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A high-performance liquid chromatographic method for the determination and control of the composition of gentamicin sulphate

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Summary

A high-performance liquid chromatographic method for routine control of the composition of gentamicin sulphate is described. The method utilizes pre-column derivatization followed by reversed-phase chromatography with fluorescence detection and uses an internal standard for quantification. Analysis of 19 samples of gentamicin from various sources indicates that the ratio of the major components and the content of minor constituents varies with the geographical origin of the sample. The results were compared with those of a microbiological assay and the B.P. nuclear magnetic resonance (nmr) spectroscopic limit test of the same samples. The microbiological assay may be influenced by biologically active impurities whilst the nmr assay was insensitive to the presence of minor components. The method described offers a more discriminating and flexible means of monitoring the composition of gentamicin and hence of providing data upon which a realistic specification for this valuable antibiotic mixture can be prepared.

Introduction

Gentamicin is a broad spectrum aminoglycoside antibiotic complex produced by *Micromonospora purpurea* and composed of 3 major components designated C_1, C_2 and C_{1a} which differ from each other in their degree of methylation (Cooper et al., 1971). Two minor components, C_{2a} and C_{2b} have also been characterized (Daniels et al., 1975).

These components have been separated by a number of chromatographic methods

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including thin-layer, column, paper and ion-exchange chromatography. Most recently high-performance liquid chromatography (HPLC) has been used by several workers. Anhalt et al. (1978) described an HPLC method using ion-pair chromatography on a reversed-phase column, post-column derivatization with *o*-phthalaldehyde and detection by fluorescene spectrophotometry. Although they obtained reproducible results, the nature of the post-column reaction coil affected the intensity of fluorescence. A method for assaying gentamicin in serum avoided this problem by using pre-column derivatization with *o*-phthalaldehyde (Maitra et al., 1977). Freeman et al. (1979) separated all 3 major components and gentamicin C_{2a} by means of pre-column derivatization with a modified *o*-phthalaldehyde reagent, followed by ion-pair reversed-phase chromatography.

Current official assays for gentamicin sulphate depend primarily upon a microbiological estimation of total potency. In the British Pharmacopoeia (1980) the ratio of major components is controlled within broad limits by means of nuclear magnetic resonance (nmr) spectroscopy. In the United States Pharmacopoeia (1980) the component ratio is measured by a combination of paper chromatography and microbiological assay. Thomas (1978) has compared these two methods with a method based on ion-exchange chromatography.

In the present paper a method similar to that of Maitra et al. (1977) but using an internal standard for quantification, has been applied to the analysis of the major components in a number of commercial samples of gentamicin sulphate from a variety of geographical sources. The results were compared with those from microbiological and nmr assays of the same samples.

Methods and materials

HPLC analyses were carried out on a Constametric I liquid chromatograph (Laboratory Control Division, Milton Roy) equipped with a Perkin Elmer 204-S fluorescence spectrophotometer detector, using a reverse-phase, chemically bonded octadecylsilane column (Spherisorb S5 ODS, Phase Separations) 10×0.4 cm i.d.). Samples were injected by means of a 25-μl loop. Peak areas were measured by a computing integrator (Perkin Elmer Sigma 10 Chromatography Data Station). Nmr analyses were performed to the method of the B.P. (1980) on a Perkin Elmer R12B 50MHz ^{1}H nmr spectrometer.

HPLC grade methanol was employed and all other reagents were analytical grade. *o*-Phthalaldehyde derivatizing reagent was prepared according to the method of Maitra et al. (1977). Samples of gentamicin sulphate were obtained from a variety of manufacturing sources and an authentic sample of gentamicin C_{2b} was provided by Schering Research Division.

Isolation of gentamicin C_1, C_2, and C_{1a}

Gentamicin base was prepared from the sulphate by passage through a column of ion-exchange resin IRA-400 (OH^- form) and eluted with water. Pure standards of the 3 major components were isolated from the mixture by column chromatography

on silica gel H (TLC grade) eluted with the lower phase of a mixture of chloroform:isopropanol:17% ammonia (2:1:1) (Cooper et al., 1971b). The identity and purity of each component was confirmed by ^1H nmr spectroscopy and field desorption mass spectrometry (Parfitt et al., 1978). Each was homogeneous by the TLC system of Wilson et al. (1973) and by HPLC.

HPLC analysis

Where sufficient material was available the commercial samples of gentamicin were assayed for sulphate by a titrimetric method (Williams, 1979). Where sufficient material was not available the samples were assumed to contain 32% (w/w) of sulphate for the purposes of calculation.

