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DETERMINATION OF STREPTOMYCIN SULFATE AND DIHYDROSTREPTOMYCIN SULFATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic, paired-ion reversed-phase high-performance liquid chromatographic method for the determination of streptomycin and dihydrostreptomycin has been developed. The method employs a microparticulate reversed-phase column (μBondapak C_{18}, LiChrosorb RP-18 or Ultrasphere Ion Pair), and a mobile phase composed of 0.02 *M* sodium hexanesulfonate and 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v) at pH 6.0 with detection by ultraviolet absorbance at 195 nm. Resolution and simultaneous quantitation of streptomycin A, dihydrostreptomycin A, streptomycin B, streptidine and process-related substances can be achieved in less than 25 min, with a relative standard deviation of *ca.* 1.1 % for the assay of either streptomycin sulfate or dihydrostreptomycin sulfate. The chromatographic bioequivalency data for streptomycin sulfate and dihydrostreptomycin sulfate were statistically identical with results obtained by the officially recognized microbiological assay. The method is designed for applicability to the analysis of other aminoglycoside antibiotics and antituberculous agents.

INTRODUCTION

Streptomycin and dihydrostreptomycin are clinically useful aminoglycoside antibiotics (Fig. 1) exhibiting comparable antimicrobial activity against a wide range of gram-negative and gram-positive bacteria, as well as mycobacteria, particularly Mycobacterium tuberculosis[1–4]. Streptomycin is produced by the microbial fermentation of *Streptomyces griseus* and was first isolated by Schatz *et al.*[5] in 1944. Dihydrostreptomycin is produced by the catalytic hydrogenation of streptomycin[6]. Numerous chemical and physical methods have been reported for the analysis of streptomycin and dihydrostreptomycin, including paper, thin-layer, and column chromatography, electrophoresis, spectrophotometry and colorimetry, fluorimetry, titrimetry, and polarography[7]. The official methods of analysis for streptomycin sulfate and dihydrostreptomycin sulfate are the USP and EP microbiological turbidimetric procedures and the EP colorimetric method[8,9]. However, none of these methods possesses a desirable combination of speed, specificity, simplicity, sensitivity, and

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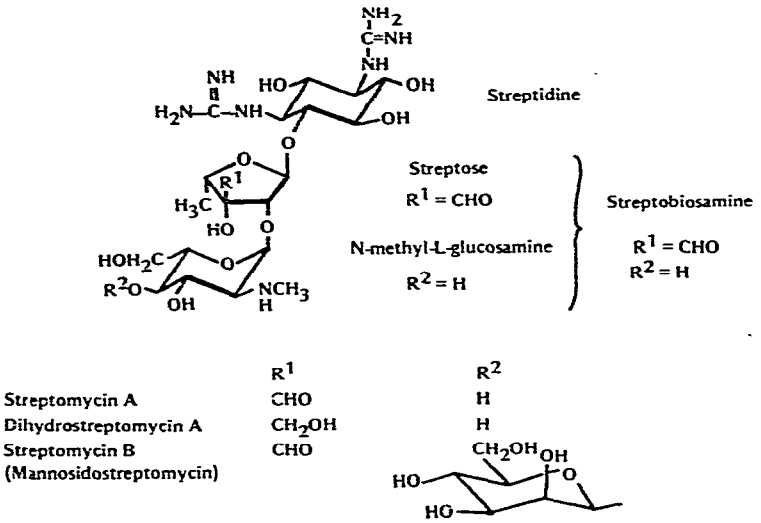

	R^1	R^2
Streptomycin A	CHO	H
Dihydrostreptomycin A	CH_2OH	H
Streptomycin B (Mannosidostreptomycin)	CHO	

Fig. 1. Structures of streptomycin and process-related substances.

precision. This paper reports the development of a high-performance liquid chromatographic (HPLC) method for separating and quantitating streptomycin A, dihydrostreptomycin A, and their biosynthetic and process-related substances in streptomycin sulfate and in dihydrostreptomycin sulfate. The technique of paired-ion, reversed-phase chromatography is employed, in combination with ultraviolet detection for measurement of the resolved sample components. Using this HPLC methodology, composition profiles of the antibiotics are obtained. Also, the method has potential application to the analysis of the antituberculous agent viomycin sulfate, and the aminoglycoside antibiotics neomycin sulfate and paromomycin sulfate.

