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Determination of spectinomycin using cation-exchange chromatography with pulsed amperometric detection

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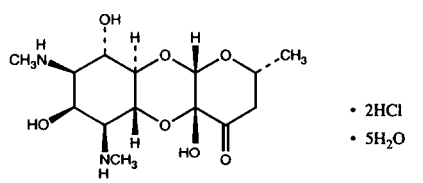
Abstract

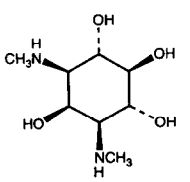
An isocratic HPLC method was developed for the determination of spectinomycin dihydrochloride pentahydrate. The method is based on cation-exchange chromatography with pulsed-amperometric detection. The method was optimised for the separation of spectinomycin, actinamine and actinospectinoic acid on a Dionex IonPac CS3 column at ambient temperature with 150 m*M* sodium acetate (pH 6) mobile phase. Detector response with respect to sodium hydroxide concentration was investigated using cyclic voltammetry. A three-step pulse sequence with a sampling potential of +0.05 V was used for detection. The k' values for spectinomycin and related impurities were insensitive to column temperature changes in the range 21–50°C and show only minor variations to pH changes in the range 5.0–6.0. The method is linear and unbiased for the determination of spectinomycin over the concentration range 25–200 μg/ml for 50-μl injections. Two-day precision data for the method show within-sample R.S.D.s in the range 0.3–1.0% and between-day R.S.D.s in the range 0.3–0.9%. The limits of detection and quantitation determined for spectinomycin are 0.02 μg and 0.06 μg on-column, respectively. The method is suitable for the analysis of drug-substance.

1. Introduction

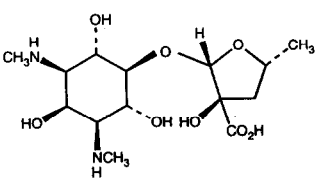
Spectinomycin is a broad-spectrum antibiotic used in the treatment of Gram negative organism infections. It is manufactured mainly as the *R-cis* form of the dihydrochloride pentahydrate salt. In aqueous and organo–aqueous solutions anomerisation occurs resulting in an equilibrium mixture of the *R-cis*, *S-cis*, *R-trans* and *S-trans* forms [1]. The keto form also hydrates readily to produce the *gem*-diol form [2,3]. Acid degradation leads to the formation of actinamine, whilst base degradation produces actinospectinoic acid [3].



R-cis spectinomycin dihydrochloride pentahydrate



actinamine



actinospectinoic acid

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Under reversed-phase equilibrium conditions, spectinomycin quickly equilibrates to a mixture of diol and keto form. The diol form has no chromophore and the keto form has no significant chromophore. Therefore, the determination of spectinomycin by HPLC with UV detection requires complex derivatisation procedures [4,5]. Also, these methods do not adequately resolve and quantify the anomers to express potency and they suffer from variability caused by lack of control of the stereoisomeric equilibrium. Although electrochemical detection using controlled potential coulometry has been applied to the determination of spectinomycin [6], the repeatability of the method is characteristically poor due to contamination of the electrode surface.

The method described in this report applies cation-exchange chromatography to elute spectinomycin anomers as a single peak and pulsed-amperometric detection for low limit of detection, good signal-to-noise ratio and low method variability.

2. Experimental

2.1. Materials

Spectinomycin dihydrochloride pentahydrate, actinamine and actinospectinoic acid were supplied by Upjohn (Crawley, UK). All other chemicals were analytical reagent grade supplied by Fisons (Loughborough, UK). Water was purified using a Millipore Milli-Q system (Waters, Watford, UK). All solvents and solutions were degassed under vacuum, purged with helium and maintained under a head of helium during use.

