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DERIVATIZATION OF SECONDARY AMINES WITH 2-NAPHTHALENESULFONYL CHLORIDE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SPECTINOMYCIN

KIYOSHI TSUJI* and KEVIN M. JENKINS
Control Analytical Research and Development, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)
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SUMMARY

A normal-phase high-performance liquid chromatographic (HPLC) method has been developed for the assay of spectinomycin hydrochloride and spectinomycin sulfate for detection at 254 nm. The method involves pre-column derivatization of secondary amines of spectinomycin with 2-naphthalenesulfonyl chloride (NSCl) using a catalyst. Lincomycin, 1-methylpyrrole, 2-acetyl-1-methylpyrrole, and 2-acetylpyrrole act as catalysts for sulfonylation of spectinomycin. Without a catalyst, the derivatization reaction forms a considerable amount of actinospectinoic acid, a degradation compound of spectinomycin, and peak area:weight ratio of the derivative is approximately 15% lower than those with the catalyst. Following derivatization the sample is extracted and chromatographed on a normal-phase silica column with detection at 254 nm. The method is applicable for the analysis of both the hydrochloride and sulfate salt forms of spectinomycin. All the known degradation compounds of spectinomycin such as actinamine, actinospectinoic acid and the biosynthesis intermediates, dihydrospectinomycin diastereoisomers, are completely separated with this method. Mass spectrometric data confirms that spectinomycin is derivatized with NSCl at the secondary amines located at positions 6 and 8 of the ring structure.

The standard curves for the HPLC assay of spectinomycin hydrochloride and sulfate are linear with correlation coefficients of 0.9997 and 0.9999, respectively over the range of 0.05 mg/ml to 0.3 mg/ml. The relative standard deviations (R.S.D.) of the HPLC assay methods for spectinomycin hydrochloride and sulfate are 0.67% and 0.86%, respectively. Spectinomycin hydrochloride and sulfate bulk drugs were assayed by the HPLC method and compared to gas–liquid chromatography and microbiological assay results. The HPLC method was used to assay spectinomycin in a veterinary formulation, Linco-Spectin® soluble powder.

The sensitivity of the HPLC assay was determined to be approximately 4 ng sample load on the column, which suggests applicability in serum and residue level studies.

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INTRODUCTION

Spectinomycin is a broad spectrum aminocyclitol antibiotic produced by *Streptomyces spectabilis* and is marketed in either hydrochloride or sulfate salt forms for the treatment of penicillin-resistant gonorrhea, and for veterinary applications as an antibacterial and antimycoplasmic agent. The antibiotic is polyfunctional with two secondary amines, three hydroxyls and a carbonyl group (Fig. 1). Spectinomycin is quite labile with the most unstable feature of the tri-ring structure being the alpha-keto-hemiketal at the 4a position. Therefore, spectinomycin is stable only within a narrow pH range. Spectinomycin and its major degradation products, actinospectinoic acid and actinamine and the biosynthesis intermediates, dihydrospectinomycin diastereoisomers, lack any UV absorbing chromophores. These properties pose difficulties in the development of an assay for the antibiotic.

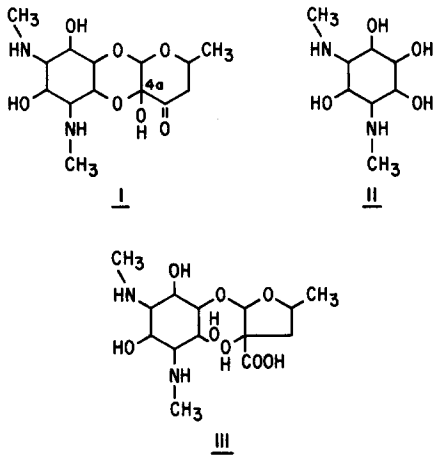

Fig. 1. Structure of spectinomycin (I), actinamine (II), and actinospectinoic acid (III).

Microbiological, gas–liquid chromatographic (GLC) and high-performance liquid chromatographic (HPLC) assay methods have been reported for determination of spectinomycin. Two different strains of *Escherichia coli* (ATCC 10536 and ATCC 29998) are being used for the turbidimetric assay of spectinomycin hydrochloride and sulfate salts, respectively[1]. The microbiological assay methods are also used for the determination of spectinomycin in the veterinary Linco-Spectin product line using a lincomycin resistant strain, ATCC 29998. The microbiological method is time-consuming, lacks precision and specificity and is subject to interference by a variety of factors.

The GLC method involves derivatization of spectinomycin with hexamethyldisilane (HMDS)[2]. Because of the polyfunctional nature of spectinomycin, careful standardization of silylation conditions are required to form quantitatively the single tetrakis-TMS-spectinomycin. The method is plagued by a decrease in the response of TMS-spectinomycin during the chromatographic run due to instability of the compound. Thus, frequent bracketing with standards is required. Degradation com-

pounds of spectinomycin, actinamine and actinospectinoic acid, usually produce two peaks due to incomplete silylation. A single peak may be produced from actinamine if the silylation time is extended; however, under such conditions, actinospectinoic acid is unstable.