EXPERIMENTAL

Apparatus

A Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Valco Model CV-6-UHPa-C-20 25-μl loop injection valve (Valco Instruments Co., Houston, TX, U.S.A.) and a Waters 450 variable-wavelength detector attached to a Varian A-25 dual-channel recorder (Varian Instruments Division, Palo Alto, CA, U.S.A.) were employed. The following commercially available prepacked reversed-phase columns were used: (a) LiChrosorb RP-18, 5 μm, 25 cm × 4.6 mm I.D. (Applied Science Labs., State College, PA, U.S.A., and Altex Scientific, Berkeley, CA, U.S.A.), (b) μBondapak C_{18}, 30 cm × 3.9 mm I.D. (Waters Assoc.), (c) Ultrasphere Ion Pair, 25 cm × 4.6 mm I.D. (Altex Scientific). An in-line guard column (25 cm × 4.6 mm I.D.) packed with either Bondapak C_{18}/Corasil (Waters Assoc.) or Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was connected between the pump and the injection valve. Upon completion of daily analysis, the guard column and analytical column were both washed with a mixture of methanol–water (50:50).

Reagents

Streptomycin sulfate (lot No. 0674-G-5) and dihydrostreptomycin sulfate (lot No. H-1) reference standards were purchased from the United States Pharmacopeial

Convention Inc. (Rockville, MD, U.S.A.). Streptidine sulfate monohydrate (lot No. 3A066-EA) and maltol (lot No. 36326-62EA) were obtained from Pfizer Quality Control Division (Groton, CT, U.S.A.). The reference sample of streptomycin B sulfate was kindly provided by Dr. R. J. Taylor of Pfizer Chemicals Division (Groton, CT, U.S.A.). Dihydrostreptomycin B sulfate was prepared by the sodium borohydride reduction of streptomycin B sulfate in water[10]. Streptobiosamine and dihydrostreptobiosamine were prepared according to the literature[11,12]. Distilled water, acetonitrile, distilled in glass (Burdick & Jackson, Muskegon, MI, U.S.A.), 1-hexanesulfonic acid, sodium salt (Regis, Morton Grove, IL, U.S.A.), sodium phosphate tribasic (Mallinckrodt, Paris, KY, U.S.A.), and 85% phosphoric acid (Mallinckrodt) were used without further purification.

Mobile phase preparation

The mobile phase was prepared by dissolving 3.8 g of sodium 1-hexanesulfonate and 9.5 g of sodium phosphate tribasic in a mixture of 850 ml of distilled water and 80 ml of acetonitrile. The solution was adjusted to pH 6.0 with phosphoric acid, diluted to one liter with distilled water, and filtered through a 5-μm, Type LS, Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to use. The amount of acetonitrile was adjusted to obtain maximum performance of the column. Decreasing the amount of acetonitrile in the mobile phase increased the elution time (*i.e.* improved resolution) of streptidine, streptomycins A and B, and dihydrostreptomycin A, while increasing the amount of acetonitrile decreased the elution time of these substances.

Chromatographic conditions

The column flow-rate was maintained at *ca.* 1 ml/min. The column temperature was maintained at 25.0 ($\pm$0.1)°C. The detector wavelength was set at 195 nm using a 0.2 absorbance units range selection. Dual-channel recorder input of 2 mV and 10 mV and a chart speed of 10 in./h were employed.