2.2. Cyclic voltammetry

Since base is essential for pulsed-amperometric detection (for oxidation) of polyhydroxy compounds [7], the effect of different concentrations of sodium hydroxide on the detector response to spectinomycin was assessed using cyclic voltammetry. This was conducted using a Metrohm VA Detector E611 with a gold working electrode, a stainless-steel auxiliary electrode and Ag/AgCl reference electrode, a Metrohm VA Scanner E612 and a Gould HR 2000 X-Y recorder. Voltammograms of four 3 m*M* solutions of spectinomycin in 150 m*M* sodium acetate and varying amounts of sodium hydroxide (12.5, 25.0, 50.0 and 100.0 m*M*) as well as of the corresponding blank solutions (without spectinomycin) were recorded. A scanning range of −0.30 to +0.10 V and a scan speed of 50 mV/s were used.

2.3. Chromatography

All chromatograms were generated using a Dionex metal-free quaternary gradient pump; a Dionex pulsed-amperometric detector with gold working electrode, a stainless-steel auxiliary electrode and Ag/AgCl reference electrode; a LDC Constametric III post-column pump; a Dionex metal-free rotary injection valve with a 50-μl injection loop; a Dionex IonPac CS3 column (250 mm × 4 mm I.D.) and Dionex CG3 guard column (25 mm × 4 mm I.D.); a Shimadzu CTO-6A column oven and a Spectra Physics SP4290 computing integrator. The system was operated using a column flow-rate of 1 ml/min; a sample injection volume of 50 μl; a column pressure of 6.2 MPa and a post-column flow-rate of 1.5 ml/min. The pulsed-amperometric detector was used with a rise-time filter setting of 3.0 s and an output range of 1 μA.

Optimisation was conducted for isocratic elution using sodium acetate mobile phase at concentrations in the range 150–250 m*M*, mobile phase pH at values in the range 5–6 and column temperature at values in the range 21–50°C. Validation was conducted for linearity of response to concentrations of spectinomycin in the range 25–200 μg/ml, limits of detection and quantitation for signal-to-noise ratios of 3 and 10, respectively, and two-day precision for triplicate injections of three solutions of spectinomycin at concentrations of approximately 75, 100 and 125 μg/ml.

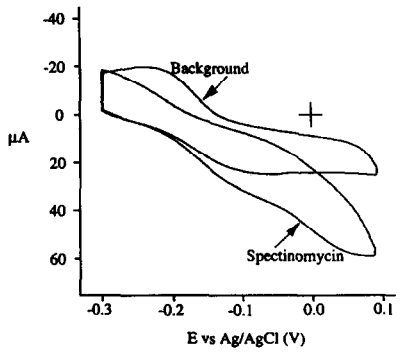

Fig. 1. Cyclic voltammogram (labelled *background*) for a solution of 150 m*M* sodium acetate and 100.0 m*M* sodium hydroxide (pH 12.6). Cyclic voltammogram (labelled *spectinomycin*) for 3 m*M* spectinomycin in 150 m*M* sodium acetate and 100.0 m*M* sodium hydroxide (pH 12.6). The scan speed was 50 mV/s. The origin of the plots is indicated by (+).

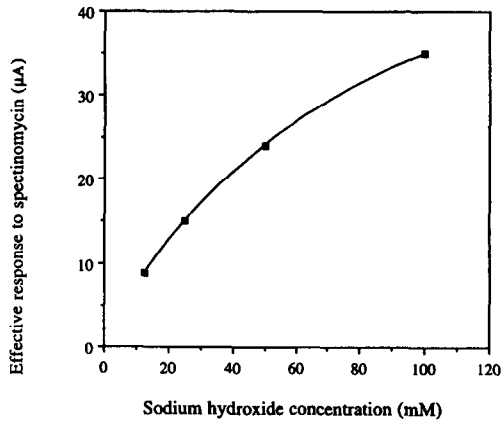

Fig. 2. Relationship between effective response to 3 m*M* spectinomycin and sodium hydroxide concentration. The effective response for spectinomycin is derived from the cyclic voltammograms by subtraction of the maximum background current from the maximum current recorded for the analyte solution.

