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DETERMINATION OF FORTIMICIN A SULFATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER DERIVATIZATION WITH 2,4-DINITROFLUOROBENZENE

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SUMMARY

Fortimicin A sulfate (astromicin sulfate), a new aminoglycoside antibiotic, is quantitated using high-performance liquid chromatography following pre-column derivatization with 2,4-dinitrofluorobenzene. The chromatographic separation of fortimicin A and the known impurities is achieved using an isocratic reversed-phase system. The column eluent is monitored by a UV detector at 350 nm, and the linearity of response for fortimicin A is demonstrated for concentrations up to 62 μg/ml. The relative standard deviation of the assay procedure is ±1.7%.

INTRODUCTION

Fortimicin A, a unique broad-spectrum aminoglycoside antibiotic, is produced by the fermentation of *Micromonospora olivoasterospora*[1,2]. The fortimicin A free base is reacted with sulfuric acid to yield a water soluble disulfate salt having a theoretical microbiological potency of 674 μg of base per mg of sulfate salt. In the fermentation process several other fortimicins are also produced in varying amounts[3]. The structures of fortimicin A[4] and related fortimicins[5,6] are shown in Fig. 1.

The potency of fortimicin A is currently determined by an agar plate-steel disc microbiological assay[7,8]. This procedure, though reliable, is very time consuming, prompting us to develop an alternate stability indicating technique for the drug. Stability indicating techniques described in the literature for aminoglycoside antibiotics include various chemical methods[9,10], column chromatography[11,12], paper chromatography[13–15], thin-layer chromatography[16–18] and gas–liquid chromatography[19–22]. However, these techniques generally suffer from either long analysis time, poor precision or lack of sensitivity. The application of high-performance liquid chromatography (HPLC) to the analysis of fortimicin A is complicated by the fact that this compound (along with most other aminoglycosides) exhibits little UV absorbance. As an alternative to UV detection, differential refractometry and photoelectric polarimetry detection have been used in the HPLC analysis of modified spectinomycin[23]. However, these detection techniques lack sensitivity.

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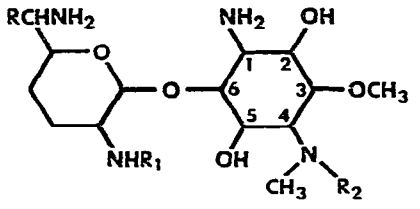


	R	R_1	R_2
Fortimicin A	CH_3	H	Glycine
B*	CH_3	H	H
D	H	H	Glycine
E*	CH_3	H	H
Isofortimicin A	CH_3	Glycine	H

*Epimeric at C3 and C4

Fig 1 Structures of fortimicins

Methods using HPLC with post-column derivatization and fluorescence detection have been reported for gentamicin[24,25], kanamycin[26] and spectinomycin[27]. When fortimicin A is chromatographed using ion-pair HPLC following by post-column derivatization with *o*-phthalaldehyde and fluorescence detection, a heavily buffered eluent is necessary to eliminate peak tailing. Since a heavily buffered eluent and a fluorescence detector is necessary in this approach, a pre-column derivatization for the HPLC assay of fortimicin A has been developed. A recent article by Tsuji *et al.*[28] describes the analysis of neomycin sulfate and other aminoglycosides using pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB) and normal-phase HPLC. This paper describes a modified pre-column DNFB derivatization procedure and reversed-phase separation for the determination of fortimicin A sulfate.

EXPERIMENTAL

Apparatus

The HPLC system consisted of an M6000A pump and U6K injector (Waters Assoc., Milford, MA, U.S.A.), an SF 770 variable wavelength UV detector (Schoeffel Instruments, Westwood, NJ, U.S.A.) and an SP4100 data handling system (Spectra-Physics, Santa Clara, CA. U.S.A.). The chromatographic separations were achieved using a Zorbax ODS (25 cm × 4.6 mm I.D.) chromatographic column (DuPont, Wilmington, DE, U.S.A.) fitted with a C_{18} (3 cm × 4.6 mm I.D.) guard column (Brownlee Labs, Santa Clara, CA, U.S.A.).