Streptomycin sulfate analysis

Streptomycin sulfate standard solution. Approximately 200 mg of dry USP streptomycin sulfate reference standard was accurately weighed into a 50-ml volumetric flask. Distilled water was added to dissolve the substance, then the flask was diluted to volume with additional distilled water. A 3-ml volume of this stock solution was then diluted to 50 ml with mobile phase and analyzed.

Streptidine sulfate monohydrate standard solution. Approximately 125 mg of streptidine sulfate monohydrate reference standard was accurately weighed into a 500-ml volumetric flask and diluted to volume with water. The substance was dissolved with the aid of sonification. A 3-ml aliquot of this stock solution was diluted to 25 ml with water, after which a 2-ml aliquot was removed and diluted to 25 ml with mobile phase and analyzed.

Sample preparation. The streptomycin sulfate sample solution was prepared and analyzed in the same manner as the standard solution.

Calculations

The streptomycin sulfate sample potency and streptidine sulfate monohydrate content are calculated by using the following equations:

$$\text{streptomycin sulfate potency } (\mu g/mg) = \frac{SA_{spl}}{SA_{std}} \times \frac{conc_{std}}{conc_{spl}} \times \text{std. potency } (\mu g/mg)$$

where SA_{spl} and SA_{std} are the streptomycin A peak heights of the sample and standard, respectively; $conc_{std}$ and $conc_{spl}$ are the final concentrations (mg/ml) of the standard and sample solutions, respectively; and std. potency is the potency of streptomycin sulfate standard.

$$\text{streptidine sulfate monohydrate } (\%) = \frac{Sd_{spl}}{Sd_{std}} \times \frac{conc_{std}}{conc_{spl}} \times 100\%$$

where Sd_{spl} and Sd_{std} are the streptidine peak heights of the sample and standard, respectively, and $conc_{std}$ and $conc_{spl}$ are the final concentrations (mg/ml) of the standard and sample solutions, respectively.

Dihydrostreptomycin sulfate analysis

Dihydrostreptomycin sulfate standard solution. Approximately 200 mg of dry USP dihydrostreptomycin sulfate reference standard was accurately weighed into a 50-ml volumetric flask, distilled water was added, the substance dissolved, and the flask was diluted to volume with distilled water. A 3-ml aliquot of this stock solution was then diluted to 50 ml with mobile phase and analyzed.

Streptidine sulfate monohydrate standard solution. The streptidine sulfate monohydrate standard solution was prepared and analyzed as described under *Streptomycin sulfate analysis.*

Trace level streptomycin sulfate standard solution. A 5-ml aliquot of the streptomycin sulfate standard solution was diluted to 50 ml with water, after which a 5-ml aliquot was removed and diluted to 50 ml with mobile phase and analyzed.

Sample preparation. The dihydrostreptomycin sulfate sample solution was prepared and analyzed in the same manner as the standard solution.

Calculations

The dihydrostreptomycin sulfate sample potency, streptidine content, and streptomycin A content are calculated by using the following equations:

$$\text{dihydrostreptomycin sulfate potency } (\mu g/mg) = \frac{DHSA_{spl}}{DHSA_{std}} \times \frac{conc_{std}}{conc_{spl}} \times \text{std. potency } (\mu g/mg)$$

where $DHSA_{spl}$ and $DHSA_{std}$ are the dihydrostreptomycin A peak heights of the sample and standard, respectively, and $conc_{std}$ and $conc_{spl}$ are the final concentrations (mg/ml) of the standard and sample solutions, respectively; std. potency is the potency of dihydrostreptomycin sulfate standard.

$$\text{streptidine sulfate monohydrate } (\%) = \frac{Sd_{spl}}{Sd_{std}} \times \frac{conc_{std}}{conc_{spl}} \times 100\%$$

$$\text{streptomycin A base (\%)} = \frac{SA_{spl}}{SA_{std}} \times \frac{conc_{std}}{conc_{spl}} \times 100\%$$

RESULTS AND DISCUSSION

The goal of this investigation was to developed a rapid, sensitive and specific chemical assay for streptomycin, dihydrostreptomycin, and process-related substances. The assay would serve as an alternative to the microbiological method and would be suitable for routine analysis (*i.e.* automation) and facile interlaboratory and compendial adoption. HPLC was selected as the most informative analytical tool in satisfying our assay criteria.

