linear gradient to 100% B was run over 10 min. Solvent composition was then held at 100% B for an additional 2 min. A constant flow-rate of 1.2 ml/min was used throughout the gradient program. Absorbance of column effluent was monitored at 405 nm with a Perkin Elmer LC-95 variable-wavelength detector. Results were calculated by a Perkin Elmer LCI-100 laboratory computing integrator.

Spectra (UV–VIS) were obtained using a Waters 990 photodiode array detector (Milford, MA, USA).

RESULTS AND DISCUSSION

Representative chromatograms of spectinomycin standard solution, plasma spiked with spectinomycin and blank turkey plasma are shown in Fig. 2A, B and C, respectively. The separation shows that the spectinomycin derivative was baseline-resolved, and that the analytical window for this analyte was excellent.

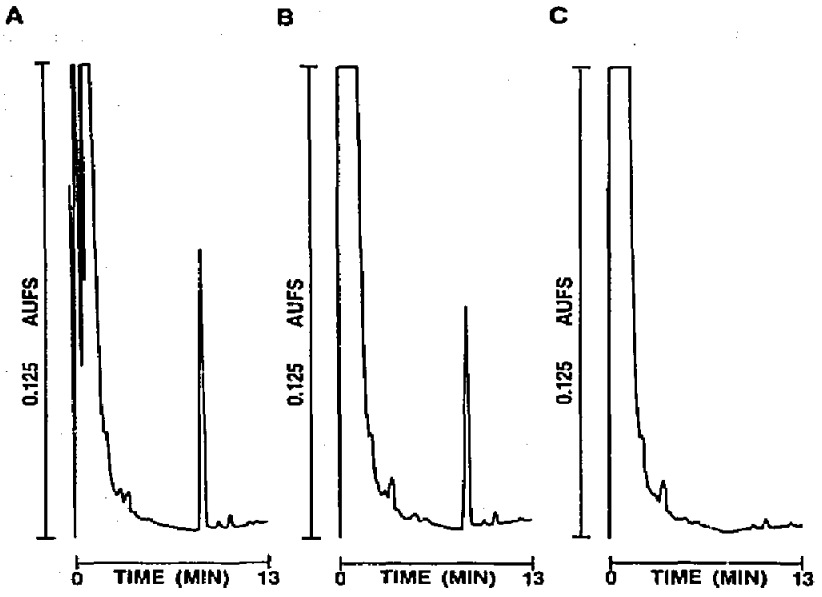

Fig. 2. Chromatogram of (A) spectinomycin standard (10 μg/ml). (B) turkey plasma spiked with 10 μg/ml spectinomycin, and (C) blank turkey plasma.

Precision of the spectinomycin method was examined by derivatization and HPLC analysis of eight aliquots of the spiked 10 μg/ml turkey plasma on one day and six on another day spiked with 1.42 μg/ml. Results are given in Table I. Recovery was estimated by comparison of the peak areas between standard solutions of spectinomycin *versus* spiked plasma at the same concentration and was equal to 87 and 83%, respectively.

TABLE I

STATISTICAL SUMMARY OF SPECTINOMYCIN ANALYSIS USING SPIKED PLASMA SAMPLES

Inter-assay variability was calculated as ± 1.24%.

Parameter	Value	
	10 μg/ml	1.4 μg/ml
Number of reapeats (n)	8	6
Range (area of the peak)	2.45–2.69	0.350–0.369
Mean	2.5463	0.3575
Standard deviation	0.0127	0.00709
Coefficient of variation (%)	3.64	1.98
Recovery (%)	87	83

TABLE II

LINEARITY OF SPECTINOMYCIN ANALYSIS USING SPIKED PLASMA SAMPLES

Spectinomycin concentration (μg/ml)	Peak area (μV s $\times$ 10^6)
2	0.34
	0.34
10	2.36
	2.50
25	4.67
	4.43
50[a]	7.66
	7.98
100[a]	14.95
	15.78

[a] Therapeutic levels.