Each commercial sample and the isolated gentamicins, C_1, C_2 and C_{1a}, were assayed for water content by the loss of weight on drying at 105°C.

HPLC separations were carried out at 30 ± 0.2°C with methanol water (79:21 v/v) containing tripotassium ethylenediaminetetracetic acid (2 g/l) as mobile phase. The mixture was filtered (membrane pore size 0.45 μm) and degassed prior to use. A flow rate of 1.6 ml/min was used. The fluorescence detector utilized and excitation wavelength of 345 nm and measured emission at 430 nm.

L-Arginine monohydrochloride (0.0375 mg/ml in water) was employed as internal standard. This solution (0.1 ml) was mixed with gentamicin (either as sulphate or base; approximately 0.14 mg/ml in water) (0.55 ml) and then *o*-phthalaldehyde reagent (0.1 ml) and ethanol (5 ml) were added. The mixture was stored in the dark for 1.5 h at room temperature and filtered prior to injection. The elution order of the derivatives of arginine and gentamicins C_1, C_2, C_{1a} and C_{2b} was determined by separate injection.

Solutions containing known concentrations of each of the gentamicins, C_1, C_2 and C_{1a}, were used to construct a calibration curve of gentamicin peak area/arginine peak area against the weight of gentamicin. Three replicates were done for each concentration and the curves were linear over the whole of the chosen range (0.01–0.1 mg/ml). Nineteen commercial samples of gentamicin sulphate were examined by this method. Six replicates of each assay were carried out and the weight of each gentamicin component was calculated from the calibration graph. In calculating the weight of each component per 100 mg of sample, allowance was made for the fact that the samples were sulphates whereas the standards were bases.

Microbiological assay

The microbiological assay was performed by a well diffusion technique similar to that of the British Pharmacopoeia (1980) using sensitivity test agar. Incubation was for 12 h. Six replicates of each of 5 concentrations of each unknown sample were examined and the potency calculated from a standard response curve constructed at the same time.

Results and conclusions

A typical chromatogram obtained by this ethod is shown in Fig. 1. The elution order is arginine, minor components, gentamicin C_{1a}, gentamicin C_2, other minor

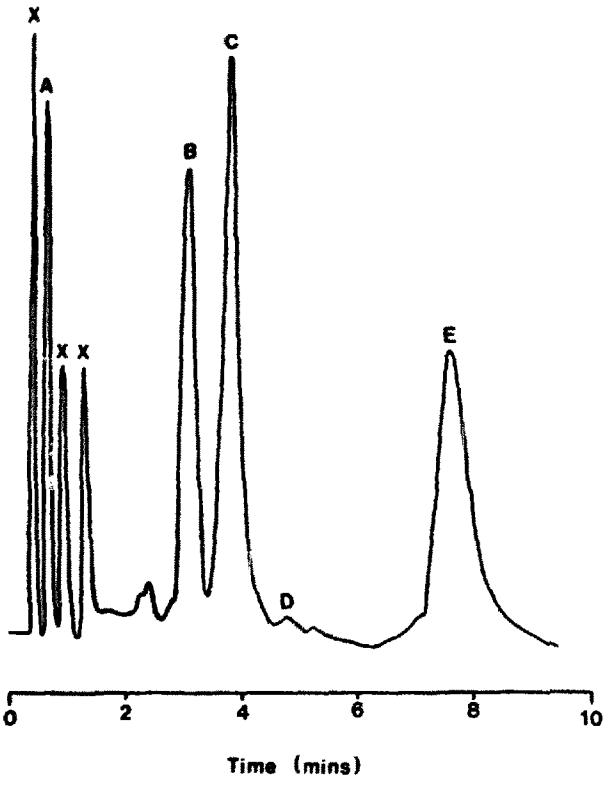

Fig. 1. High-performance liquid chromatogram of a commercial sample of gentamicin sulphate (country of origin, U.S.A.) with arginine as internal standard. The mixture was derivatized with *o*-phthalaldehyde prior to injection. Peak A is L-arginine, B is gentamicin C_{1a}, C is gentamicin C_2, D is gentamicin C_{2b} and E is gentamicin C_1. Peaks marked × are due to the derivatizing reagent and other small peaks are minor constituents of the gentamicin complex.

components including gentamicin C_{2b}, and gentamicin C_1. It is noteworthy that this order differs from that reported by Maitra et al. (1977) using a similar HPLC system. Their method differs in several details from that reported here.