Several HPLC methods for the assay of spectinomycin have been reported; the lack of UV absorbing chromophores necessitates either derivatization[3,4] or end adsorption mode[5] of detection. Myers and Rindler[3] developed an HPLC method for monitoring fermentation suspension which uses reverse phase ion-pairing chromatography followed by post-column oxidative degradation and derivatization for fluorometric detection. The post-column derivatization HPLC method suffers from instrumental down time due to the complexity of the system. An HPLC method using pre-column derivatization has been developed by Kane[4]. This method uses derivatization with thiosemicarbazide for detection at 278 nm. Following derivatization, the sample is chromatographed using a reversed-phase column with an ion-pairing technique. Although this method produces results which are comparable with GLC and microbiological methods, the derivatization procedure requires placing the samples in an oil bath at 85°C for 4.5 h. The extreme length of derivatization time makes this assay unsuitable for use in quality control laboratories. Also spectinomycin degradation products would not be derivatized for detection by this method. A polar bonded phase HPLC assay for a spectinomycin has been reported by Ledden *et al.*[5]. The method uses a LiChrosorb Amino Spheri-5 column with end-absorption detection at 214 nm. This assay method uses an acetonitrile–phosphate buffer mobile phase with chromatography at 45–50°C to reduce significant peak tailing. The peak resolution of the assay is extemely sensitive to acetonitrile concentration. Chromatographic detection of the degradation product, actinamine, cannot be achieved due to the very low absorptivity of actinamine at 214 nm.

Based on the problems associated with the available methods for the assay of spectinomycin, it is desirable to develop an HPLC assay method with short derivatization time, good stability of the derivative, ease of derivatization with high sensitivity for applications in trace impurity monitoring and detection of minor degradation products. The HPLC method reported in this paper meets the above requirements by forming a derivative using 2-naphthalenesulfonyl chloride (NSCl), followed by normal-phase chromatography and sensitive detection at 254 nm.

EXPERIMENTAL

Instruments

A Perkin-Elmer series 4 solvent delivery system (Perkin-Elmer, Norwalk, CT, U.S.A.), a Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), or an LDC minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to pump the mobile phase at a flow-rate of about 1 ml/min. Analysis was performed using a 5-μm irregular-particle silica column (LiChrosorb SI-100 or SI-60, 250 × 4.6 mm I.D.) at ambient temperature. A 40-μl sample of derivatized spectinomycin was injected quantitatively onto a column using a Waters WISP 710B injector. The column effluent was monitored at 254 nm using either a LDC spectromonitor D variable wavelength detector with an attenuation setting of 0.1 absorbance units full scale (a.u.f.s.) or with a LDC UV III monitor fixed wavelength detector with an atten-

uation setting of 0.064 a.u.f.s. Quantitation of peak area responses was performed by the VAX computer system (Digital Equipment Corp., Maynard, MA, U.S.A.), a Hewlett-Packard 3390A integrator (Hewlett-Packard, San Diego, CA, U.S.A.), and/or a Shimadzu Chromatopac E1A integrator (Shimadzu, Kyoto, Japan). A Cahn electronic balance (Model 21, Cahn, Division of Ventron Corporation, Cerritos, CA, U.S.A.) was used to weigh spectinomycin powders.

Reagents

Butyl chloride, acetonitrile, ethyl acetate, isopropanol and tetrahydrofuran were all UV grade, distilled in glass, obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetic acid was analytical reagent grade (Mallinckrodt, St. Louis, MO, U.S.A.) and 2-naphthalenesulfonyl chloride (NSCl) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Analytical reagent grade sodium bicarbonate (Mallinckrodt) was used to prepare buffer solutions. Actinospectinoic acid, actinamine and dihydrospectinomycin diastereoisomers were obtained from the Upjohn company, Kalamazoo, MI, U.S.A.

The derivatization solution was prepared by dissolving NSCl in acetonitrile to obtain a concentration of 10 mg/ml. An internal standard, methylprednisolone acetate, at 0.2 mg/ml may then be added to the derivatization reagent. The reagent was prepared fresh daily prior to derivatization. The buffer solution was prepared by weighing 4.2 g of sodium bicarbonate in 1 l or double distilled water to obtain a 0.05 *M* bicarbonate solution with a pH of approximately 8.0. A catalyst, either lincomycin hydrochloride at a concentration of 150 mg/ml or 1-methylpyrrole at 100 μl/l was then added to the buffer solution (buffer B).

The mobile phase composed of butyl chloride (50% water saturated)–tetrahydrofurane–ethyl acetate–isopropanol–acetic acid (860:37:30:25:50) was used to assay spectinomycin. For the impurity assay, a 0.2 convex gradient curve available on the Perkin-Elmer Series 4 solvent delivery system was used to deliver mobile phase A to B in 20 min. Mobile phase A is composed of chloroform–acetonitrile–acetic acid (95:4.4:0.6) and mobile phase B is composed of chloroform–acetonitrile–acetic acid (65:30:5).

Reference standard and sample preparations

Spectinomycin reference standard solution. Approximately 25 mg of spectinomycin dihydrochloride pentahydrate reference standard or 30 mg of spectinomycin sulfate tetrahydrate reference standard were accurately weighed "as is" into a 100-ml volumetric flask. The buffer solution (buffer B) was then added to volume.

Spectinomycin hydrochloride and sulfate samples. Approximately 25 mg of spectinomycin hydrochloride or 30 mg of spectinomycin sulfate samples were accurately weighed "as is" into a 100-ml volumetric flask. The buffer solution (buffer B) was then added to volume.

Linco-Spectin soluble powder samples. Approximately 1.05 g of Linco-Spectin soluble powder were accurately weighed "as is" into a 250-ml volumetric flask. The buffer solution (buffer B) was added to volume. A 10.0-ml volume of the solution was pipetted into a 100-ml volumetric flask and diluted to volume with the buffer solution.