Chromatographic assay and development optimization

Because of the characteristic ionic, water-soluble nature of streptomycin and dihydrostreptomycin, a paired-ion reversed-phase HPLC mode of separation was explored. Recently, this technique has been successfully applied to the analysis of various types of therapeutic agents[13–19]. Initial developmental work using a μBondapak C_{18} column, a heptanesulfonic acid–acetate buffer mobile phase, and refractive index detection resulted in resolution of streptomycin A, dihydrostreptomycin A, and streptidine and thereby demonstrated the feasibility of the paired-ion reversed-phase approach. Further work focused on evaluation of column type and on optimization of mobile phase variables, such as concentration of buffer, organic modifier, type of counter-ion, and pH.

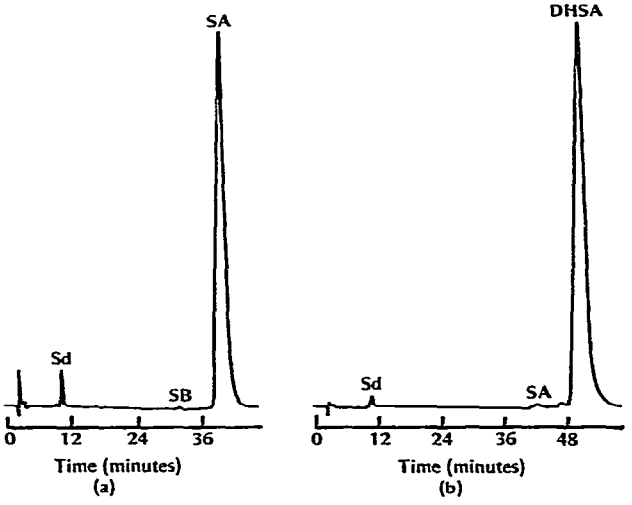

Fig. 2. Paired-ion HPLC chromatograms of (a) USP streptomycin sulfate and (b) USP dihydrostreptomycin sulfate using a LiChrosorb RP-18 (5 μm) column. Mobile phase: 0.02 *M* sodium hexanesulfonate, 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v), pH 6.0. Flow-rate: 0.8 ml/min. Sample size: 10 μg. Detector: UV at 195 nm, and 0.1 a.u.f.s. Peaks: Sd = streptidine; SB = streptomycin B; SA = streptomycin A; DHSA = dihydrostreptomycin A.

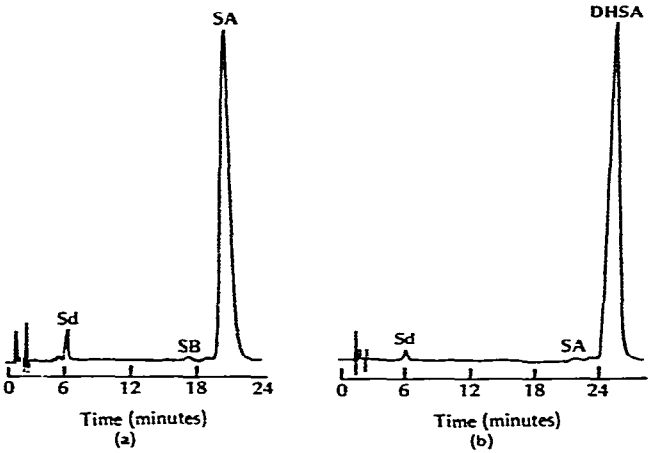

Fig. 3. Paired-ion HPLC chromatograms of (a) USP streptomycin sulfate and (b) USP dihydrostreptomycin sulfate using a μBondapak C_{18} (10 μm) column. Mobile phase: 0.02 *M* sodium hexanesulfonate, 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v), pH 6.0. Flow-rate: 1.3 ml/min. Sample size: 5 μg. Detector: UV at 195 nm, and 0.2 a.u.f.s. Peaks as in Fig. 2.