(i) Maitra et al. carried out the derivatization reaction with the gentamicin adsorbed onto silicic acid. They did not discuss the time course of the reaction. Thus it is possible that the fluorescent derivatives obtained by these workers were not identical to those in the present study.

(ii) Although both studies used a C-18 chemically bonded reverse-phase column, these were supplied by different manufacturers. Several workers, in studies involving polynuclear aromatic hydrocarbons (Ogan and Katz, 1980; Amos, 1981), steroids (Nice and O'Hare, 1978), and polynuclear aromatic epoxide adducts to nucleosides (Panthananickal and Marnett, 1981), have observed differences in selectivity in C-18 chemically bonded stationary phases from different commercial sources.

(iii) Maitra et al. performed their studies at ambient column temperature, whereas the present work was conducted at 30°C. However, we found that although the use of an elevated temperature improved resolution, it did not alter elution order.

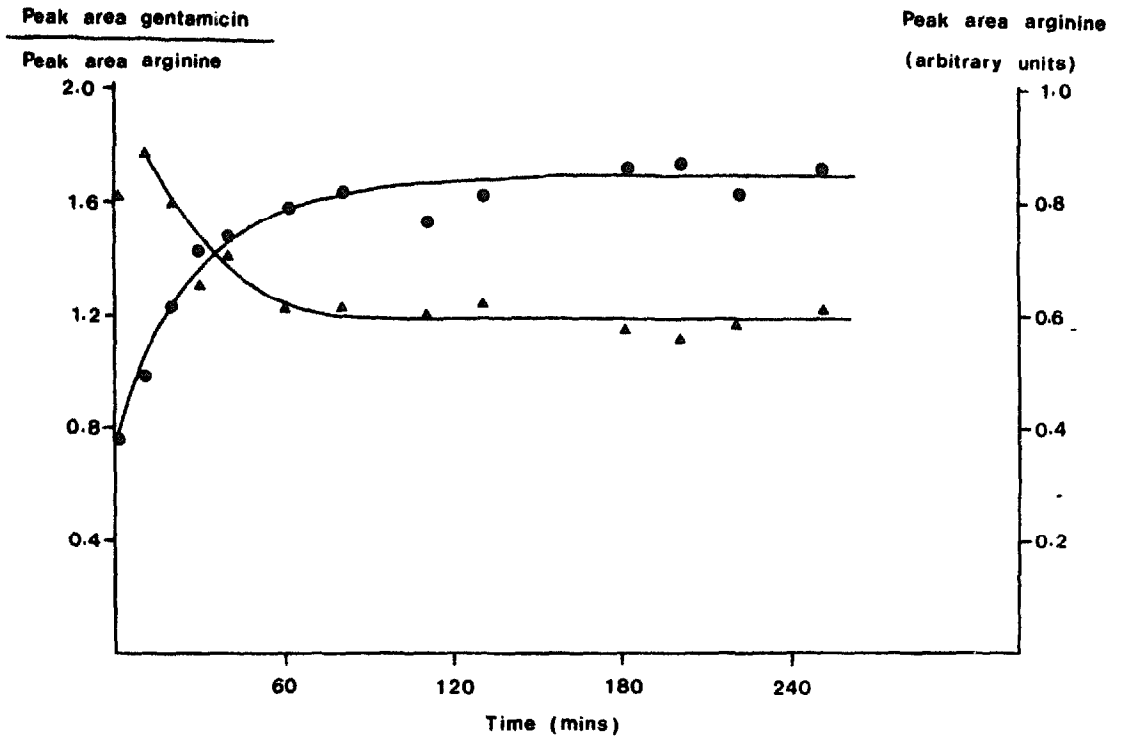

Fig. 2. The effect of derivatization reaction time upon the HPLC peak area of arginine, ▲———▲, and the peak area ratio of gentamicin/arginine, ●———●. The reaction was carried out in the dark at 20°C.

The stability of the gentamicin and arginine derivatives was investigated in order to establish optimum conditions for the assay. Derivatized arginine was not stable to light, the peak area declining rapidly with time. Fig. 2 illustrates that even when stored in the dark, the arginine derivative undergoes some decomposition but that after one hour both the peak area of the arginine derivative and the gentamicin/arginine peak area ratio became more stable. The assay method utilizes this plateau region.