Derivatization

Volumes of 10 ml each of the spectinomycin reference standard solution or sample solutions were quantitatively transferred into a 50-ml volumetric flask. A 10-ml quantity of the derivatization solution was added to the flask and the flask was tightly stoppered to avoid any sample loss during derivatization. The flask was shaken and placed in a 100°C silicone oil bath for 10 min to form the derivative. The amount of NSCl represents more than 50 fold in excess of the amount required for derivatization. The flask was then cooled to room temperature and the mobile phase solution containing approximately 0.03 mg/ml methylprednisolone acetate (as internal standard) was added to volume. The internal standard may be added to the derivatization solution for ease of operation. The flask was shaken vigorously for 10 min. The contents were then transferred to a 50-ml centrifuge tube and centrifuged at low speed (< 300 *g*) for 3–5 min to obtain a clear upper organic layer. Portions of the organic layer are then chromatographed.

RESULTS AND DISCUSSION

Derivatization of spectinomycin

Both the lack of UV absorbing chromophores and the instability of spectinomycin in solution presented problems. The tricyclic ring structure of spectinomycin has three major classes of functional groups: secondary amines, hydroxyl and carbonyl groups (Fig. 1). The derivatization reagents evaluated for reaction with these groups were NBD chloride (4-chloro-7-nitrobenzo-2,1,3-oxadiazole)[6], MDPF [1-methoxy-2,4-diphenyl-3(2H)-furanone][7], dansyl chloride, fluorescamine[8], and 2-diphenylacetyl-1,3-indandione-1-hydrazone[9]. Use of these reagents followed conditions described in the literature with some modification to accommodate the structure of spectinomycin. Unfortunately, these reagents were unsuccessful in forming a detectable derivative of spectinomycin.

Derivatization of spectinomycin with 2-naphthalenesulfonyl chloride (NSCl) was then attempted. NSCl reacts by nucleophilic substitution with hydroxyl or amine groups to form esters or amides, respectively. There are three hydroxyl groups and two secondary amines on the spectinomycin molecule. Samples of 2 ml of spectinomycin hydrochloride (0.5 mg/ml) dissolved in various buffers, such as bicarbonate, phosphate, borate and Tris buffers, were reacted with 3 ml of NSCl reagent (10 mg/ml in acetonitrile) by heating at 50°C for 30 min. After cooling, 3 ml of methylene chloride was added. Following vigorous shaking and centrifugation the lower organic layers were spotted on silica thin-layer chromatographic plates. The plates were developed using butyl chloride–tetrahydrofuran–methanol–acetic acid (950:70:35:30). After development the plates were examined under UV light.

The best evidence for the formation of a spectinomycin derivative was with the use of either a 0.05 *M* bicarbonate or 0.1 *M* phosphate buffer. These derivatized samples were subsequently chromatographed on a silica column. A large peak eluted which was not observed in the reagent blank. Since the peak area of spectinomycin derivatized in phosphate buffer was far smaller than that of the bicarbonate buffer, the sodium bicarbonate buffer (0.05 *M*) was utilized for further study.

Optimization of chromatographic conditions

Initially, reversed-phase columns, *e.g.* LiChrosorb C_{18}, C_8, C_4, C_2 were used in an attempt to chromatograph spectinomycin; however, the efforts were abandoned due to interference of the unreacted derivatization reagent and inadequate peak resolution. Further attempts to chromatograph spectinomycin utilized the LiChrosorb 5-μm silica column Si-100 (Brownlee Labs, Santa Clara, CA, U.S.A.) or Si-60 (E. Merck, Darmstadt, F.R.G.) with a mobile phase containing chloroform, acetonitrile and acetic acid. Although a satisfactory chromatogram was obtained by use of this mobile phase, the mobile phase system was not pursued further due to instability in the base-line and short column life probably caused by impurities present in the solvent used. A mobile phase of butyl chloride (50% water saturated)–tetrahydrofuran–methanol–acetic acid (850:70:35:30) was successfully selected. Further modification of the mobile phase composition was necessary to optimize the chromatographic condition due to extensive tailing of the major spectinomycin peak.

Mobile phase optimization. Due to its ruggedness and availability, the LiChrosorb column was used to develop the HPLC assay for spectinomycin. Optimization of the mobile phase for a LiChrosorb column was achieved using a Perkin-Elmer Series 4 solvent delivery system. The mobile phase components studied were butyl chloride, tetrahydrofuran, acetic acid, isopropanol and ethyl acetate. Butyl chloride (50% water saturated) and tetrahydrofuran were kept at a constant ratio of 23:1 throughout the study to allow observation on the effect of the modifiers (acetic acid, isopropanol, ethyl acetate). The retention time, theoretical plates and tailing factor for the spectinomycin peak were calculated to examine the effect of each modifier.

The results, summarized in Fig. 2, demonstrate that both isopropanol and acetic acid changed retention behaviour while ethyl acetate was less effective. Both isopropanol and acetic acid caused substantial increases in theoretical plates as their percentage compositions were increased. Isopropanol concentration was judged to be critical to yield high theoretical plates, while ethyl acetate had a negligible effect. The modifier most effective in reducing peak tailing was ethyl acetate, while isopropanol slightly increased the peak tailing (Fig. 3). Acetic acid had virtually no effect on peak tailing. Based on the above information, the optimum mobile phase composition was determined to be butyl chloride (50% water saturated)–acetic acid–tetrahydrofuran–ethyl acetate–isopropanol (860:50:37:30:25), for chromatography of spectinomycin.

