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DETERMINATION OF THE COMPONENT RATIO OF COMMERCIAL GENTAMICINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING PRE-COLUMN DERIVATIZATION

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SUMMARY

Commercial samples of gentamicin from different origins were analyzed by paired-ion high-performance liquid chromatography (HPLC) on a C_{18} bonded phase. The procedure uses pre-column derivatization with a *o*-phthalaldehyde–mercaptoacetic acid reagent and UV detection (350 nm). The ratios of the four gentamicin components (C_1, C_{1a}, C_{2a} and C_2) were determined and compared with the compositions obtained by an independent method based on ^{13}C NMR spectrometry. Quantitation by HPLC, based on peak heights and peak areas, was performed with the aid of an external standard, which was an artificial mixture of the four components. The latter were prepared by separation of the gentamicins C_1, C_2 + C_{2a} and C_{1a} by chromatography on silica gel, followed by chromatography of the C_2 + C_{2a} fraction on a cellulose phosphate column.

INTRODUCTION

Gentamicin is an aminoglycoside antibiotic produced by *Micromonospora purpurea*[1]. It is a complex mixture of three major components (C_1, C_{1a} and C_2) and several minor components including C_{2a} and C_{2b} (Fig. 1). Gentamicin C_{2a} was identified as a 6′-C-epimer of C_2[2]; gentamicin C_{2b}, a 6′-N-methylgentamicin C_{1a}, is identical with the antibiotic XK-62-2, also known as sagamicin[3–5]. According to Byrne *et al.*[2] commercial gentamicin contains only 4% of the C_{2a} and C_{2b} components. It has recently been shown[6] that the C_{2a} component represents a significant proportion (from 6 to 18%) of the gentamicin C complex. Thus, C_{2a} can no longer be considered as a minor component and the analysis of commercial samples should not be limited to C_1, C_{1a} and C_2.

Several methods based on bonded phase high-performance liquid chromatography (HPLC) have been developed for the assay of this antibiotic in serum[7–16] and also for determination of the component ratio in commercial samples and pharmaceutical preparations[17–20]. Separation of the C_2 and the C_{2a} components by HPLC was reported in two of these papers[16,18]. Both procedures use a pre-column deriv

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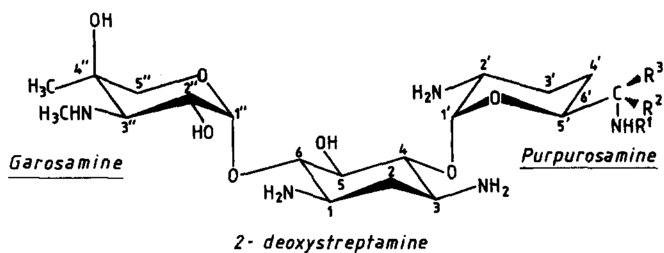

		R_1	R_2	R_3
gentamicin	C_1 :	Me	H	Me
gentamicin	C_2 :	H	H	Me
gentamicin	C_{1a} :	H	H	H
gentamicin	C_{2a} :	H	Me	H
gentamicin	C_{2b} :	Me	H	H

Fig. 1. Structure of gentamicin C components.

atization with *o*-phthalaldehyde (OPA) in the presence of a mercaptan. In our hands the procedure reported by Freeman *et al.*[18] gave a better resolution of the C_2 and C_{2a} components than that reported by Marples and Oates[16]. Thus, the Freeman method was chosen for determination of the component ratio of various commercial gentamicin samples of known origin and also of some reference preparations. A similar study has recently shown the variation in composition of 38 gentamicin samples available for clinical use[6]. Although the brand names are given for most products, this information does not indicate the primary producer.

It should be noted that differentiation of C_2 and C_{2a} was also obtained in our laboratory by paired-ion HPLC of underivatized gentamicin[21]. The method was not applied in this study because separation of the two components by this procedure is time-consuming (at least 50 min for complete analysis).

EXPERIMENTAL

Materials and reagents

Gentamicin samples were obtained from Essex Laboratories (Heist o/d Berg, Belgium; affiliated with Schering, U.S.A.), Pierrel (Milan, Italy) and Chinoin (Budapest, Hungary). Samples of Bulgarian, Chinese and Hungarian origin were gifts from Gist Brocades (Delft, The Netherlands) and Phenix Pharmaceuticals (Antwerp, Belgium). Gentamicin C_{2b} (sagamicin) and antibiotic JI 20B were obtained from the Kyowa Hakko Kogyo (Tokyo, Japan).

o-Phthalaldehyde (OPA), mercaptoacetic acid (MAA) and sodium heptanesulphonate were purchased from Janssen Chimica (Beerse, Belgium). The Amberlite CG-50 resin was obtained from Serva (Heidelberg, F.R.G.) and the Whatman cellulose phosphate P11 from Whatman (Ferrières, France). Water distilled in glass was used for the preparation of mobile phases. Methanol (E. Merck) was distilled before use. The OPA–MAA reagent was prepared according to the procedure of Freeman *et al.*[18].