3. Results and discussion

The cyclic voltammograms (example shown in Fig. 1) show that the maximum current for the blank solutions is reached at approximately −0.05 V. The values obtained for the maximum current measured for the blank solutions (Table 1) varied with each experiment conducted. The lowest (25 μA) and highest (72.5 μA) values obtained were for the blank solutions containing 100.0 m*M* sodium hydroxide and 50.0 m*M* sodium hydroxide, respectively. For the 3 m*M* spectinomycin solutions, the maximum current measured occurred at +0.1 V for those solutions containing 12.5, 25.0 or 100.0 m*M* sodium hydroxide. The cyclic voltammogram for the spectinomycin solution containing 50.0 m*M* sodium hydroxide showed a maximum response at −0.03 V which decreases by 4 μA at 0 V and show no further significant decrease at 0.1 V. This solution gave the largest value of 96.5 μA for current measured and the corresponding blank solution gave the largest background current measured (72.5 μA). The largest effective response to spectinomycin was 35 μA (Table 1). This value was obtained for the 3 m*M* spectinomycin solution containing 100.0 m*M* sodium hydroxide after subtraction of the current measured for the corresponding blank solution. A plot of the effective response to spectinomycin *versus* concentration of sodium hydroxide is shown in Fig. 2. The solid line for the plot is interpolated to a simple curve. A similar response curve was obtained in a comparable study reported for carbohydrates [8].

The results show that under the conditions

Table 1
Maximum measured current for blank solutions and spectinomycin solutions derived from the cyclic voltammograms

Sodium hydroxide concentration (m*M*)	Maximum blank solution current (μA)	Maximum sample solution current (μA)	Effective response to analyte (μA)
12.5	37.5	46.3	8.8
25.0	26.3	41.3	15.0
50.0	72.5	96.5	24.0
100.0	25.0	60.0	35.0

used for the cyclic voltammetry, an increase in amperometric response to spectinomycin with respect to increasing sodium hydroxide concentration is obtained. This indicates that even though spectinomycin degrades in alkaline solution, its amperometric detection in sodium hydroxide can be achieved. At sodium hydroxide concentrations between of 25–100 m*M* the current due to the background for potential values between −0.1 to +0.1 V does not interfere with the detection of spectinomycin. Using pulsed amperometry, a measuring potential between −0.1 to +0.1 V and a sodium hydroxide concentration between 25–100 m*M* should provide detection of spectinomycin following chromatographic separation with sodium acetate. However, sodium hydroxide must be added post-column to avoid on-column degradation of spectinomycin.

With cyclic voltammetry, the measured current for spectinomycin in 100 m*M* sodium hydroxide rose sharply to 58.5 μA at +0.05 V, at an approximate rate of 192 μA/V. Beyond +0.05 V the increase in current to 60 μA at +0.1 V was at a much slower rate. For pulsed-amperometric detection of spectinomycin, a measuring potential of +0.05 V was used, since beyond this potential the increase in amperometric response is minimal. Additionally, at higher measuring potentials, interference due to oxidation of unknown components that may be present in real samples would be greater. A measuring potential of +0.05 V during pulsed-amperometric detection of spectinomycin should not give an unduly high background current since the cyclic voltammograms show no significant change in the current for the blank solutions between −0.05 and +0.10 V.

A three-step pulse-sequence (Fig. 3) was used for the pulsed-amperometric detection of spectinomycin, actinospectinoic acid and actinamine following separation by cation-exchange chromatography and post-column addition of 100.0 m*M* sodium hydroxide (33.3 m*M* effective concentration). The pulse-sequence used +0.05 V sampling potential with a 100-ms delay-time and 380-ms measuring-time. The oxidation potential to clean the electrode was +0.60 V for 120 ms