The standard curve for the HPLC assay of spectinomycin using duplicate spiked plasma samples at levels of 2, 10, 25, 50 and 100 μg/ml was linear, $y = 0.148x + 0.551$ with a correlation coefficient of 0.9981. Data are shown in Table II.

Surprisingly, during development of the derivatization procedure two peaks (see Fig. 3b) were noted to appear early in the process. Both peaks showed UV–VIS spectra indicative of 2,4-DNPH derivatives (Fig. 3). The spectrum of peak 2 was shifted further to the visible region indicating higher proportion of the chromophore. The lower polarity of peak 2 supports this observation.

A reaction mixture consisting of 900 μl of 2,4-DNPH in acetonitrile (5 mg/ml), 100 μl of spectinomycin solution in 1X PBS buffer (100 μg/ml) and two different TFA concentrations (0.3 and 6%) incubated at 55°C for 0.5 h gave us two peaks with a peak 1/peak 2 area ratio of 8:2 (Table III). Observations were made again after an additional 0.5 h at 55°C, after leaving overnight at room temperature and after an additional 1 h at 60°C. Heating the sample at 55°C sped up the reaction considerably: the peak 1 area was decreased while the peak 2 area increased. Kinetics support the formation of bis derivative through mono derivative.

Up to 2 h heating at either 60 or 70°C did not cause total transformation of peak 1 to peak 2. Increased concentration of the TFA facilitated faster production of peak 2. The beneficial effect of TFA reached a plateau at 0.7% final concentration in the reaction mixture. Conditions described in the Experimental section facilitated formation only of peak 2.

To avoid additional residual production of peak 2 without increasing the heating time, acetone was used to quench the excess derivatization reagent. The acetone derivative was much more polar and appeared at the void. Samples treated