Column type

Though both Altex LiChrosorb RP-18 and Waters μBondapak C_{18} columns were used and performed satisfactorily, the μBondapak C_{18} column was chosen for the analysis procedure because it was more amenable to routine use. An Altex Ultrasphere Ion Pair column was also been evaluated and was found to be an attractive alternative to the μBondapak C_{18}. Representative HPLC chromatograms of streptomycin sulfate and dihydrostreptomycin sulfate obtained with the LiChrosorb RP-18,

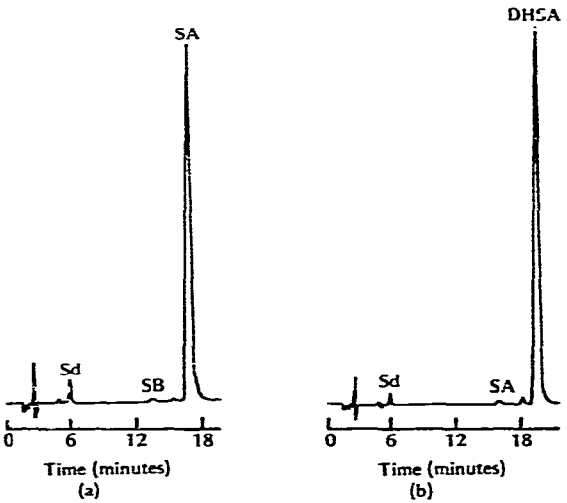

Fig. 4. Paired-ion HPLC chromatograms of (a) USP streptomycin sulfate and (b) USP dihydrostreptomycin sulfate using an Ultrasphere Ion Pair (5 μm) column. Mobile phase: 0.02 *M* sodium hexanesulfonate, 0.025 *M* tribasic sodium phosphate in acetonitrile–water (10:90, v/v), pH 6.0. Flow-rate: 1.3 ml/min. Sample size: 5 μg. Detector: UV at 195 nm, and 0.2 a.u.f.s. Peaks as in Fig. 2.

μBondapak C_{18}, and Ultrasphere Ion Pair columns using the optimal mobile phase described below are presented in Figs. 2, 3 and 4, respectively.

Mobile phase

To achieve maximum assay sensitivity, an acetonitrile–water mobile phase consisting of a phosphate–phosphoric acid buffer in lieu of the aforementioned acetate–acetic acid buffer was chosen to allow for far-ultraviolet (UV) detection. The high UV transparency of the phosphate buffer at *ca.* 195 nm permitted the simultaneous detection of streptomycin A, dihydrostreptomycin A, and several process-related substances. Following these assay improvements, a systematic study was made of the mobile phase variables responsible for assay selectivity and efficiency as characterized by the appropriate capacity factors. The mobile phase variables examined were: concentration of trisodium phosphate and acetonitrile, type and concentration of counter-ion, and pH. The following observations were made.

Effect of mobile phase trisodium phosphate and acetonitrile concentration upon the capacity factor (k'_{SA}) of streptomycin A. Increasing the concentration of either trisodium phosphate or acetonitrile causes a reduction in k'_{SA} and, in general, the capacity factors of all of the process-related substances (*i.e.* streptomycin B, dihydrostreptomycin A, streptidine, etc.). Of these two mobile phase variables, the acetonitrile concentration had a more pronounced effect on the resolution of streptomycin A and the aforementioned substances. Use of a buffer salt in the mobile phase was necessary, as its absence resulted in excessive streptomycin A and dihydrostreptomycin A retention times (*i.e.*, >45 min). Buffer concentration below 0.01 *M* resulted in skewed or tailing component peaks and poor component resolution. A similar phenomenon was observed by Anhalt[16] in his developmental work on a paired-ion HPLC method for assaying gentamycin in serum.