Reagents

Acetonitrile and methanol used were distilled in glass UV grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Phosphoric acid was ACS grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). Aniline was ACS grade from Fisher Scientific

(Fair Lawn, NJ, U.S.A.). Aniline hydrochloride was prepared by reacting 7 ml of aniline dissolved in 300 ml of hexane with 6 ml of concentrated hydrochloric acid. The precipitated salt (9 g theoretical yield) was filtered, washed with ethyl acetate and dried under vacuum in a desiccator.

2,4-Dinitrofluorobenzene (DNFB) was of 98% minimum purity from Eastman-Kodak (Rochester, NY, U.S.A.). The DNFB derivatization reagent was prepared by dissolving 2 ml of DNFB in 200 ml of methanol. This 1% (v/v) reagent was prepared fresh daily. Dibasic and monobasic potassium phosphate were AR grade from Mallinckrodt (St. Louis, MO, U.S.A.). A 1% potassium phosphate (dibasic) pH 9.0 buffer was prepared by dissolving 10.0 g in 1 l of distilled water. The pH of the solution was adjusted to 9.0 with either phosphoric acid or 50% aqueous potassium hydroxide. A 0.02 *M* potassium phosphate (monobasic) pH 3.0 buffer was prepared by dissolving 8.165 g in 3 l of distilled water. The pH of the solution was adjusted to 3.0 with phosphoric acid.

Internal standard preparation

An accurately weighed 100-mg portion of aniline hydrochloride was dissolved and diluted to 100 ml in 1% potassium phosphate (dibasic) pH 9.0 buffer.

Fortimicin A sulfate sample and standard preparation

Into separate 50-ml volumetric flasks, 100-mg portions of fortimicin A sulfate sample and reference standards were accurately weighed. The fortimicin A sulfate was dissolved and diluted to volume in 1% potassium phosphate (dibasic) pH 9.0 buffer.

Derivatization procedure

Treating sample and standard solutions identically, a 5-ml portion of each preparation was pipetted into separate 200-ml volumetric flasks. A 4-ml portion of the internal standard preparation and 20 ml of 1% DNFB solution were added to each flask and the flask openings were covered with aluminum foil. The solutions were heated at 85°C in a water bath for 1 h, with occasional swirling. After heating, 100 ml of acetonitrile was added to each solution (the potassium phosphate precipitated at this point). The flasks were shaken to dissolve the derivatized fortimicin A and 60 ml of 0.02 *M* potassium phosphate (monobasic) pH 3.0 buffer was added. On mixing, a clear solution resulted and the contents of each flask were diluted to volume with acetonitrile. A portion of each solution was filtered through a 0.4-μm polycarbonate membrane (Nuclepore, Pleasanton, CA, U.S.A.) prior to injection into the liquid chromatograph.

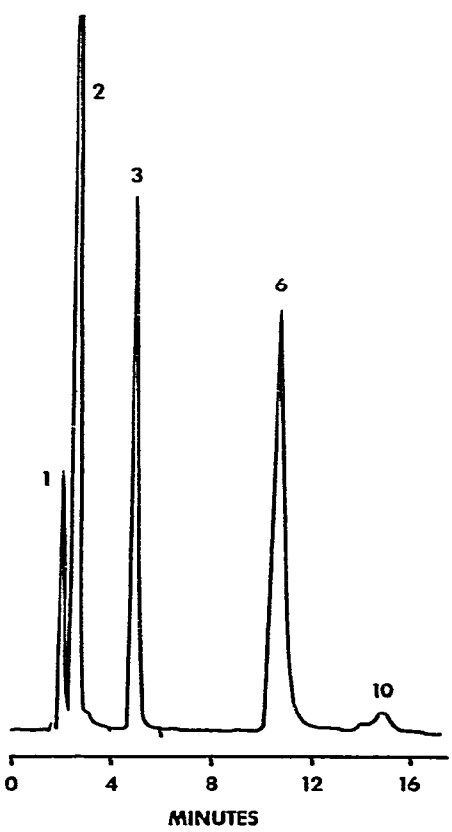
Chromatographic conditions

HPLC mobile phase: 0.02 *M* potassium phosphate (monobasic) pH 3.0 buffer–acetonitrile (35:65). A 700-ml portion of the aqueous buffer was added to a 2-l volumetric flask and diluted to volume with acetonitrile. Flow-rate: 2.0 ml/min. Pressure: 1600 p.s.i. Detector: 350 nm at 0.04 a.u.f.s., attenuation at 32 on the integrator. Chart speed: 0.5 cm/min.

