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GENTAMICIN C-COMPONENT RATIO DETERMINATION BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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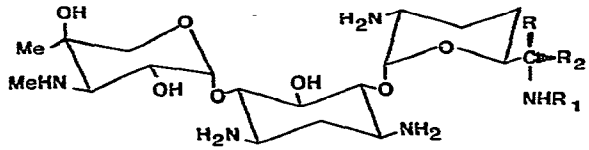
SUMMARY

A rapid and reproducible high-pressure liquid chromatographic determination of gentamicin has been developed. The analysis is performed by a combination of paired-ion chromatography, post-column derivatization and fluorescence detection. The results show gentamicin to be composed of three major components, C_1, C_2 and C_{1a}, and several minor components. The quantitative results are compared to those obtained by a microbiological method and are in excellent agreement. This technique is applicable to other aminoglycoside analyses.

INTRODUCTION

Gentamicin, an aminoglycoside antibiotic produced by the fermentation of *Micromonospora purpurea*, was introduced in 1969 and has a broad spectrum of activities against both gram-positive and gram-negative bacteria. It is composed of three major components, gentamicins C_1, C_2 and C_{1a}, and several minor components including gentamicins A, A_1, B, B_1, C_{2a} and C_{2b} (ref. 1).



Gentamicin C_1: $R = R_1 = Me$; $R_2 = H$

Gentamicin C_2: $R = Me$; $R_1 = R_2 = H$

Gentamicin C_{2a}: $R = R_1 = H$; $R_2 = Me$

Gentamicin C_{2b}: $R = R_2 = H$; $R_1 = Me$

Gentamicin C_{1a}: $R = R_2 = R_1 = H$

Chromatographic methods for analysis of the gentamicin complex have been reported[2–5]. Since these methods do not possess a combination of speed, specificity, sensitivity or precision, the most widely used method for analysis has been a microbiological assay. Microbiological assay, however, is time consuming and lacks specificity.

A rapid and reliable liquid chromatographic assay of each of the three major components of gentamicin has been developed. Most of the minor components are clearly separated and easily identified on each of the chromatograms studied. The procedure utilizes three relatively new techniques of liquid chromatography: (1) fluorescence detection; (2) post-column derivatization; and (3) ion-pairing chromatography. Reagents used in the separation are novel, and the post-column reaction apparatus is composed of commercially available components only slightly modified for the analysis. Detection is based on the reaction of *o*-phthalaldehyde with primary amines to give fluorescent products[6,7]. Recommended reagents for the analysis are all easily obtainable and require no special handling. The use of a similar technique for the assay of gentamicin in serum has recently been reported by one of us[8].

A second liquid chromatographic method of analysis utilizing more conventional chromatography has also been investigated. This method employs normal-phase chromatography and refractive index detection. Since this method incorporates a large volume of aqueous base as the mobile phase, the silica gel column is quickly degraded. This condition and the limited sensitivity make this technique less desirable than the ion-pairing method. However, this method has been acceptably applied to large-scale preparative chromatography[5].

Results from the liquid chromatographic analysis compared favorably with an assay performed according to the USP microbiological method.

EXPERIMENTAL

Reagents

The purified gentamicin components C_1, C_{1a} and C_2 were kindly provided by J. Allan Waitz (Schering Co., Bloomfield, N.J., U.S.A.). Gentamicin sulfate (USP reference standard) was obtained from Schering Co., *o*-phthalaldehyde (Fluoropa, manufactured by Durrum) from Pierce (Rockford, Ill., U.S.A.), 2-mercaptoethanol from Sigma (St. Louis, Mo., U.S.A.), methanol from Burdick & Jackson Labs., (Muskegon, Mich., U.S.A.) and sodium pentanesulfonate from Eastman-Kodak (Rochester, N.Y., U.S.A.). Water was deionized and glass-distilled. All other chemicals were of reagent grade. *o*-Phthalaldehyde reagent (OPA) was prepared by the method of Benson and Hare[7] with the exception that 5% Brij was added to reduce precipitation of polysulfide in the detector. Solutions of antibiotic were freshly prepared in distilled water at a concentration of 500 μg/ml.

Apparatus and chromatography for method I

A Waters M6000A (Waters Assoc., Milford, Mass., U.S.A.) pump was used to deliver the mobile phase. A Ferrand (Valhalla, N.Y., U.S.A.) Model RF-2 fluorometer equipped with 350-nm (excitation) and 450-nm (emission) filters was used to detect the product formed by continuous-flow, post-column derivatization with OPA reagent. The OPA reagent was delivered with a second Waters M6000A pump.

A zero dead volume T union (R. S. Crum, Inc., Springfield, N.J., U.S.A.) was used to introduce the OPA reagent into the chromatographic stream, and a reaction coil comprising a length of PTFE tubing (1.5 m × 0.7 mm I.D.) coiled to a 6-mm diameter was used between the mixing T union and detector. Analysis was performed using a Hibar LiChrosorb RP-8 column (25 cm × 3.0 mm I.D.) (E. Merck, Darmstadt, G.F.R.) with a mean particle size of 7 μm. 2-μl samples containing 500 μg/ml of antibiotic were injected using a Waters U6K injector.

The mobile phase contained 0.015 *M* sodium pentanesulfonate, 0.2 *M* sodium sulfate and 0.1% acetic acid in water. The mobile phase flow-rate was 1.5 ml/min at 3000 p.s.i., and OPA was introduced at 0.5 ml/min at 300 p.s.i. Reagents and mobile phase were filtered and degassed prior to use.

Apparatus and chromatography for method II

This procedure utilized a Waters M6000A pump, a μ-Partisil column (30 cm × 3.9 mm I.D.) (Waters Assoc.), a Waters refractive index detector and the U6K injector system. The mobile phase was composed of water–methanol–diethylamine (60:40:0.5). The mobile-phase flow-rate was 1 ml/min at 3000 p.s.i.