Effect of concentration and type of counter-ion upon k'_{SA}. When the alkylsulfonic acid counter-ion molar concentration is equal to or greater than the trisodium phosphate molar concentration, increasing the counter-ion concentration increases k'_{SA} as well as the capacity factors of streptomycin sulfate process-related substances. When the counter-ion concentration is less than the trisodium phosphate concentration, skewing or tailing of the streptomycin A peak occurs. The type of counter-ion (*i.e.* pentane-, hexane-, or heptanesulfonic acid) used in the mobile phase has a predictable effect upon k'_{SA}, that is, the retention time of streptomycin A increases in the following order: pentane sulfonate, hexane sulfonate, heptane sulfonate. Hexanesulfonic acid was selected in preference to pentanesulfonic acid and heptanesulfonic acid as the preferred mobile phase counter-ion, because it offered optimal resolution of the components of interest within a reasonable chromatographic analysis time (*i.e.*, 30 min or less). A 0.02 *M* counter-ion concentration was selected to obtain assay linearity for both streptomycin sulfate and dihydrostreptomycin sulfate at the sample concentration used for analysis.

Effect of pH on k'_{SA}. Employing the HPLC conditions cited in Fig. 2, the effect of pH on k'_{SA} and capacity factors of streptomycin sulfate process-related substances was examined. As illustrated in Fig. 5, the mobile phase pH has a significant effect upon k'_{SA} and the capacity factors of dihydrostreptomycin A, streptomycin B, and streptidine. A mobile phase pH of 6 was chosen to maximize component resolution and solution stability of the streptomycin and dihydrostreptomycin samples.

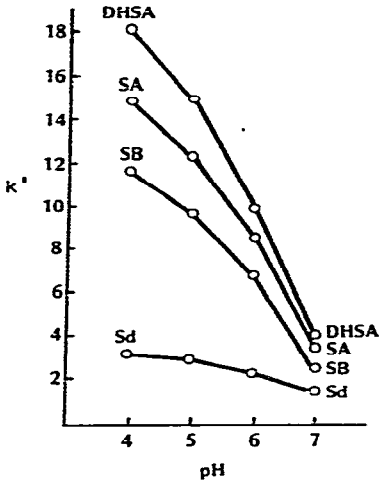

Fig. 5. Influence of pH of the mobile phase on capacity factors of streptomycins and process-related substances.

The information gathered in this systematic study of mobile phase variables proved to be invaluable in arriving at the optimal HPLC conditions for streptomycin sulfate and dihydrostreptomycin sulfate analysis. Based on the above observations, the chosen optimal mobile phase was 0.02 *M* sodium hexanesulfonate and 0.025 *M* tribasic sodium phosphate in acetonitrile–water (8:92, v/v) at pH 6.0. HPLC chromatograms generated with such a mobile phase are presented in Figs. 2–4. Retention data for streptomycin A, dihydrostreptomycin A, and corresponding process-related substances are summarized in Table I.

TABLE I

RELATIVE RETENTIONS OF STREPTOMYCIN A, DIHYDROSTREPTOMYCIN A AND PROCESS-RELATED SUBSTANCES

Compound	*Relative retention*
Streptobiosamine	void*
Dihydrostreptobiosamine	void*
Streptidine	0.30
Maltol**	0.45
Streptomycin B	0.80
Streptomycin A	1.00
Dihydrostreptomycin B	1.05
Dihydrostreptomycin A	1.20

* Under the cited chromatographic conditions, streptobiosamine and dihydrostreptobiosamine exhibited peak UV absorptivities only in the column void volume region of the chromatogram.

** Maltol (3-hydroxy-2-methyl-4H-pyran-4-one) is formed from the streptose portion of both streptomycin A and streptomycin B when either compound is heated in the presence of dilute alkali.