RESULTS AND DISCUSSION

Attempts to chromatograph fortimicin, derivatized as described by Tsuji *et al.*[28], using a normal-phase HPLC system failed to resolve the various isomers. The reversed-phase system described here gave optimum resolution of isofortimicin A and fortimicin A while maintaining reasonable retention times for the fortimicin E and B isomers. Chromatograms of a typical fortimicin A sulfate bulk drug, an internal standard preparation and a distilled water blank derivatized as described in the text are presented in Figs. 2–4. The derivatized internal standard and fortimicin A peaks are well resolved from unreacted 2,4-dinitrofluorobenzene when monitored at 350 nm. At 254 nm, a loss of resolution occurs between the unreacted 2,4-dinitrofluorobenzene and the internal standard. Fig. 5 is a chromatogram in which a fortimicin A sulfate bulk drug was spiked with known impurities (each minor component at 4–10% of the drug level) prior to derivatization. The fused peaks eluting between the fortimicin A and fortimicin E are unknowns from the added impurities.

In the derivatization procedure, the effects of solution pH, buffer system, reac-



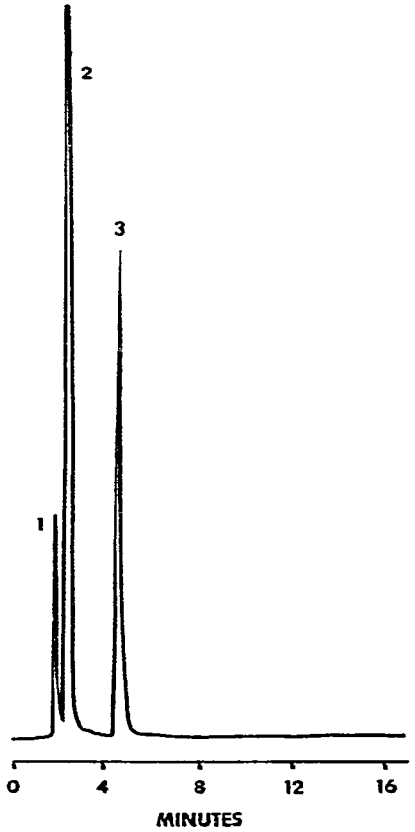
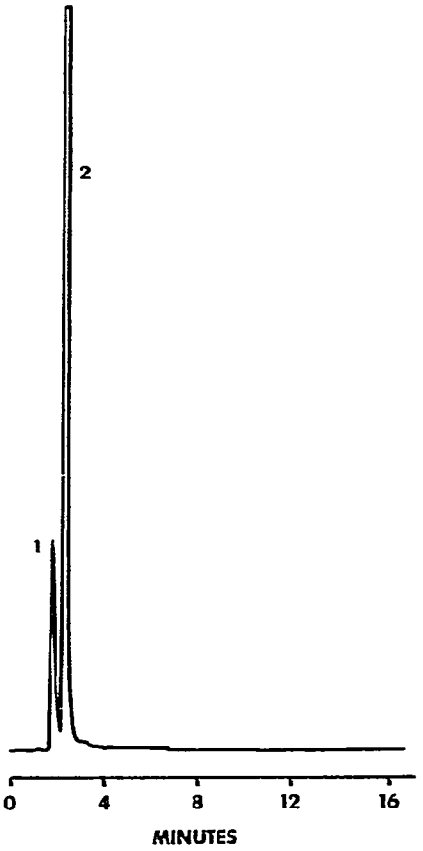


Fig. 2. Typical chromatogram of a fortimicin A sulfate bulk drug. Peaks: 1, 2 = DNFB and reaction products; 3 = internal standard; 6 = fortimicin A and 10 = fortimicin B.

Fig. 3. Chromatogram of aniline hydrochloride derivatized with DNFB. Peak identities: same as Fig. 2.