Separation of the gentamicin C components

Amberlite CG-50 (Type I, 100–200 mesh) was suspended in water and freed from fine particles by decantation. The slurry was adjusted to pH 9 with ammonium hydroxide and poured into a glass column (20 × 4 cm). A solution of commercial gentamicin sulphate (8 g, 058/R) in water (30 ml) was adjusted to pH 9 with ammonium hydroxide and poured onto the resin. Ammonium sulphate and minor components were eluted (3 ml/min) with 0.175 *M* ammonium hydroxide (2000 ml). Elution with 0.2 *M* ammonium hydroxide (3000 ml) afforded 4.3 g of the gentamicin C free base, which was separated into its components by chromatography on a silica gel column according to Cooper *et al.*[22]. The column (3 cm I.D.) was prepared from a silica (250 g) slurry in the lower phase of isopropanol (IPA)–chloroform–17% ammonium hydroxide (1:2:1). Elution was carried out with the lower phase of IPA–chloroform–25% ammonium hydroxide (1:2:1) at a flow-rate of 180 ml/h. The effluent was divided into three fractions according to the chromatographic pattern observed with thin-layer chromatography (TLC) according to Wilson *et al.*[23] and HPLC according to Freeman *et al.*[18].

Fraction I (1 g) contained C_1 as major component, II (0.8 g) was a mixture of C_2 and C_{2a} and III (0.7 g) contained C_2, C_{2a} and C_{1a}. Each of these fractions was further separated on a 150-g silica gel column, yielding a C_1 fraction (0.94 g), a $C_2 + C_{2a}$ fraction (1.2 g) and a C_{1a} fraction (0.24 g).

The $C_2 + C_{2a}$ fraction was converted into its hydrochloride and separated (three analyses of 400 mg) on a Whatman cellulose phosphate column (15 × 3 cm I.D.). Elution with 2.15 *M* sodium chloride (25 ml/h) was monitored by continuous measurement of the optical rotation using a Thorn-NPL Type 243 polarimeter equipped with a flow-cell (2 × 0.4 cm I.D.) according to Thomas and Tappin[24]. Gentamicin C_2 was found between 525 and 750 ml of the effluent, the C_{2a} peak between 750 and 1000 ml. Fractions containing C_2 and C_{2a} were evaporated to dryness and extracted several times with boiling methanol. The methanol extracts containing the gentamicin components were freed from sodium chloride by conversion of the gentamicin into the Schiff bases. The procedure is described for the C_2 component.

The residue obtained upon evaporation of the methanol extract was dissolved in water, and adjusted to pH 11 with sodium hydroxide. The solution was evaporated to dryness and the residue was taken up in absolute ethanol (40 ml) containing benzaldehyde (1.05 g). The suspension was heated for 30 min at 50°C and the solvent evaporated. The residue was extracted with dichloromethane, the filtered extract was evaporated to dryness, dissolved in diethyl ether (35 ml) and extracted (3 × 25 ml) with 0.1 *M* hydrochloric acid. The aqueous layer was adjusted to pH 9 with ammonium hydroxide and freed from ammonium chloride with the aid of Amberlite GC-50 resin. This afforded 400 mg of gentamicin C_2 (free base) and also 130 mg C_{2a} (free base).

The free bases of the four components were dissolved in water ($\approx$ 30 mg/ml) adjusted to pH 5 with sulphuric acid, filtered through a Whatman glass fibre filter (GF/B), evaporated to a small volume and freeze-dried. The freeze-dried material was triturated with absolute methanol and dried overnight *in vacuo* over P_2O_5. Samples were allowed to equilibrate with the moisture of the atmosphere for 20 h and kept in closed vials.

Contamination with other compounds was not observed by TLC[23], HPLC[18], ^{1}H and ^{13}C NMR. The NMR spectra determined for C_1, C_{1a} and C_2 were identical to those reported[3,4,25–27]. NMR spectra of C_{2a} (not previously reported) were in agreement with the proposed structure and will be published elsewhere[28].

Proton NMR spectra of the four components were also recorded in the presence of a known amount of benzyl alcohol. Integrations of the phenyl signal and the anomeric proton signal of the purpurosamine fragment ($\delta \approx 5.88$ ppm) were used for determination of the free base content of each of the components.