Derivatization

pH effect. To establish the optimum pH for derivatization of spectinomycin, spectinomycin samples were dissolved in bicarbonate buffer at pH 7.0, 8.0, 9.0 and 10.0, and were derivatized. No derivative was formed at pH 7.0 but derivatives were formed in all other pH conditions. Due to the instability of spectinomycin in solutions above pH 8.5, pH 8.0 buffer was used for further assay development. No significant increase in the rate of derivatization was observed when pH 9.0 or pH 10.0 buffers were used.

Reagent ratio. The derivatization reagent solution prepared contained 10 mg of NSCl per ml or 44 m*M* in acetonitrile. Spectinomycin hydrochloride concentration was 0.25 mg/ml or 0.75 m*M*. The excess reagent is typical for initial screening of the derivatization procedures. The sample-reagent ratios (mole:mole) of 1:10, 1:20, 1:30,

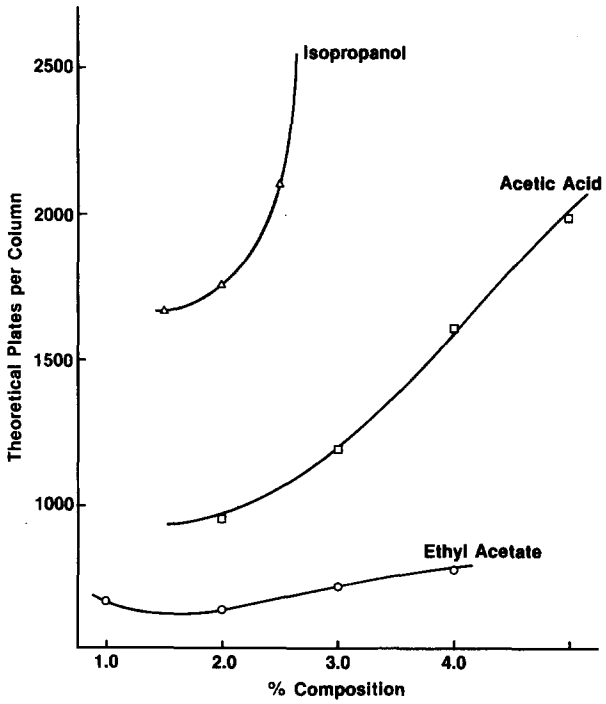

Fig. 2. Theoretical plates as influenced by mobile phase composition.

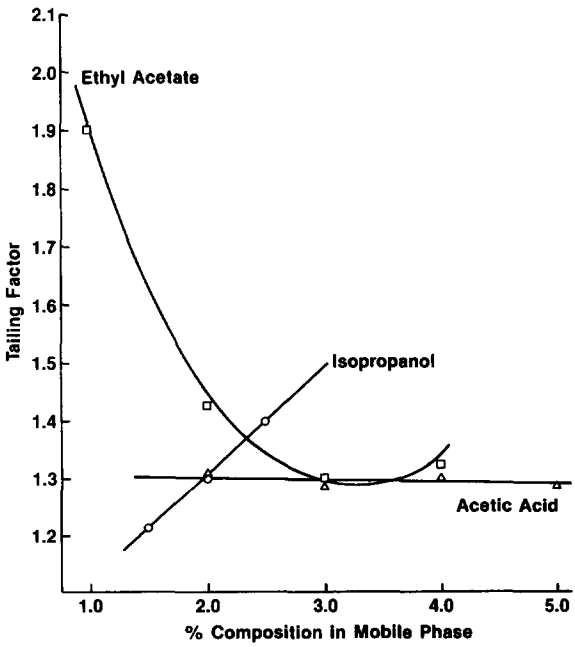

Fig. 3. Effect of mobile phase composition on peak tailing factor.

1:40, 1:50 and 1:100 were then examined in order to optimize the sample–reagent ratio. Spectinomycin peak area increased to the reagent ratio of 1:30 and plateaued thereafter to 1:100. Increasing the reagent ratio beyond 1:30 did not affect the chromatographic performance.

Derivatization temperature and time. The major difficulty encountered with the development of an assay for spectinomycin was to optimize the derivatization conditions to achieve complete derivatization and yet to reduce the formation of actinospectinoic acid and actinamine, two degradation compounds of spectinomycin. During the development of the assay method, inclusion of 1 mole of lincomycin to 1 mole of spectinomycin during derivatization was found to increase the spectinomycin peak area while minimizing the formation of actinospectinoic acid. The relative standard deviation (R.S.D.) was also reduced to less than 1% (Tables I and II). To study the effect of lincomycin, bicarbonate buffer solution containing various quan-

TABLE I

PRECISION OF THE SPECTINOMYCIN HYDROCHLORIDE HPLC ASSAY METHOD USING LINCOMYCIN AS A CATALYST FOR DERIVATIZATION

Sample No.	*Weight (mg)*	*Peak area*		*Area: weight ratio*
		Spectinomycin	*Internal std.*	
1	24.96	7 008 594	6 093 052	0.04608
2	25.16	7 101 322	6 102 122	0.04625
3	25.34	7 130 314	6 099 957	0.04613
4	24.84	7 013 729	6 117 898	0.04615
5	24.99	7 134 896	6 109 671	0.04673
6	25.34	7 124 575	6 112 901	0.04599
7	25.37	7 106 045	6 127 402	0.04571
			Average	0.04615
			Relative S.D.	0.67%