RESULTS AND DISCUSSION

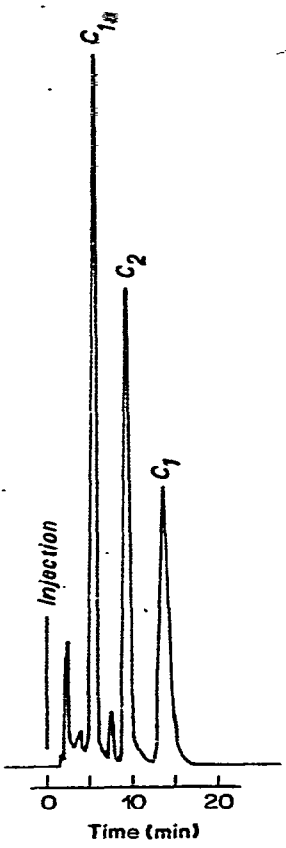
Fig. 1 is representative of the chromatograms obtained by method I. The elution order was C_{1a}, C_2, C_1. Polar impurities appear prior to these three components. Fig. 2 presents the results from method II. Note that the elution order has changed to C_2, C_{1a}, C_1, a reversal of C_2 and C_{1a} compared to the other method. The order and relative position of each component was determined using pure components injected separately. In method I, the retention times can be shortened by the addition of sodium sulfate and/or methanol. Greater retention times may be obtained by addition of greater amounts of sodium pentanesulfonate.

Component ratios of ten samples of gentamicin sulfate were evaluated using method I. These results were compared to those obtained using the official USP microbiological analysis. The results indicate an excellent agreement for the two techniques in almost all cases (Table I). Data from replicate ($N = 7$) assays by method I of USP Reference Standard are presented in Table II. The maximum coefficient of variation (CV) was 2.6%.

The results obtained from method II indicate this technique could be used for qualitative separation of the gentamicin C complex. However, column degradation during prolonged usage negated its usefulness as a reliable quantitative method of analysis. Recently, Sancilio *et al.*[5] investigated the usefulness of this method for preparative chromatographic separation, and small quantities of ultra-pure components of each of the three major gentamicin C components were obtained. The results from these experiments will be presented at a later time.

The geometry of the post-column reaction coil was also studied. Substitution of a stainless-steel coil of 3 mm in diameter gave increased response. All results presented in this report, however, were obtained using the reaction coil described above which was 6 mm in diameter.

In summary, rapid and reliable chromatographic systems for the analysis of gentamicin have been developed. One of these methods is a viable alternative to the



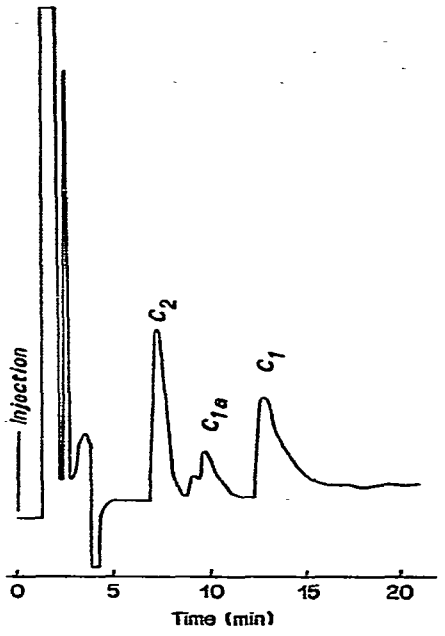


Fig. 1. Representative chromatogram of gentamicin by method I.

Fig. 2. Representative chromatogram of gentamicin by method II.

TABLE I

COMPARISON OF COMPONENT RATIOS FOR TEN BATCHES OF GENTAMICIN DETERMINED BY HPLC AND MICROBIOLOGICAL ASSAY

Batch	*HPLC assay (%)*			*Microbiological assay (%)*		
	C_1	C_2	C_{1a}	C_1	C_2	C_{1a}
1	35.6	35.6	28.8	34.6	33.5	31.9
2	34.5	34.9	30.6	33.5	34.0	32.5
3	36.6	36.0	27.4	33.6	37.0	29.4
4*	37.2	42.1	20.8	33.9	40.5	25.8
5	36.4	34.6	29.0	37.4	33.4	29.2
6	36.7	34.0	29.2	36.3	35.0	28.7
7	35.7	35.4	28.9	35.3	33.8	30.9
8	34.8	35.1	30.1	34.8	34.8	30.4
9	34.3	36.5	29.3	32.9	34.1	33.0
10	35.2	36.2	28.6	35.0	36.4	28.6

* USP Reference Standard.

TABLE II

REPLICATE ANALYSES OF COMPONENT RATIO FOR THE USP REFERENCE STANDARD OF GENTAMICIN SULFATE

Component	*Analysis number*							*Av. ± S.D.*	*CV (%)*
	1	*2*	*3*	*4*	*5*	*6*	*7*		
C_{1a}	20.4	21.2	20.0	21.1	21.0	21.4	20.3	20.8 ± 0.5	2.6
C_2	41.9	41.4	42.5	42.4	42.9	41.3	42.0	42.1 ± 0.6	1.4
C_1	37.7	37.4	37.5	36.5	36.1	37.3	37.7	37.2 ± 0.6	1.7

microbiological method used routinely for the analysis of gentamicin and gives comparable results. It has also been observed that this same methodology can be adapted to the quantitative analysis of other similar antibiotics. Additional work with neomycin, netilmicin, sisomicin and kanamycin has been completed and will be presented in a subsequent publication.

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