HPLC equipment

Chromatographic equipment consisted of a Waters M-45 (Waters Assoc., Milford, MA, U.S.A.) pump, a Valco CV-6-UHPa-N60 injection valve (Valco, Houston, TX, U.S.A.) with 10-μl loop and a Waters M-440 UV detector equipped with a 350-nm filter. Detector signals were processed by and recorded on a Hewlett-Packard 3990A recording integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Columns (10 × 0.46 cm) were laboratory-packed with 5-μm ODS-Hypersil (Shandon, U.K.), 5-μm LiChrosorb RP-8 (E. Merck, Darmstadt, F.R.G.), 5-μm RoSil C18HL DA (Alltech Europe, Eke, Belgium) or 5-μm Spherisorb S5ODS (Phase Separations, U.K.). The 10-μm LiChrosorb RP-18 material was packed in a 25 × 0.46 cm column. The number of theoretical plates measured for the C_2 peak varied from 1400 to 1600 for the 10-cm columns.

Mobile phases

Sodium heptanesulphonate (5 g) was dissolved in a mixture of distilled water (280 ml) and glacial acetic acid (50 ml). The solution was diluted to 1 l with methanol. With the Hypersil column thermostatted at 21°C this mobile phase gave a complete analysis in about 16 min at a flow-rate of 1 ml/min. The resolution and retention times observed for the Hypersil column can be reproduced for the other columns by increase or decrease of the water content of the mobile phase by a few per cent.

Chromatographic procedure

Gentamicin sulphate samples were dissolved in water at a concentration of 1 mg/ml. A 0.25-ml volume of this solution, 0.1 ml of the OPA–MAA reagent[18] and 0.25 ml isopropanol were heated in a water-bath at 60°C for 15 min, filtered through a Whatman GB/F (1-μm) glass fibre filter and injected (10 μl) immediately. Peak areas and peak heights were measured by the integrator as shown in Fig. 2. The compositions of the gentamicin samples were calculated by comparison with a chromatogram of reference mixture I (Table I), which is taken as external standard.

Response factors relative to gentamicin C_1, based on peak area, RA_i, and peak height, RH_i, were determined for each of the four components. These factors are given by the formulae

$$RA_i = \frac{A_i}{X_i} \cdot \frac{X_1}{A_1} \quad \text{and} \quad RH_i = \frac{H_i}{X_i} \cdot \frac{X_1}{H_1}$$

where A_i and H_i are the area and height percentages measured from chromatograms of the external standard and X_i is the corrected component ratio given in Table I.

The subscript 1 refers to the C_1 component. The compositions of the gentamicin samples listed in Tables II and III were obtained by dividing the peak areas and peak heights by the appropriate relative response factors. Values thus obtained were adjusted so that their sum equals 100%. The sequence of analysis was one external standard run prior to each series of five gentamicin samples. The composition of these samples was calculated with the aid of the response factors (mean values), determined from the external standard chromatograms, recorded just before and just after the series of five samples.

RESULTS AND DISCUSSION

Elution sequence of the gentamicin components

The reaction of gentamicin with OPA in the presence of mercaptoethanol (ME) has frequently been used[7,8,11,15–17,19] in pre- and post-column derivatization of gentamicins. The reagent converts primary amino functions into substituted isoindoles[29]. This increases the lipophilic nature of the gentamicins (which permits retention by a C_{18} bonded phase) and also introduces fluorophores in the molecule. In the Freeman method ME is replaced by MAA. This modification and also the presence of sodium heptanesulphonate in the mobile phase is believed to be responsible for an improved separation of the C_2 and C_{2a} components. The replacement of ME by MAA also allows UV detection at 330 nm (and also at 350 nm).

The order of elution obtained in almost all HPLC procedures[6,8,11,16,18], using pre-column derivatization with an OPA reagent, is: C_1, C_{1a}, (C_{2a}), C_2. The C_1 component which contains only three primary amino functions (and thus three isoindoles upon derivatization) is less strongly retained than the other three components containing four primary amines. A different elution order (C_{1a}, C_2, C_1) has been described by Kraisintu *et al.*[19]. We applied the method of Maitra *et al.*[8] on a μBondapak C_{18} column (as reported in the original paper) and on a Spherisorb S5ODS column and confirmed the C_1, C_{1a}, C_2 sequence with both columns. Using the Kraisintu[19] procedure (which is a slight modification of the original Maitra[8] derivatization) and a Spherisorb S5ODS column we found the same sequence (C_1, C_{1a}, C_2), which is in contradiction to the data given in the Kraisintu paper. Partial derivatization of the C_{1a} and C_2 components could be an explanation for this discrepancy.

It should be noted that, with a derivatization similar to that of Maitra *et al.*[8] and Kraisintu *et al.*[19] but with a higher concentration of ethylenediaminetetraacetate (EDTA) in the mobile phase, Marples and Oates[16] could separate the C_2 peak as C_{2a} and C_2 peaks, an observation which was confirmed in our laboratory.