TABLE II

PRECISION OF SPECTINOMYCIN SULFATE HPLC ASSAY METHOD USING LINCOMYCIN AS A CATALYST FOR DERIVATIZATION

Sample No.	*Weight (mg)*	*Peak area*		*Area: weight ratio*
		Spectinomycin	*Internal std.*	
1	30.09	8 006 931	6 255 806	0.04254
2	30.10	8 118 949	6 280 293	0.04295
3	30.23	8 131 563	6 260 424	0.04297
4	30.09	7 981 918	6 263 523	0.04235
5	30.19	8 065 522	6 290 699	0.04247
6	30.30	8 270 463	6 285 377	0.04343
7	30.19	8 030 751	6 251 506	0.04255
			Average	0.04275
			Relative S.D.	0.89%

tities of lincomycin hydrochloride (0, 0.1, 0.2, 0.3 and 0.5 mg/ml) were prepared and used to derivatize spectinomycin. Data indicated that the spectinomycin peak area: weight ratio increased from 0 to 0.1 mg lincomycin/ml and plateaued thereafter to 0.50 mg/ml. The 0.15 mg/ml concentration of lincomycin, corresponding to a mole ratio of approximately 0.5:1 of spectinomycin, was chosen for further study.

A reaction time–temperature study was performed to determine the optimum derivatization condition. Samples of spectinomycin were dissolved in the buffer solution containing approximately 0.15 mg/ml lincomycin hydrochloride. Derivatization reactions were carried out at 23, 40, 60, 80, and 100°C for 0.5, 1.5, 3, 5, 6, 10, 15, 20, 30, 45 and 60 min at each of the five temperatures. The results, summarized in Fig. 4, indicated that the peak area:weight ratio for derivatized spectinomycin was maximum when derivatized at 100°C and that the reaction plateaued from 5 to 30 min. Thus, the derivatization temperature and time of 100°C and 10 min were chosen for further study.

Catalytic action of lincomycin. The formation of actinospectinoic acid during derivatization without lincomycin was concluded to be the result of a dramatic pH shift occurring during derivatization (Fig. 4). Bicarbonate buffer loses its buffering capacity within 2–6 min of heating at 100°C, resulting in a rapid pH shift to the acidic range. This drop in pH of the reaction solution causes degradation of spectinomycin to form actinospectinoic acid. As shown in Fig. 4, pH measurements taken during the derivatization process demonstrated that lincomycin has no effect on the buffering capacity of the reactant solution. It was therefore postulated that lincomycin may be acting as a catalyst to increase the reaction rate, thereby derivatizing all of the spectinomycin in solution before the pH shifts. Efforts to stabilize pH of the reactant solution by use of varieties of buffers while maintaining the maximum peak-area were unsuccessful.

In an attempt to elucidate the possible catalytic effect of the amino moiety of lincomycin, the compounds, 2-acetyl-1-methylpyrrole, 2-acetylpyrrole, N-acetyl-L-

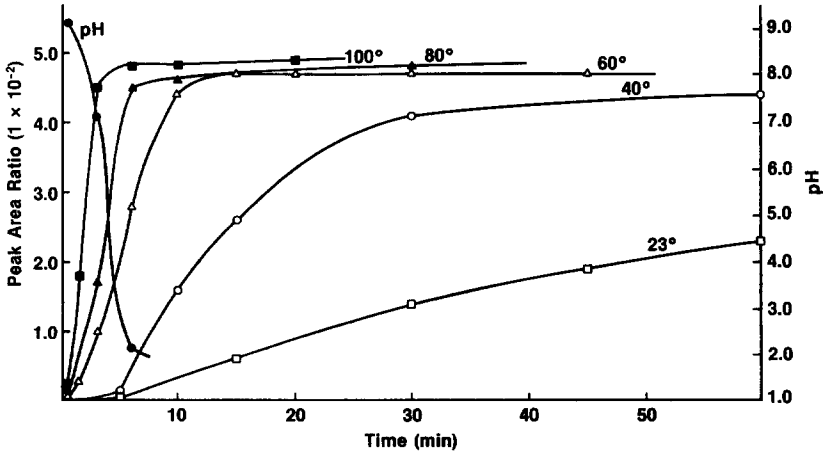

Fig. 4. Derivatization time–temperature and pH change during formation of naphthalenesulfonyl spectinomycin.

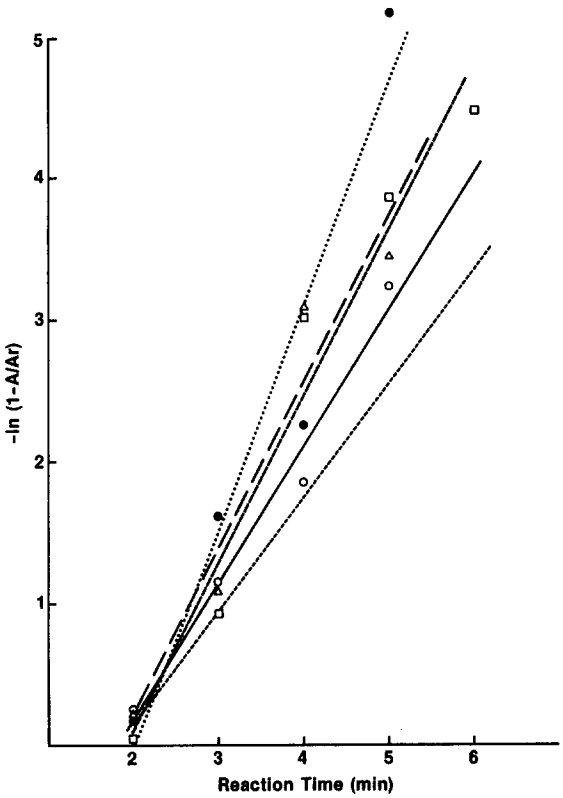

Fig. 5. Effect of catalyst on sulfonylation of spectinomycin when reacted at 100°C. ●···●, 1-methylpyrrole; □——□, 2-acetylpyrrole; △---△, 2-acetyl-1-methylpyrrole; ○——○, lincomycin; ----, bicarbonate buffer alone.