Linearity

Assay linearity for streptomycin A in streptomycin sulfate and dihydrostreptomycin A in dihydrostreptomycin sulfate was established within a concentration range of 0.024–0.24 mg/ml. Trace level assay linearity for streptidine, streptomycin A, and streptomycin B was established over a $1.0–3.0 \cdot 10^{-3}$ mg/ml concentration range.

Precision

The assay precision of the HPLC method was determined for five individual weights of a streptomycin sulfate sample and five individual weights of a dihydrostreptomycin sulfate sample. The relative standard deviation of streptomycin A determination and dihydrostreptomycin A determination was 1.1%. The precision of streptidine determination in both streptomycin sulfate and dihydrostreptomycin sulfate, of streptomycin B determination in streptomycin sulfate, and of streptomycin A determination in dihydrostreptomycin sulfate at approximately the 1% concentration level were 1.9%, 1.7% and 2.0%, respectively. The precision studied over a four day period was comparable.

Detection and sensitivity

The mobile phase selection (acetonitrile and phosphate buffer) permitted detection of both streptomycin A and dihydrostreptomycin A and related substances at *ca.* 200 nm or shorter wavelengths. This choice of mobile phase composition simplified the assay development work markedly by eliminating the need to develop a suitable post-column chemical derivatization detector. At a wavelength of 195 nm, the detection limit for streptomycins A and B, and dihydrostreptomycin A in streptomycin

TABLE II

ANALYSIS OF STREPTOMYCIN SULFATE

Sample	*Potency (μg/mg)*		*Streptidine sulfate monohydrate (%)*
	Turbidimetric (microbiological)	*HPLC*	
1	739	743	1.09
2	726	731	1.25
3	732	736	1.32
4	724	730	1.30
5	719	724	1.41
6	709	696	1.58
7	693	691	1.80
8	715	698	1.55
9	719	731	1.15
10	727	718	1.38
11	737	727	1.22
12	737	747	1.61
13	741	741	1.36
14	765	762	1.03
15	762	750	1.06
Average	730	728	

sulfate and dihydrostreptomycin sulfate dry powder products, is *ca.* 2 μg/ml or 50 ng/25 μl injection. Streptidine assay sensitivity of at least 0.5 μg/ml (*i.e.* 12.5 ng/25 μl injection) can be expected in the analysis of similar streptomycin and dihydrostreptomycin samples.

Assay equivalency with microbiological methodology

The assay equivalency of the HPLC method was examined by analyzing fifteen samples each of streptomycin sulfate and dihydrostreptomycin sulfate and comparing the calculated HPLC results with those obtained by the turbidimetric microbiological assay methodology (Tables II and III). The results demonstrate that the HPLC assay is equivalent to the bioassay.

TABLE III

ANALYSIS OF DIHYDROSTREPTOMYCIN SULFATE

Sample	*Potency (μg/mg)*		*Streptidine sulfate monohydrate (%)*	*Streptomycin A sulfate (%)*
	Turbidimetric (microbiological)	*HPLC*		
1	680	670	0.81	0.34
2	736	717	0.63	0.69
3	720	722	0.53	0.14
4	706	703	0.44	0.25
5	655	665	0.64	0.12
6	723	734	0.46	0.30
7	721	721	0.49	0.51
8	727	739	0.44	0.19
9	717	713	0.42	0.69
10	728	727	0.75	0.29
11	731	728	0.32	0.42
12	742	737	0.44	0.38
13	753	735	0.42	0.11
14	702	706	1.60	0.39
15	699	703	1.53	0.83
Average	716	715		

The capability of simultaneously determining streptidine, streptomycin A and B, and dihydrostreptomycin A played a decisive role in designing this paired-ion reversed-phase HPLC assay. High assay sensitivity for trace level determination of streptidine and streptomycin B in streptomycin sulfate and of streptidine and streptomycin A in dihydrostreptomycin sulfate is of interest in monitoring studies of streptomycin A biosynthesis and dihydrostreptomycin production (through hydrogenation of streptomycin A) to ascertain optimal conditions for maximum product yield and quality. Use of a dual channel recorder allows the determination of streptidine, streptomycin A, (and streptomycin B) at the 1% or lower concentration level as demonstrated by the data in Tables II and III.