The C_{2b} component is not mentioned in the publication of Freeman *et al.*[18]. In our experience this component is located in the ascending part of the C_1 peak. Antibiotic JI 20B (dihydroxy C_{2a}), which is a precursor[30] of C_{2a}, C_2 and C_1, is located in the descending part of the C_1 peak. The ^{13}C NMR analyses performed in our laboratory on three Italian, one Hungarian and two American samples showed that the C_{2b} content is lower than 2% (the limit of detection).

Quantitation of the components

For quantitation of the four gentamicin components (C_1, C_{1a}, C_2 and C_{2a}) it is necessary to know their specific responses in the chromatographic procedure. This

TABLE I

COMPONENT RATIOS OF REFERENCE MIXTURES OF GENTAMICIN SULPHATE

Sum of components = 100% in each case.

Mixture	*% C_1*	*% C_{1a}*	*% C_{2a}*	*% C_2*
I				
Uncorrected	30.0	20.0	10.0	40.0
Corrected*	30.1	18.6	9.8	41.5
^{13}C NMR**	30.1 (0.1)	18.3 (0.6)	10.1 (0.3)	41.5 (0.6)
II				
Uncorrected	40.0	30.0	10.0	20.0
Corrected	40.7	28.3	10.0	21.0
^{13}C NMR**	40.4 (0.3)	29.1 (0.2)	9.5 (0.5)	21.0 (0.5)

* Corrected for the relative base content of the components, 0.968, 0.897, 0.950 and 1.00 respectively for C_1, C_{1a}, C_{2a} and C_2.

** Standard deviation (five determinations) given in parentheses.

implies that the pure components and a reference mixture of known composition are available. The pure components were obtained by separation of the gentamicins C_1, C_2 + C_{2a} and C_{1a} by chromatography on silica gel[22], followed by chromatography of the C_2 + C_{2a} fraction on a cellulose phosphate column[24]. The purity and identity of the four gentamicins were checked by ^{1}H NMR, ^{13}C NMR, TLC[23] and HPLC[18]. The area of the main peak observed in HPLC represented at least 96% of the total area for all four components. The free bases were converted into their sulphate salts and the free base contents of the latter were determined by ^{1}H NMR using benzyl

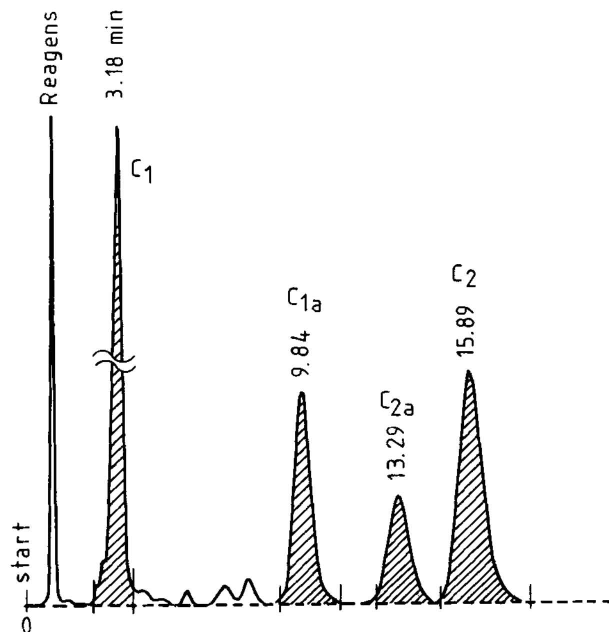

Fig. 2. Chromatogram of commercial gentamicin sulphate (USQ-3-GMF-N-6020) obtained with a 5-μm Hypersil ODS column (10 × 0.46 cm).

alcohol as internal standard. Two reference mixtures were prepared and their compositions corrected for the free base content (Table I). The component ratios of the two reference mixtures were also determined by a ^{13}C-NMR method developed in our laboratory[28]. Comparison of the data is shown in Table I. It is seen that component ratios obtained by this independent method were in good agreement with the values given.

Most of the analyses described in the present paper were carried out on a 10-cm 5-μm Hypersil ODS column, as reported in the original Freeman method. Quantitation based on peak area, investigated during this study, required an improved separation between the reagent peak and the C_1 component, which was obtained by increasing the water content of the mobile phase from 250 ml/l (as in the original procedure) to 280 ml/l. This resulted in an increase in the retention time of the components. A typical chromatogram is given in Fig. 2. Similar chromatograms were also obtained with other C_{18} bonded phases (see Experimental), which indicates that the choice of the stationary phase is not critical. Addition of an internal standard (nonylamine) as in the original method was omitted, since analyses given in the present work are limited to component ratio determinations.