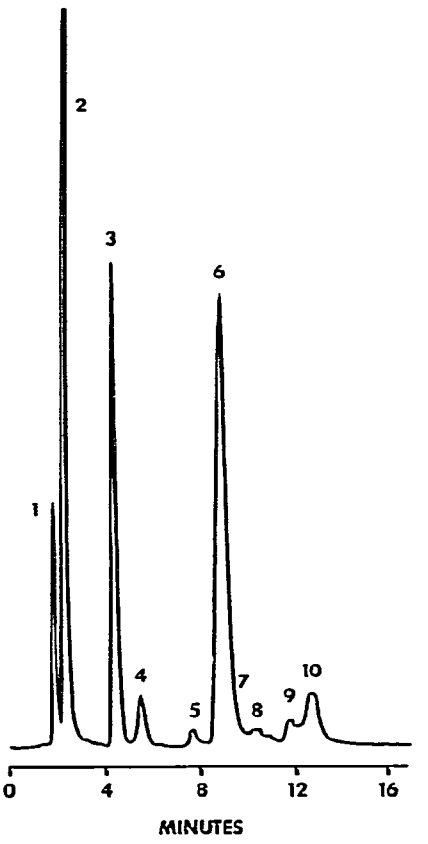


Fig 4. Chromatogram of a derivatization blank. Peak identities: same as Fig 2

Fig. 5. Chromatogram of fortimicin A sulfate containing added impurities. Peaks: 1, 2 = DNFB and reaction products; 3 = internal standard; 4 = fortimicin D; 5 = isofortimicin A; 6 = fortimicin A; 7, 8 = unknown; 9 = fortimicin E; 10 = fortimicin B

tion time and excess DNFB were investigated. The reaction conditions stated in the text were chosen to insure quantitative derivatization of the parent drug and associated minors with slight variations in the sample and reagent preparations. When fortimicin A sulfate was derivatized at pH 9–10, essentially the same peak area response of the reaction product was observed. Three pH 9 buffer systems were investigated: 1% potassium phosphate (dibasic), 1% THAM and 0.75% sodium borate. Under identical conditions, essentially the same reaction rate was observed for fortimicin A in each system.

When fortimicin A sulfate was reacted with DNFB at 85°C in 1% potassium phosphate (dibasic) pH 9.0 buffer, the reaction was complete at 15 min. The longer reaction time stated in the method ensures complete conversion of the drug and associated minors. Similarly, amounts of DNFB solution ranging from 25–100% of that stated in the procedure gave identical results for fortimicin A.

The derivatization procedure described in the text differs from that reported by Tsuji *et al.*[28], in that the derivatized aminoglycoside is not extracted. After reaction,

the derivatized drugs are solubilized with the addition of acetonitrile and the solution pH is adjusted to approximately 6.5 with the addition of potassium phosphate buffer. The final sample solution contains aqueous potassium phosphate–acetonitrile–methanol (69:111:20). Solution stability for derivatized fortimicin A has been demonstrated for at least 20 h at room temperature.

Method validation

When 39.8 to 123.3 mg weights of fortimicin A sulfate standard were taken through the procedure (final concentrations of 19.9–61.6 μg/ml) a linear response was obtained using both peak areas and peak heights. In both cases the correlation coefficients were 0.9999 and both response *vs.* concentration plots essentially passed through the origin.

Precision data were collected using a typical lot of fortimicin A bulk drug by three analysts over a five-day period and these data are summarized in Table I.

TABLE I
PRECISION OF THE FORTIMICIN A SULFATE ASSAY

Analyst	*Day*	*Potency "as is" (μg/mg)**	*% Theory (100% = 674)*
1	1	584	86 7
1	1	584	86 7
2	2	590	87.5
3	3	605	89.8
3	3	594	88 1
1	4	589	87.4
1	4	588	87.2
2	5	566	84.0
2	5	588	87 2
	Mean	588	87 2
	Standard deviation	±10.3	±1.5
	Relative standard deviation	±1 7%	±1.7%

* The above lot of bulk drug yielded "as is" potencies of 581 μg/mg (three day average) by microbiological assay

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