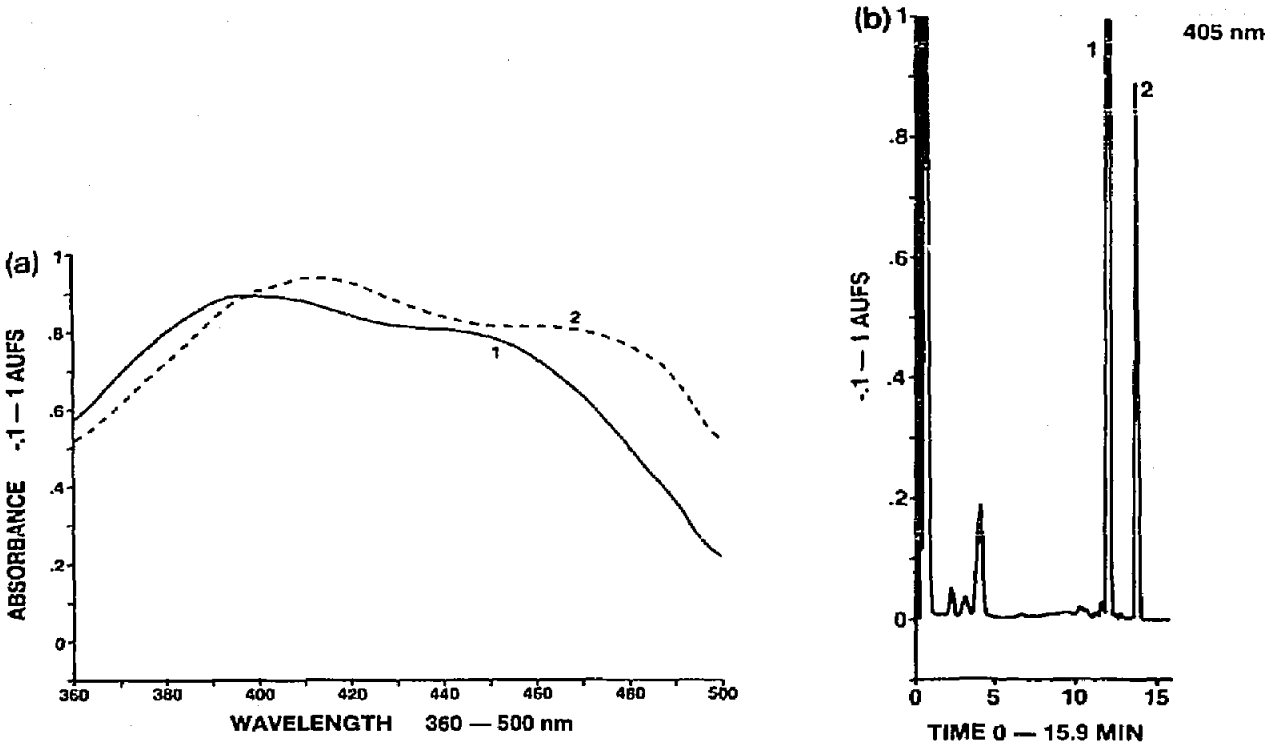

Fig. 3. (a) UV–VIS spectra acquired for porposed mono-hydrazone peak 1 and bis-hydrazone peak 2. (b) Typical chromatogram of standard solution of spectinomycin incubated for only 5 min at 70°C during derivatization. Mobile phase A and B were the same as described in Experimental. Gradient conditions: 2 min 100% A, 12 min to 100% B, 10 min 100% B. Flow-rate: 1.2 ml/min.

this way were stable at room temperature for up to three days while being monitored.

Spectinomycin solubility was a factor which had to be considered in formulating the derivatization solution. At higher acetonitrile concentrations, spectino-

TABLE III

KINETICS OF DERIVATIZATION REACTION OF SPECTINOMYCIN STANDARD

Time/temperature	TFA concentration (%)	Area peak 1	Area peak 2
0.5 h, 55°C	0.3	0.00506	0.00125
	0.6	0.01234	0.00331
Additional 0.5 h, 55°C	0.3	0.01175	0.00305
	0.6	0.02910	0.01002
Overnight, 25°C	0.3	0.04736	0.04136
	0.6	0.05623	0.08491
Additional 1 h, 60°C	0.3	0.02076	0.09683
	0.6	0.01788	0.15215

mycin precipitated. At lower acetonitrile concentrations, the rate of derivatization was lowered.

The short HPLC cartridge allowed the use of efficient gradient runs with minimal re-equilibration time. By holding solvent composition at 100% B at the end of the run, we were able to remove neutral lipids from the column after each sample injection. Avian plasma is high in neutral lipids, which can build up on the column over time and cause separation and back-pressure problems.

Based on information obtained during method development (formation of the two derivatives) we would like to hypothesize at this time that the most probable structure of peak 2 is the bis-hydrazone of spectinomycin which could resemble an osazone-like derivative [5] if opening of the ring at carbon 4 (see Fig. 1) takes place during our procedure. Suspected mono-hydrazone (carbonyl 3) and bis-hydrazone (carbonyl 3 + hydroxyl of the carbon 4) derivatives were collected and injected into the 4800 quadrupole mass spectrometer with Finnigan-MAT thermospray. Unfortunately, mass spectrometric (MS) results indicated that the compounds were very unstable in the thermospray environment, and the masses of the parent ions could not be obtained. A further attempt will be made to characterize the derivatives when access to electrospray-MS (ES-MS) or fast atom bombardment MS (FAB-MS) becomes available.

It is worth noting that only a small portion of the available sample (20 μl from 480 μl) was injected. Full analytical potential of this method will be further explored when we investigate residual spectinomycin in treated birds. Up to 200 μl of incompletely derivatized plasma samples containing high levels spectinomycin were injected during collection of peaks 1 and 2 for MS studies and no deterioration to resolution or to high background was observed. The amount of derivatized spectinomycin presented in Fig. 2A represents 20 ng of spectinomycin, and peak height reaches 0.07 absorbance units.

CONCLUSION

An original, fast and inexpensive method for the HPLC determination of spectinomycin in turkey plasma has been developed and provides a reliable analytical procedure for use in animal studies. The method was developed (Tables I and II) for practical purposes to monitor therapeutic levels (50–100 μg/ml) of spectinomycin in turkey plasma [6,7].

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