Reference mixture I was used as an external standard for analysis of the gentamicin samples. The response factors relative to C_1 based on peak area, RA_i, and peak height, RH_i, were calculated from external reference chromatograms (see Experimental). The RA_i values (means from 20 analyses); 1 for C_1, 1.34 for C_{1a}, 1.35 for C_{2a} and 1.25 for C_2, reflect the number of isoindole moieties introduced into the gentamicin components (three for C_1 and four for C_{1a}, C_{2a} and C_2). If complete derivatization, equal stability of the derivatized gentamicins and equal molar absorptivity, ε, of the isoindole moieties is assumed, the RA_i values should be 1, 1.42, 1.37 and 1.37 respectively for C_1, C_{1a}, C_{2a} and C_2. The compositions of the gentamicins listed in Tables II and III were obtained by dividing the peak areas and peak heights by the appropriate response factor. Values thus obtained were adjusted so that their sum equals 100%.

It is seen that there is a systematic difference between the analyses based on peak areas and those on peak heights. The C_1 contents are higher, and those of the other three components lower, when the analysis is based on peak areas. When analyses by HPLC are compared with those obtained by the independent ^{13}C NMR method[28], it appears that compositions based on peak heights are in a better agreement with ^{13}C NMR analysis than those based on peak areas. It is believed that the resolution in the C_1 region of the chromatogram is not sufficient for analysis by the peak area method. Inspection of the chromatogram (Fig. 2) show first of all a tailing of the reagent peaks and also the presence of interfering substances in the vicinity of the C_1 peak which give a larger contribution to the area of this peak than to its height. Thus, the peak height method is preferred, unless a better resolution can be obtained of the beginning of the chromatogram.

For some of the samples, the composition, as determined by the official Food and Drug Administration (FDA) method[31] (paper chromatographic separation followed by bioassay), was available. Comparison with HPLC data (based on peak heights) shows that the FDA method overestimates the C_{1a} content (from 1.8 to 15.2%). The C_2 + C_{2a} content is slightly underestimated (up to 5.2%). Deviations for the C_1 content range from 1.8 to 8.9%. Analysis by the FDA method of the

TABLE II

COMPOSITION OF COMMERCIAL SAMPLES OF GENTAMICIN SULPHATE

Some of components taken as 100% in each case. Standard deviations are given in parentheses.

Origin and batch number	*Analysis*	*% C_1*	*% C_{1a}*	*% C_{2a}*	*% C_2*	*% (C_2 + C_{2a})*	*No. of analyses*
Italy							
GST/679 (1983)*	HPLC area	35.0 (0.3)	15.4 (0.2)	11.4 (0.3)	38.1 (0.4)	49.5	5
	HPLC height	33.8 (0.2)	15.9 (0.1)	11.4 (0.2)	38.9 (0.3)	50.3	5
	^{13}C NMR	32.9 (0.4)	14.7 (0.2)	11.1 (0.7)	41.3 (0.3)	52.4	5
080/13 (1983)*	HPLC area	35.5 (0.1)	20.0 (0.2	11.0 (0.4)	33.5 (0.6)	44.5	11
	HPLC height	33.7 (0.3)	20.4 (0.2)	11.5 (0.2)	34.4 (0.3)	45.9	11
	^{13}C NMR	34.6 (0.4)	19.8 (0.9)	10.6 (0.5)	35.0 (0.1)	45.6	5
	Bioassay**	35.5	23.5	–	–	41.1	
0.73/R (1982)*	HPLC area	32.9 (0.3)	18.2 (0.2)	10.6 (0.2)	38.2 (0.2)	48.8	10
	HPLC height	30.3 (0.6)	19.0 (0.3)	10.6 (0.3)	40.1 (0.6)	50.7	10
	^{13}C NMR	30.8 (0.3)	19.5 (0.5)	9.4 (0.2)	40.3 (0.1)	49.7	5
	Bioassay**	31.1	22.1	–	–	46.8	
058/R (1982)*	HPLC area	36.3 (0.5)	16.6 (0.2)	9.8 (0.2)	37.4 (0.5)	47.2	4
	HPLC height	33.5 (0.5)	17.9 (0.1)	9.6 (0.2)	39.0 (0.5)	48.6	4
	Bioassay	33.7	19.6	–	–	46.7	
026 (1979)*	HPLC area	32.0 (0.2)	15.5 (0.1)	11.9 (0.2)	40.6 (0.2)	52.5	4
	HPLC height	30.5 (0.2)	16.1 (0.1)	11.8 (0.1)	41.6 (0.2)	53.4	4
	Bioassay**	28.0	23.0	–	–	49.0	
U.S.A.							
USQ-3-GMF-N-6020 (1983)*	HPLC area	39.5 (0.5)	17.1 (0.2)	11.7 (0.2)	31.7 (0.2)	43.4	7
	HPLC height	38.5 (0.2)	17.3 (0.1)	12.1 (0.2)	32.1 (0.2)	44.2	7
	^{13}C NMR	39.3 (0.3)	15.5 (0.3)	12.0 (0.1)	33.2 (0.7)	45.2	5
	Bioassay**	30.4	32.5	–	–	39.5	
SZ-6MC-2-L-1006 (1983)*	HPLC area	36.4 (0.3)	22.3 (0.2)	11.2 (0.2)	30.1 (0.4)	41.3	5
	HPLC height	35.2 (0.2)	22.6 (0.3)	11.1 (0.1)	31.1 (0.2)	42.2	5
	Bioassay**	30.1	31.5	–	–	40.5	4