Calibration curves prepared with standards were used to determine the absolute weights of gentamicins C_{1a}, C_2 and C_1 in the unknown samples. A series of 21 determinations performed on a single sample gave co-efficients of variation for gentamicins C_{1a}, C_2 and C_1 of 2.64, 2.18 and 1.23, respectively. Nineteen samples of commercial gentamicin from a variety of manufacturers and geographical sources were analyzed by this method (Table 1) and by a biological assay. Table 2 compares the total weights of the major components with the biological potency for each sample. These tables take no account of minor constituents for which no standards are available, though it is clear that insome samples the major components comprise less than 80% of the total weight. Such samples exhibited many small peaks on the chromatograms attributable to minor constituents (Fig. 3). It is notable that the biological potencies of these samples were not significantly different from that of the standard, suggesting that one or more of the minor constituents may possess biological activity. The chemical structures and biological activities of the minor

TABLE 1

CONTENT OF GENTAMICIN C COMPONENTS IN 19 COMMERCIAL SAMPLES OF GENTAMICIN DETERMINED BY HPLC

Sample	Country of origin	Water content (%)	Major components (mg/100 mg)			
			C_{1a}	C_2	C_1	Total
1	U.S.A.	3.8	23.7	37.9	35.3	96.7
2	U.S.A.	N.D.	23.6	36.0	14.6	74.2
3	U.S.A.	N.D.	13.0	41.6	39.8	94.4
4	U.S.A.	N.D.	15.3	45.6	37.2	98.1
5	U.S.A.	N.D.	19.8	50.4	32.1	102.3
6	Italy	N.D.	15.3	50.0	32.5	97.8
7	Hungary	N.D.	17.1	48.0	32.3	97.4
8	China	N.D.	22.0	39.7	20.3	82.0
9	Switzerland	3.7	27.8	28.5	25.2	81.5
10	Switzerland	3.8	25.4	29.3	26.5	81.2
11	Switzerland	3.8	25.0	24.7	24.9	74.6
12	Switzerland	3.2	21.7	28.6	24.7	75.0
13	Italy	3.8	13.1	54.7	16.2	90.0
14	Italy	3.7	19.1	54.8	15.9	89.8
15	Italy	3.7	19.3	55.3	16.0	90.6
16	Italy	4.1	19.2	54.6	16.1	89.9
17	Italy	3.9	19.2	54.4	16.2	89.8
18	Italy	4.1	19.3	55.3	16.3	90.9
19	U.S.A.	4.6	26.6	43.4	19.9	89.9

[a] N.D. = Not Determined.

TABLE 2

COMPARISON OF HPLC ASSAY AND BIOLOGICAL ASSAY OF GENTAMICIN SAMPLES

Sample	HPLC–total gentamicin C_1, C_{1a} and C_2 (mg/100 mg)	Standard deviation	Biological assay, potency	Standard deviation
1	96.7	4.2	100.0 [a]	
2	74.2	4.0	99.0	4.2
3	94.4	0.8	96.1	6.9
4	98.1	1.8	96.5	5.0
5	102.3	2.6	100.9	4.0
6	97.8	3.4	98.1	4.3
7	97.4	2.2	97.0	5.2
8	82.0	5.0	95.9	5.5
9	81.5	1.4	99.7	4.4
10	81.2	0.7	93.6	5.4
11	74.6	2.5	97.6	8.0
12	75.0	0.9	100.6	1.3
13	90.0	0.9	100.5	3.8
14	89.8	1.4	97.7	2.0
15	90.6	1.0	93.3	5.0
16	89.9	1.0	93.6	7.7
17	89.8	1.9	101.8	6.9
18	90.9	1.0	95.1	4.4
19	89.9	2.3	91.7	5.0

[a] This sample complied with B.P. specifications and was used as the standard for biological assays.