proline, 1-methylpyrrole and 1-methylpyrrolidine, representing a portion of the lincomycin structure, 2-acetylpyridine and 4-(dimethylamino)pyridine, were employed as catalysts in the sulfonylation reaction. The catalytic action of 4-(dimethylamino)-pyridine[10,11], and imidazole[12] on acylation reactions are well documented. These chemicals were dissolved in the bicarbonate buffer at a 1:1 mole ratio with spectinomycin and the rate of formation of the derivative was compared to that of bicarbonate buffer with and without lincomycin. Spectinomycin samples were dissolved in each separate buffer–reagent solution and the aliquots derivatized at 100°C for 2, 3, 4, 5 and 6 min. The peak-area:weight ratio was calculated and $-\ln(1 - A/Ar)$ (where A is the observed peak-area:weight and Ar is the maximum peak area:weight) was plotted *versus* the derivatization time (Fig. 5). The rate of reaction indicted that there was more than a 2-fold increase in the rate of derivatization in the presence of lincomycin (1.66 *vs.* 0.76 for with and without lincomycin, respectively) and that the rates of reaction with 2-acetyl-1-methylpyrrole, 2-acetylpyrrole, and 1-methylpyrrole were superior to that of lincomycin. N-Acetyl-L-proline, 2-acetylpyridine, 4-(dimethylamino)pyridine and 1-methylpyrrolidine were ineffective. Ineffectiveness of the latter two compounds may be due to their high pKa values [pK_a of 10.3 and 10.1 for 1-methylpyrrolidine and 4-(dimethylamino)pyridine, respectively]. These two compounds would not be protonated in the reaction solution (pH 8.0).

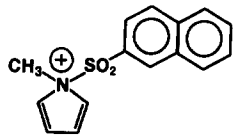

Fig. 6. Postulated structure of activated sulfonyl–ammonium complex of 1-methylpyrrole.

The data obtained above indicates that the catalytic action of lincomycin in sulfonylation of spectinomycin resides at the pyrrolidinyl moiety. Based on this data it is postulated that the catalytic action may be due to formation of an activated sulfonyl–ammonium complex (fig. 6) analogous to the well known acyl–ammonium complexes[10–12].

Extraction of derivative. Since normal-phase HPLC is selected to chromatograph spectinomycin it is necessary to extract the derivative into an organic layer following derivatization. Methylene chloride was initially used for this extraction but the solution formed an emulsion which was difficult to break even by centrifugation. Extraction with the mobile phase yielded the best recovery and improved the chromatography.

Identification of naphthalenesulfonyl derivatives. Column fractions corresponding to spectinomycin, actinamine, and actinospectinoic acid were collected and analyzed by mass spectrometry. Examination of the electron impact mass spectrum of derivatized spectinomycin indicated the presence of a relatively strong molecular ion peak at *m/e* 713 ($M^+ + 1$) indicating the formation of an intact disubstituted naphthalenesulfonyl-spectinomycin molecule. The presence of a peak at *m/e* 523 (M^+) indicated a monosubstituted form which may have formed during ionization. Mass spectrometric analysis of the actinamine fraction indicated a strong molecular ion peak at *m/e* 587 ($M^+ + 1$) indicating an intact disubstituted derivative of actinamine molecule.

Stability of spectinomycin in buffer solution

In order to make the assay rugged for quality control laboratory use, it was necessary to determine the stability of spectinomycin when dissolved in the pH 8.0 bicarbonate buffer. Spectinomycin hydrochloride and sulfate samples were dissolved in pH 8.0 buffer and left to stand for 5 min, 1 h, 2 h, 3 h, 5 h, 7 h, 24 h and 48 h at room temperature prior to derivatization and chromatography. The results showed that both spectinomycin hydrochloride and sulfate are stable for nearly 7 h and that a significant drop in spectinomycin peak-area:weight ratio occurred after 24 h at room temperature.

Internal standard

Although both hydrocortisone acetate and prednisolone acetate may be acceptable as internal standards, methylprednisolone acetate was found to be superior since it elutes earlier than the other two steroids without interfering with the chromatography of spectinomycin, its impurities, or its degradation compounds (Fig. 7). The negative peak found just after the major, solvent front peak is due to the acetonitrile used to dissolve the derivatization reagent. The internal standard (0.2 mg/ml) may be included in the derivatization reagent for ease of operation. A typical chro-