GMC-7-M-9012 (1977)*	HPLC area	39.0 (0.3)	28.1 (0.2)	7.4 (0.1)	25.4 (0.3)	32.8	3
	HPLC height	37.1 (0.1)	29.1 (0.2)	7.2 (0.1)	26.6 (0.2)	33.8	3
	Bioassay**	29.7	36.3	–	–	33.0	
GMC-7-M-6103 (1977)*	HPLC area	38.0 (0.4)	29.4 (0.3)	8.4 (0.2)	24.2 (0.3)	32.6	3
	HPLC height	35.5 (0.2)	30.8 (0.1)	8.4 (0.1)	25.2 (0.2)	33.6	3
	Bioassay**	26.6	37.9	–	–	32.0	
GMC-5-M-4-1	HPLC area	47.5 (0.5	17.9 (0.1)	6.2 (0.2)	28.4 (0.3)	34.6	3
	HPLC height	44.3 (0.2)	19.3 (0.3)	6.2 (0.1)	30.2 (0.3)	36.4	3
Hungary							
83-06-147 (1983)*	HPLC area	36.2 (0.3)	22.0 (0.1)	11.6 (0.2)	30.1 (0.3)	41.7	9
	HPLC height	34.4 (0.3)	22.5 (0.1)	12.3 (0.1)	30.8 (0.4)	43.1	9
	^{13}C NMR	34.6 (0.5)	21.8 (1.3)	12.0 (0.9)	31.6 (0.7)	43.6	5
83-06-148 (1983)*	HPLC area	38.5 (0.5)	18.6 (0.2)	11.7 (0.2)	31.2 (0.4)	42.9	3
	HPLC height	36.4 (0.2)	19.5 (0.2)	12.0 (0.1)	32.1 (0.3)	44.1	3
83-06-145 (1983)*	HPLC area	37.7 (0.3)	20.8 (0.3)	11.7 (0.1)	29.9 (0.2)	41.6	4
	HPLC height	35.9 (0.4)	21.0 (0.4)	12.1 (0.1)	31.1 (0.3)	43.2	4
78-10-55 (1979)*	HPLC area	35.6 (0.3)	16.7 (0.3)	12.0 (0.2)	35.8 (0.2)	47.8	4
	HPLC height	33.6 (0.4)	17.2 (0.4)	12.4 (0.1)	36.0 (0.2)	49.3	4
	Bioassay**	35.8	18.6	–	–	45.6	
China							
8009025	HPLC area	43.2 (0.4)	23.3 (0.2)	13.1 (0.2)	20.4 (0.2)	33.5	5
	HPLC height	40.1 (0.4)	24.4 (0.2)	13.8 (0.2)	21.7 (0.3)	35.5	5
	Bioassay**	36.3	29.2	–	–	34.5	
Bulgaria							
No batch No.	HPLC area	42.8 (0.5)	25.9 (0.3)	9.0 (0.2)	22.3 (0.3)	31.3	3
	HPLC height	40.8 (0.3)	26.7 (0.2)	9.1 (0.1)	23.5 (0.2)	32.6	3
168 (1983)*	HPLC area	39.5 (0.3)	27.1 (0.3)	9.3 (0.3)	24.1 (0.2)	33.4	3
	HPLC height	36.8 (0.3)	28.4 (0.1)	9.3 (0.1)	25.5 (0.3)	34.8	3

* Date mentioned on the certificate of analysis

** As given by the manufacturer (FDA method).

TABLE III

COMPOSITION OF REFERENCE STANDARDS OF GENTAMICIN SULPHATE

Sum of four components taken as 100%. Standard deviations are given in parentheses.