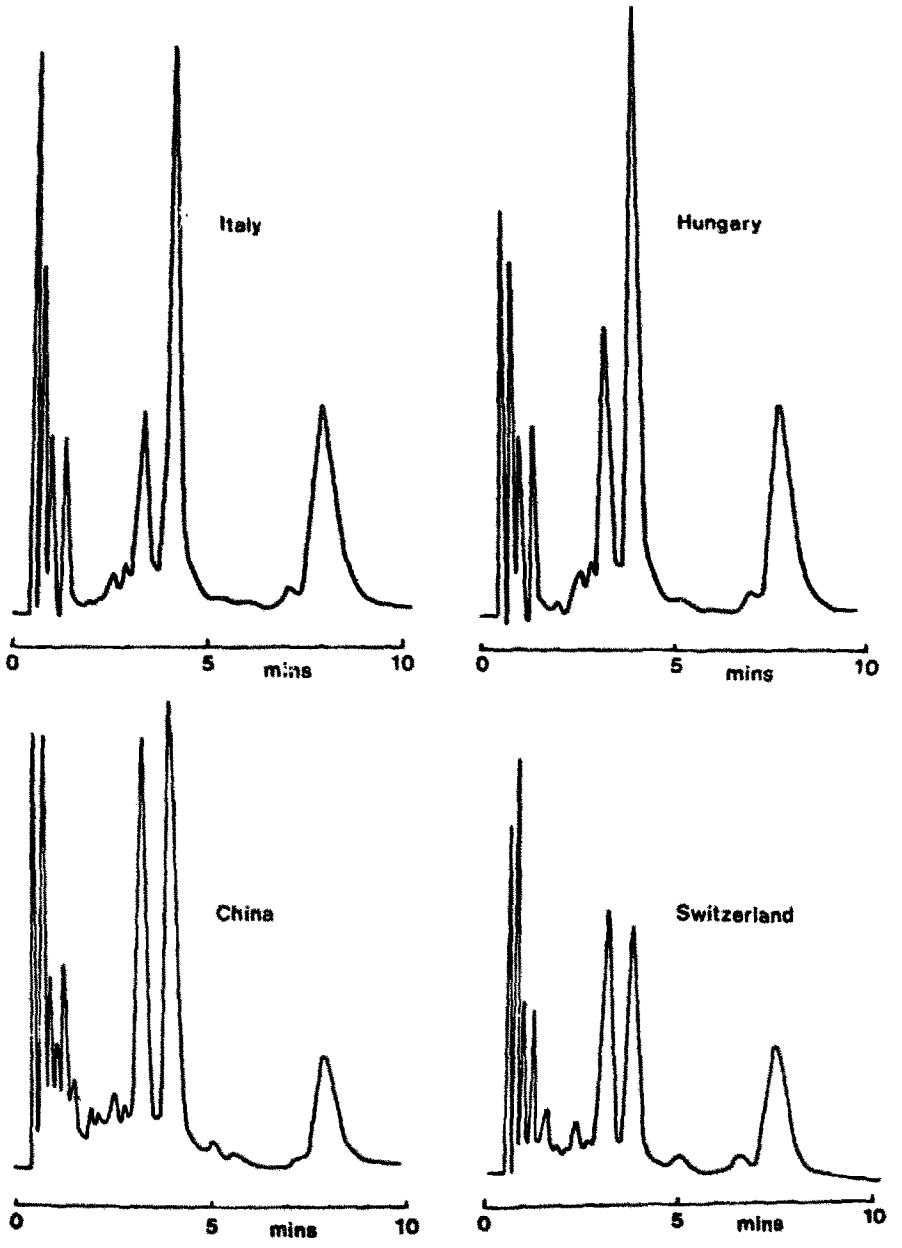

Fig. 3. High-performance liquid chromatograms of commercial samples of gentamicin sulphate obtained from a variety of manufacturing sources.

components are currently being investigated and will be reported.

Table 1 and Figs. 1 and 3 indicate that the component profiles of different batches of gentamicin vary much more widely than prevously reported (Wilson et al., 1972; Thomas and Tappin, 1974; Anhalt et al., 1978; Thomas, 1978). In addition to different ratios of major components, the minor components also differed from sample to sample with up to 8 peaks present in some cases. The geographical origin of the sample is a major source of variability. Thus all 7 Italian samples and the

single Hungarian sample are characterized by a high gentamicin C_2 content. The 4 Swiss samples contain substantial amounts of minor components including gentamicin C_{2b}, whilst material of Chinese origin which also has a high content of minor compounds has a different profile of both major and minor components. These differences may well reflect differences in the strain of producer organism, fermentation conditions or work-up procedure in the various manufacturing plants. Studies on the biological properties of gentamicins C_1, C_{1a} and C_2 suggest that these compounds differ only slightly in both antimicrobial activity and toxicity (Weinstein et al., 1967). Thus the observed variations in the major component ratios may be of little therapeutic significance, whereas the presence of minor components of unknown biological activity could be far more significant.