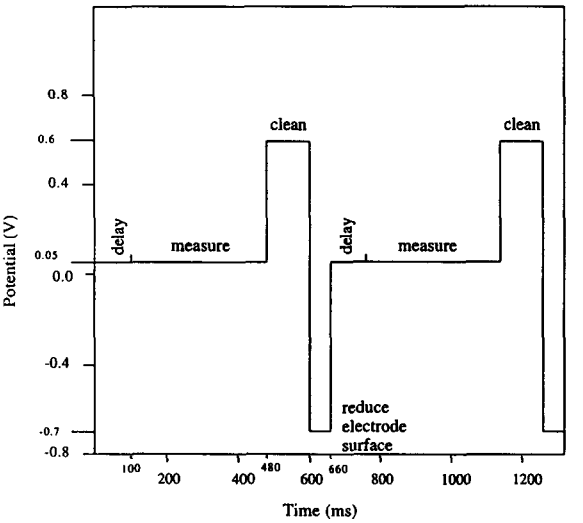

Fig. 3. Pulse sequence for the detection of spectinomycin, actinospectinoic acid and actinamine using a gold working electrode, a stainless-steel auxiliary electrode and an Ag/AgCl reference electrode.

and the reduction potential to activate the electrode surface was −0.70 V for 60 ms. The separation of the components of interest was optimised with respect to mobile phase (sodium acetate) concentration, mobile phase pH and column temperature. The k' values (Table 2) for spectinomycin, actinamine and actinospectinoic for the sodium acetate mobile phase at concentrations in the range 150–250 m*M* show that the best separation is achieved at 150 m*M* sodium acetate. Satisfactory separation of the three components is achieved at all concentrations of sodium acetate investigated and the time for analysis is significantly reduced with increasing concentrations of sodium acetate. However, resolution of other minor unknown impurities that elute close to actinospectinoic acid and spectinomycin is only adequately accomplished at 150 m*M* sodium acetate concentration. The mobile phase pH over the narrow range of 5.0 to 6.0 does not interfere significantly with the resolution of the components as shown by the k' values (Table 2). Mobile phase pH outside these values caused acid- and base-catalysed degradation of spectinomycin. The results in Table 2 also show that k' is independent of changes in temperature over the range 21–50°C. Fig. 4

Table 2
Changes in k' with respect to changes in pH, column temperature and sodium acetate concentration

	k'		
	Actinospectinoic acid	Spectinomycin	Actinamine
pH			
5.0	0.20	4.65	5.59
5.5	0.20	4.38	5.39
6.0	0.20	5.39	5.26
Temperature (°C)			
21	0.24	4.26	5.42
30	0.31	4.29	5.52
40	0.31	4.29	5.59
50	0.31	4.26	5.73
Sodium acetate (mM)			
150	0.39	8.18	9.77
200	0.23	4.32	5.34
250	0.11	3.07	3.75

shows a typical chromatogram for a 50-μl injection of a solution containing spectinomycin, actinamine and actinospectinoic acid, each at a concentration of 50 μg/ml in 150 mM sodium acetate. The sample was chromatographed using 150 mM sodium acetate mobile phase (pH 6) and 21°C column temperature. At the sample concentration used, the detector response to all three components is above 300 nA. The peaks labelled 1 and 2 are minor impurities found in actinospectinoic acid. The peak labelled 3 is a minor impurity found in actinamine. The analysis time for the method is 15 min. Under the chromatographic conditions applied throughout this study the spectinomycin anomers consistently eluted as a single peak. The co-elution of the anomers allows easy quantification of the potency of spectinomycin based on a single major component.