Standard	*Analysis*	% C_1	% C_{1a}	% C_{2a}	% C_2	% $(C_2 + C_{2a})$	*No. of analyses*
International reference preparation (WHO) (1967	HPLC area	36.6 (0.4)	23.6 (0.3)	6.3 (0.2)	35.5 (0.5)	41.8	7
	HPLC height	33.3 (0.2)	25.0 (0.2)	6.2 (0.1)	35.4 (0.3)	41.6	7
	Bioassay*	24.7	38.9			36.4	
USP reference standard H	HPLC area	45.3 (0.3)	24.5 (0.3)	7.3 (0.1)	22.7 (0.3)	30.0	8
	HPLC height	43.5 (0.3)	25.2 (0.3)	7.5 (0.1)	23.5 (0.1)	31.0	8
	Bioassay**	36.9	31.5			31.6	
	^{13}C NMR	44.7 (0.2)	26.2 (1.3)	7.1 (0.9)	22.0 (0.3)	29.1	5
BP chemical reference substance (# 1091)	HPLC area	38.3 (0.4)	18.0 (0.2)	14.2 (0.4)	29.5 (0.2)	43.7	7
	HPLC height	37.0 (0.2)	18.4 (0.1)	14.7 (0.3)	29.9 (0.2)	44.6	7
Artificial mixture Reference mixture II (Table I)	HPLC area	39.7 (0.3)	28.6 (0.2)	10.2 (0.2)	21.5 (0.3)	31.7	12
	HPLC height	40.0 (0.2)	28.6 (0.1)	10.0 (0.1)	21.5 (0.3)	31.5	12
	^{13}C NMR	40.4 (0.3)	29.1 (0.2)	9.5 (0.5)	21.0 (0.5)	30.5	5

* As given in ref. 32.

** As stated on the label.

International reference preparation[32] and of the USP reference standard (H) shows the same divergence from our HPLC analyses.

Composition of commercial gentamicin samples

The compositions of the commercial gentamicins (Tables II and III) show considerable variation, as previously observed by White *et al.*[6]. The C_1 content is located between 30 and 40% for most of the samples; the C_{1a} content is generally between 16 and 22%. Exceptions are the two Bulgarian, the Chinese and two American samples (GMC-7-M-9012 and GMC-7-M-6103) produced in 1977, where the percentage of C_{1a} ranges from 25 to 30%. All samples collected recently contain 9–14% C_{2a}. Less C_{2a} (6–9%) was found in American samples produced in 1977 or earlier. The highest C_2 content (about 40%) was found in Italian samples. The percentages of C_2 were lower (about 30%) for American and Hungarian preparations and still lower (20–26%) for the Chinese and Bulgarian gentamicins analysed during this study.

Most samples considered in the present study fall within the FDA limits for gentamicin; 20–50% for C_1, 15–40% for C_{1a} and 20–50% for C_2. The percentages of C_2, considered as the sum of C_2 and C_{2a}, of the Italian gentamicins are near or just outside the upper C_2 limit of 50%. The composition of Italian samples reported in a paper by Kraisintu *et al.*[19] is quite different from that given in the present study.

The analysis of gentamicin published in the British Pharmacopoeia (BP), Addendum 1983[33] is based on the method of Freeman *et al.*[18]. In this method the areas of the peaks due to C_1, C_{1a}, C_2 and C_{2a} are expressed as a percentage of the sum of the areas of the four components. It should be remembered that the area response factors are not identical for the four components, which means that analysis accord-

TABLE IV

COMPARISON OF AREA PERCENTAGES AND COMPONENT RATIOS AS DETERMINED BY THE FREEMAN PROCEDURE (PEAK HEIGHT METHOD)

Origin and batch number	*Analysis*	*% C_1*	*% C_{1a}*	*% C_{2a}*	*% C_2*	*% $(C_2 + C_{2a})$*	*No. of analyses*
Italy (080/13)	BP method*	29.9	22.5	12.4	35.4	47.5	11
	HPLC height**	33.7	20.4	11.5	34.4	45.9	11
USA (USQ-3-GMF-N-6020)	BP method	33.7	19.4	13.3	33.4	46.7	7
	HPLC height	38.5	17.3	12.1	32.1	44.2	7
Hungary (83-06-147)	BP method	30.5	24.8	13.1	31.6	44.7	9
	HPLC height	34.4	22.5	12.3	30.8	43.1	9
China (8009025)	BP method	36.3	27.0	15.0	22.0	37.0	5
	HPLC height	40.1	24.4	13.8	21.7	35.5	5
Bulgaria (168)	BP method	32.9	30.8	10.8	25.5	36.3	3
	HPLC height	36.8	28.4	9.3	25.5	34.8	3

* British Pharmacopoeia method[33] (areas of the C_1, C_{1a}, C_{2a} and C_2 peaks expressed as the percentages of the sum of the four peak areas).

** See Table III.

ing to the BP method does not indicate the actual composition of the gentamicin sample. This is illustrated in Table IV in which area percentages according to the BP method are compared with the component ratios (as calculated by the peak height method) for a number of gentamicin samples. It is seen that the BP area percentages of C_1 are 4–5% lower than the actual C_1 content. Area percentages are consequently higher for the other three components.

It can be concluded that the Freeman procedure is a convenient method for determination of the component (C_1, C_{1a}, C_{2a}, C_2) ratios of gentamicin samples, provided a reference standard of known composition is available.