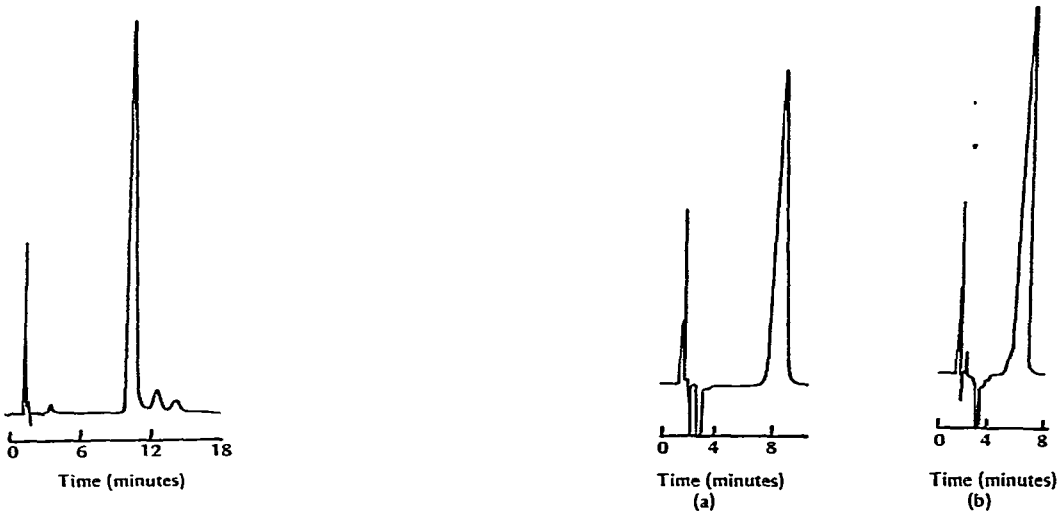

Fig. 6. Paired-ion HPLC chromatogram of viomycin sulfate using chromatographic conditions as described in Fig. 3 except with a two-fold flow-rate.

Fig. 7. Paired-ion HPLC chromatograms of (a) neomycin sulfate (0.25 mg) and (b) paromomycin sulfate (0.25 mg) using a μBondapak C_{18} (10 μm) column. Mobile phase: 0.016 *M* sodium hexanesulfonate, 0.02 *M* tribasic sodium phosphate in acetonitrile–water (17:83, v/v) pH 3.5. Flow-rate: 1.5 ml/min. Detector: refractive index, 8 × range, 10 mV recorder span.

Applicability to the analysis of other aminoglucosides and antituberculous agents

The HPLC conditions described here, without modification, have been applied directly to the analysis of the antituberculous agent, viomycin (Fig. 6). With only a minor mobile phase modification and by employing refractive index detection, the aminoglucosides paromomycin and neomycin can be similarly analyzed by this method (Fig. 7).

CONCLUSION

A rapid, accurate and sensitive paired-ion reversed-phase HPLC assay for separating and quantitating streptomycin A, dihydrostreptomycin A and their biosynthetic and process-related intermediates has been developed. The simplicity of the method should allow facile interlaboratory use. Furthermore, it is suitable for automation and has the potential for compendial adoption. It should be considered as an acceptable alternative to the microbiological method in routine analyses. The method is applicable to the analysis of streptomycin and dihydrostreptomycin finished products, other aminoglycoside antibiotics, and antituberculous agents. Assay sensitivity is adequate for pharmacological studies to determine either streptomycin A or dihydrostreptomycin A in biological matrices.

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