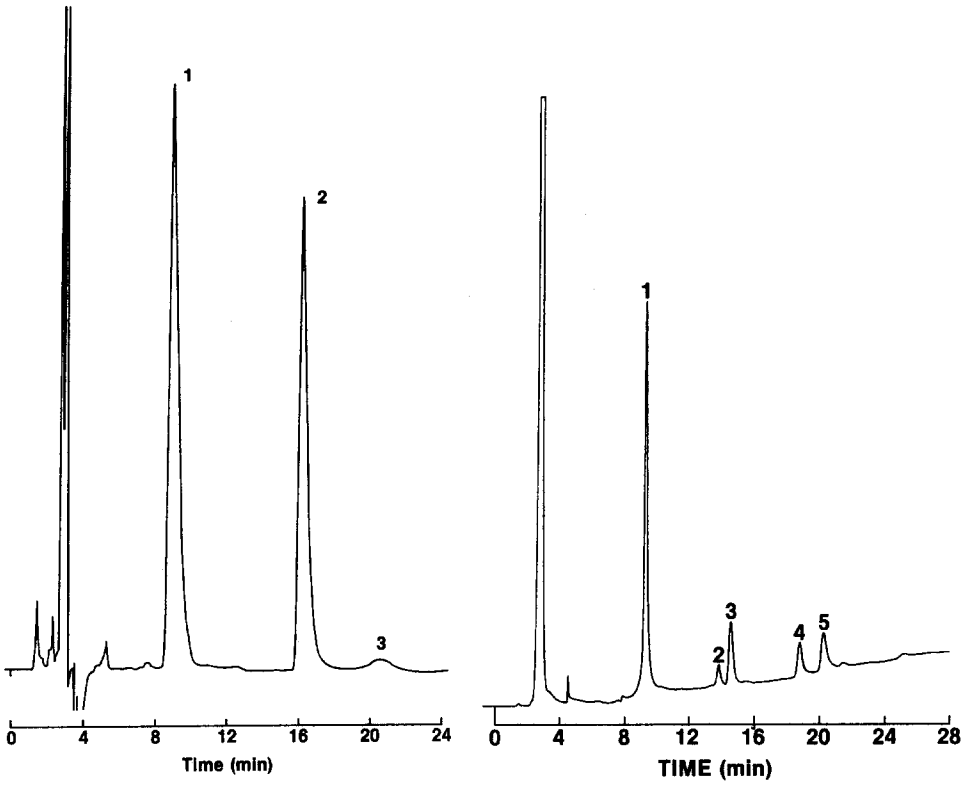

Fig. 7. Typical HPLC chromatogram of spectinomycin. Peak identification: 1 = spectinomycin, 2 = internal standard, 3 = actinospectinoic acid. Mobile phase, butyl chloride (50% water saturated)–tetrahydrofurane–ethyl acetate–isopropanol–acetic acid (860:37:30:25:50). Column: LiChrosorb Si-60, 5 μm, 250 × 4.0 mm.

Fig. 8. HPLC chromatogram indicating separation of spectinomycin, its impurities, and degradation compounds. Peak identification: 1 = spectinomycin, 2 = actinospectinoic acid, 3 = 4(*S*)-dihydrospectinomycin, 4 = actinamine, and 5 = 4(*R*)-dihydrospectinomycin. Mobile phases A to B in 20 min using a 0.2 convex gradient curve available on the Perkin-Elmer Series 4 solvent delivery system. Mobile phase A, chloroform–acetonitrile–acetic acid (95:4.4:0.6), mobile phase B, chloroform–acetonitrile–acetic acid (65:30:5). Column: LiChrosorb Si-60, 5 μm, 250 × 4.0 mm.

matogram of spectinomycin hydrochloride with the internal standard methylprednisolone acetate is shown in Fig. 7. Retention times for the spectinomycin and the internal standard were approximately 10 and 18 min, respectively.

Chromatography of impurities and degradation products

A chromatogram indicating separation of spectinomycin, its impurities and degradation compounds is shown in Fig. 8. Small impurity peaks eluted at 22 and 26 min are associated with a 4(*S*)-dihydrospectinomycin sample used. Impurity peaks which eluted at 24 and 27 min are associated with a 4(*R*)-dihydrospectinomycin sample used.

The two degradation products of spectinomycin, actinospectinoic acid and actinamine, were derivatized and chromatographed. Actinospectinoic acid is formed

from spectinomycin under basic pH conditions and actinamine is formed under acidic conditions. Standard curves of actinamine and actinospectinoic acid were constructed using the concentration which corresponded to 0 to 5% (0, 0.1, 0.2, 0.4, 0.7, 1.3, 2.5, and 5%) of the spectinomycin peak area at midpoint. The curves were linear with the correlation coefficient of 0.9992 and 0.9998 for actinamine and actinospectinoic acid, respectively. The precision of the impurity assay was determined by analyzing seven samples of spectinomycin spiked with actinospectinoic acid and actinamine at 2.5% and 5%, respectively of spectinomycin. An impurity peak which eluted immediately after the actinamine peak is associated with an actinamine sample used. The R.S.D of the HPLC impurity assay was 17.2% and 14.3% for actinospectinoic acid and actinamine, respectively.

Assay sensitivity

Sensitivity of the assay was determined by preparing a solution containing 504 ng spectinomycin/ml. A 10.0 ml aliquot of the diluted solution was derivatized with 10.0 ml of reagent and then extracted in 5 ml of butyl chloride. Due to partitioning of acetonitrile between butyl chloride and the aqueous buffer, the actual organic extraction volume was 12 ml, yielding a final concentration of the spectinomycin of 420 ng/ml. This extracted solution was then diluted 1 to 5 with butyl chloride and centrifuged to obtain a clear solution with a concentration of 84 ng spectinomycin/ml. A 50-μl injection of this final solution was chromatographically monitored at 0.002 and 0.001 absorbance units full scale (a.u.f.s.). The height of the spectinomycin peak was approximately four and six times the baseline noise, respectively. The amount of spectinomycin sample injected on the column was by weight 4.2 ng. This level of sensitivity suggests applicability of the HPLC method for serum and residue level studies.