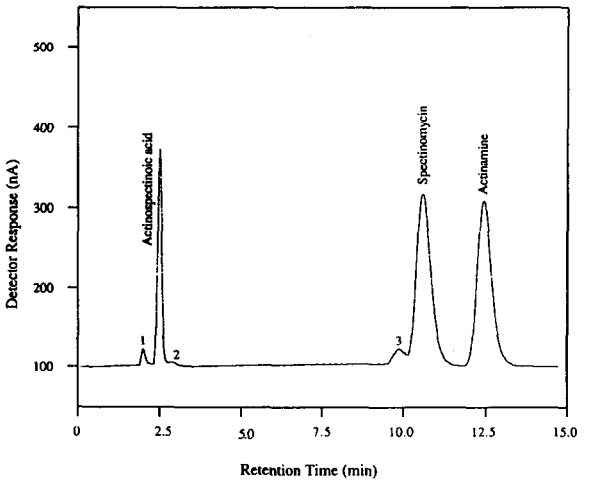

Fig. 4. Chromatogram for a 50-μl injection of a solution containing spectinomycin, actinamine and actinospectinoic acid. Each are at a concentration of 50 μg/ml in 150 mM sodium acetate. The sample was chromatographed using 150 mM sodium acetate mobile phase (pH 6) and 21°C column temperature. The peaks labelled 1, 2 and 3 are minor impurities in actinospectinoic acid and actinamine.

Least squares linear regression for spectinomycin at concentrations between 25–200 μg/ml *versus* peak area response gave an equation for the line of $y = -1.27 \cdot 10^{-4} + 4313.6x$ and $r^2 = 1$. The response to spectinomycin over the concentration range examined is therefore linear and unbiased, thus, supporting a proposal for nominal sample concentrations of 50–100 μg/ml for weight based assay and 100–200 μg/ml for determination of impurities. Results for the two-day precision study for spectinomycin solutions at concentrations of 74.78, 99.81 and 124.87 μg/ml (Table 3) show within-sample R.S.D.s in the range 0.3–1.0% and between-day R.S.D.s in the range 0.3–0.9%. The limit of detection of spec-

Table 3
Two-day peak area precision data for solutions of spectinomycin

	Spectinomycin concentration (μg/ml)		
	Sample 1 74.78	Sample 2 99.81	Sample 3 124.87
Day 1			
	308 411	408 518	573 924
	307 512	407 296	581 563
	309 131	410 719	580 987
Mean	308 351	408 844	575 491
R.S.D. (%)	0.3	0.4	1.0
Day 2			
	310 512	410 823	582 134
	312 306	409 513	583 479
	309 151	408 791	579 352
Mean	310 656	409 709	581 655
R.S.D. (%)	0.5	0.3	0.4
Overall mean	309 503	409 276	578 573
Overall R.S.D. (%)	0.6	0.3	0.9

tinomycin was determined as 0.02 μg on-column and the limit of quantitation was estimated as 0.06 μg on-column.

4. Conclusion

The results of these studies show that the method developed resolves spectinomycin and its known degradation products. Under the conditions of the method the anomers of spectinomycin elute as a single peak, thus reducing the variability seen with non-ion-exchange methods. The method is linear and unbiased between 25–200% of a nominal sample concentration of 100 μg/ml and shows a limit of detection of 20 ng on-column.

Acknowledgement

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References

[1] D.R. White, R.D. Birkenmeyer, R.C. Thomas, S.A. Mizsak and V.H. Wiley, *Aminocyclitol Antibiotics* (*ACS Symposium Series*, No. 125), American Chemical Society, Washington, DC, 1980, p.111.
[2] P.F. Wiley, A.D. Argoudelis and H. Hoeksema, *J. Am. Chem. Soc.*, 85 (1963) 2652.
[3] W. Rosenbrook Jr., *Jpn. J. Antibiot.*, 32 (Suppl.) (1979) S211.
[4] H.N. Myers and J.V. Rindler, *J. Chromatogr.*, 176 (1979) 103.
[5] K. Tsuji and K.M. Jenkins, *J. Chromatogr.*, 333 (1985) 365.
[6] L. Elrod Jr., J.F. Bower and S.L. Messener, *Pharm. Res.*, 5 (1988) 664.
[7] R.D. Rocklin and C.A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577.
[8] T. Soga, Y. Inoue and K. Yamaguchi, *J. Chromatogr.*, 625 (1992) 151.