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REFERENCES

1 M. J. Weinstein, G. M. Luedemann, E. M. Oden and G. M. Wagman, *Antimicrob. Agents Chemother.*, (1963) 1.
2 K. M. Byrne, A. S. Kershner, H. Maehr, J. A. Marquez and C. P. Schaffner, *J. Chromatogr.*, 131 (1977) 191.
3 P. J. L. Daniels, C. Luce, T. L. Nagabhushan, R. S. Jaret, D. Schumacher, H. Reimann and J. Ilavsky, *J. Antibiot.*, 28 (1975) 35.
4 R. S. Egan, R. L. Devault, S. L. Mueller, M. T. Levenberg, A. C. Sinclair and R. S. Stanaszek, *J. Antibiot.*, 28 (1975) 29.
5 R. Okachi, I. Kawamoto, S. Takasawa, M. Yamamoto, S. Sato, T. Sato and T. Nara, *J. Antibiot.*, 27 (1974) 793.
6 L. O. White, A. Lovering and D. S. Reeves, *Ther. Drug Monit.*, 5 (1983) 123.
7 J. P. Anhalt, *Antimicrob. Agents Chemother.*, 11 (1977) 651.
8 S. K. Maitra, T. T. Yoshikawa, J. L. Hansen, T. Nilsson-Ehle, W. J. Palin, M. C. Schotz and L. B. Guze, *Clin. Chem.*, 23 (1977) 2275.
9 G. W. Peng, M. A. F. Gadalla, A. Peng, V. Smith and W. L. Chiou, *Clin. Chem.*, 23 (1977) 1838.
10 W. L. Chiou, R. L. Nation, G. W. Peng and S. H. Huang, *Clin. Chem.*, 24 (1978) 1847.
11 S. E. Bäck, I. Nilsson-Ehle and P. Nilsson-Ehle, *Clin. Chem.*, 25 (1979) 1222.
12 N.-E. Larsen, K. Marinelli and A. Møller Heilessen, *J. Chromatogr.*, 221 (1980) 182.
13 D. M. Barends, J. S. F. van der Sandt and A. Hulshoff, *J. Chromatogr.*, 182 (1980) 201.
14 D. M. Barends, C. L. Zwaan and A. Hulshoff, *J. Chromatogr.*, 222 (1981) 316.
15 H. Kubo, T. Kinoshita, Y. Kobayashi and K. Tokunaga, *J. Chromatogr.*, 227 (1982) 244.
16 J. Marples and M. D. G. Oates, *J. Antimicrob. Chemother.*, 10 (1982) 311.
17 J. P. Anhalt, F. D. Sancilio and T. McCorke, *J. Chromatogr.*, 153 (1978) 489.
18 M. Freeman, P. A. Hawkins, J. S. Loran and J. A. Stead, *J. Liquid Chromatogr.*, 2 (1979) 1305.
19 K. Kraisintu, R. T. Parfitt and M. G. Rowan, *Int. J. Pharmaceutics*, 10 (1982) 67.
20 T. A. Getek, A. C. Haneke and G. B. Selzer, *J. Assoc. Offic. Anal. Chem.*, 66 (1983) 172.
21 P. J. Claes and H. Vanderhaeghe, *J. Liquid Chromatogr.*, submitted for publication.
22 D. J. Cooper, M. D. Yudis, H. M. Marigliano and T. Traubel, *J. Chem. Soc., C*, (1971) 2876.
23 W. L. Wilson, G. Richard and D. W. Hughes, *J. Chromatogr.*, 78 (1973) 442.
24 A. H. Thomas and S. F. Tappin, *J. Chromatogr.*, 97 (1974) 280.
25 J. B. Morton, R. C. Long, P. J. L. Daniels, R. W. Tkach and J. H. Goldsten, *J. Amer. Chem. Soc.*, 95 (1973) 7464.
26 D. J. Cooper, H. M. Marigliano, M. D. Yudis and T. Traubel, *J. Infect. Dis.*, 119 (1969) 342.
27 B. E. Rosenkrantz, J. R. Greco, J. C. Hoogerheide and E. M. Oden, *Anal. Profiles Drug. Subst.*, 9 (1980) 295.

28 R. Busson and H. Vanderhaeghe, in preparation.
29 P. S. Simons, Jr. and D. F. Johnson, *J. Org. Chem.*, 43 (1978) 2886.
30 R. T. Testa and B. C. Tilley, *J. Antibiot.*, 29 (1976) 140.
31 *Code of Federal Regulations,* Title 21 Food and Drugs, Part 444.20a, Her Majesty's Stationery Office, London, 1983.
32 J. W. Lightbown, *Bull. W.H.O.*, 47 (1972) 343.
33 *British Pharmacopoeia 1980,* Addendum 1983, The Office of Federal Registers, National Archives and Records Service, Washington, DC, 1981, p. 235.