Linearity and precision

The linearity of the HPLC assay was determined by analysis of six levels of spectinomycin hydrochloride and sulfate ranging from 20% to 200% of the proposed mid-point of the standard curve (25 mg). The plot of area ratio to concentration was linear with a correlation coefficient of 0.9997 and 0.9999 for hydrochloride and sulfate, respectively and the intercept was not significantly different from zero.

The precision of the HPLC assay was determined by the use of seven individually weighed and derivatized spectinomycin hydrochloride and sulfate samples. The relative standard deviation of the assay for the determination of spectinomycin hydrochloride and sulfate were 0.67% and 0.86%, respectively (Tables I and II).

Assay of bulk drugs

Eight lots of spectinomycin hydrochloride were assayed and compared to the microbiological and GLC results (Table III). The average HPLC result was 660 μg/mg while the average GLC and microbiological results were 652 and 645 μg/mg, respectively. A paired t-test at the 96% confidence level supported the hypothesis that there was no significant difference between the HPLC and GLC assay methods. However, statistical analysis of the HPLC and microbiological data indicated that the 2% difference between the methods was statistically significant. This finding was considered to be not of concern since the microbiological data were based on a single assay point.

TABLE III

ASSAY OF SPECTINOMYCIN HYDROCHLORIDE BULK DRUG (μg/mg). COMPARISON OF HPLC, GLC AND MICROBIOLOGICAL ASSAY METHOD

Lot. No.	*HPLC*			*GLC*	*Microbiol.*
	Day 1	*Day 2*	*Av.*		
1	654	657	655.5	650	649
2	656	648	652.0	664	659
3	665	661	663.0	654	650
4	670	667	668.5	644	640
5	670	660	665.0	656	648
6	660	644	652.0	652	623
7	675	662	668.5	648	650
8	656	654	655.0	650	642
		Average	659.9	652.3	645.1
		Relative S.D.	1.0%	0.9%	1.5%

Nine lots of spectinomycin sulfate were assayed and the results were compared to microbiological results (Table IV). The average HPLC result was 641 μg/mg while the average microbiological result was 655 μg/mg. The results by HPLC were approximately 2% lower than the results by the microbiological method. A paired *t*-test supported the hypothesis that there was significant difference between the two assay methods. Since the HPLC data were 2% higher for spectinomycin hydrochloride and yet 2% lower for spectinomycin sulfate when compared to the microbiological assay data, these differences were considered acceptable in view of uncertainty and variability associated with the microbiological assay method.

Assay of Linco-Spectin soluble powder

Six lots of Linco-Spectin soluble powder were assayed by the HPLC method

TABLE IV

ASSAY OF SPECTINOMYCIN SULFATE BULK DRUG (μg/mg). COMPARISON BETWEEN HPLC AND MICROBIOLOGICAL ASSAY METHOD

Lot No.	*HPLC*				*Microbiological*			
	Day 1	*Day 2*	*Day 3*	*Av.*	*Day 1*	*Day 2*	*Day 3*	*Av.*
A	646	653	–	650	658	652	664	658
B	635	638	647	640	659	634	671	655
C	636	664	645	648	668	667	653	663
D	643	647	–	645	671	667	651	663
E	613	626	624	621	643	645	633	640
F	618	640	–	629	656	654	632	647
G	648	662	650	653	650	640	668	653
H	–	643	650	647	666	665	681	671
I	624	636	635	632	645	637	651	644
			Average	641				655
			Relative S.D.	1.7%				1.5%

TABLE V

THREE-WAY CROSS-OVER STUDY FOR THE ASSAY OF SPECTINOMYCIN (mg/g) IN LINCO-SPECTIN

Lot No.	*HPLC*		*Microbiological turbidimetric assay*
	NSCl at 254 nm	*End-absorption at 214 nm*	
1	444	449	471
2	444	467	474
3	448	438	465
4	452	447	476
5	447	438	478
6	448	445	472
Average	447	447	473

TABLE VI

RECOVERY OF SPECTINOMYCIN WHEN SPIKED IN PLACEBO OF LINCO-SPECTIN SOLUBLE POWDER

Amount added (mg)	*Amount recovered (mg)*	*Recovery (%)*
9.9050	10.153	102.5
19.809	20.185	101.9
29.714	30.130	101.4
39.618	38.826	98.0
49.523	48.830	98.6
	Average	100.5

and the results were compared to those of microbiological method. The average results of the HPLC (NSCl and end-absorption[5]) and microbiological assay methods were 447, 447, and 473 mg/ml, respectively (Table V). The results demonstrated no statistically significant difference between the two HPLC methods. However, there was a significant difference between either HPLC method and microbiological method at the 95% confidence level.

Although a lincomycin resistant strain of *E. coli* (ATCC 29998) is used to assay spectinomycin in a lincomycin–spectinomycin combination product (Linco-Spectin), microbiological response is variable and is not always predictable. We have confidence in the results of the HPLC methods because of their near 100% recovery of spectinomycin when spiked in the placebo of Linco-Spectin (Table VI) and nearly identical results obtained by two distinctly different HPLC methods with two completey different sample preparation procedures.

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