Where sufficient material was available the samples were also examined by nmr spectroscopy according to the method of the B.P. (1980) (Table 3). Samples 1, 9, 10, 11, 12, 13 and 19 all complied with the B.P. requirements though samples 9–12 inclusive (the Swiss samples) all had a relatively high content of minor components. Samples 14–18 inclusive failed to comply due to a high $\delta 1.25/\delta 1.35$ ratio consistent with their high proportion of gentamicin C_2.

Current official methods for the assay of gentamicin sulphate assess total potency by a microbiological method. The broad limits imposed on the gentamicin C component ratio by the B.P. nmr limit test will detect and exclude large deviations from the 'usual' ratio. However, this method is relatively insensitive to the presence of minor components. We believe that the rapid and sensitive HPLC method described here offers a more flexible and useful means of assessing and thereby controlling the constitution of the gentamicin complex.

TABLE 3

METHYL PEAK HEIGHT RATIOS DETERMINED ACCORDING TO THE B.P. nmr METHOD

Sample	Peak height ratios	
	$\delta 1.2/\delta 1.35$ B.P. limits (0.200–0.260)	$\delta 2.75/\delta 2.95$ B.P. limits (0.260–0.440)
1	0.233	0.371
9	0.225	0.337
10	0.227	0.337
11	0.242	0.353
12	0.232	0.338
13	0.250	0.277
14	0.266	0.294
15	0.276	0.281
16	0.270	0.294
17	0.274	0.290
18	0.276	0.293
19	0.260	0.312

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References

Amos, R., Evaluation of bonded phases for the high performance liquid chromatographic determination of polycyclic aromatic hydrocarbons in effluent waters. J. Chromatogr., 204 (1981) 469–478.

Anhalt, J.P., Sancilio, F.D. and McCorkle, T., Gentamicin C-component ratio determination by high pressure liquid chromatography. J. Chromatogr., 153 (1978) 489–493.

Cooper, D.J., Daniels, P.J.L., Yudis, M.D., Marigliano, H.M., Cuthrie, R.D. and Bukhari, S.T.K., The gentamicin antibiotics: Part III: The gross structures of the gentamicin C_2 components. J. Chem. Soc. C. (1971b) 3126–3129.

Cooper, D.J., Yudis, M.D., Marigliano, H.M. and Traubel, T., The gentamicin antibiotics: Part II: Separation and degradation of the gentamicin C components. J. Chem. Soc. C. (1971a) 2876–2879.

Daniels, P.J.L., Luce, C., Nagabhushan, T.L., Jaret, R.S., Schumacher, D., Reimann, H. and Illavsky, J., Gentamicin antibiotics: 6; gentamicin C_{2b}, an aminoglycoside antibiotic produced by *Micromonospora purpurea* mutant JI-33. J. Antibiotics, 28 (1975) 35–41.

Freeman, M., Hawkins, P.A., Loran, J.S. and Stead, J.A., The analysis of gentamicin sulphate in pharmaceutical specialties by high performance liquid chromatography. J. Liquid Chromatogr., 2 (1979) 1305–317.

Maitra, S.K., Yoshikawa, T.T., Hansen, J.L., Nilsson-Ehle, I., Palin, W.J., Schotz, M.C. and Guze, L.B., Serum gentamicin assay by high performance liquid chromatography. Clin. Chem., 23 (1977) 2275–2278.

Nice, E.C. and O'Hare, M.J., Selective effects of reversed-phase column packings in high performance liquid chromatography of steroids. J. Chromatogr., 166 (1978) 263–267.

Ogan, K. and Katz, E., Retention characteristics of several bonded-phase liquid chromatography columns for some polycyclic aromatic hydrocarbons. J. Chromatogr., 188 (1980) 115–127.

Panthananickal, A. and Marnett, L.J., Comparison of commercial reversed-phase high performance liquid chromatographic columns for the separation of benzo(α)pyrene diolepoxide-nucleic acid adducts. J. Chromatogr., 206 (1981) 253–265.

Parfitt, R.T., Games, D.E., Rossiter, M., Rodgers, M.S. and Weston, A., Chemical ionisation and field desorption mass spectrometry of the gentamicins. Biomed. Mass Spectrom., 3 (1976) 232–234.

The British Pharmacopoeia 1980, pp. 208–209, Pharmaceutical Press, London.

Thomas, A.H., Comparison of 3 methods of evaluating the composition of gentamicin sulphate. J. Pharm. Pharmacol., 30 (1978) 378–379.

Thomas, A.H. and Tappin, S.D., Separation of gentamicin complex by ion exchange column chromatography. J. Chromatogr. 97 (1974) 280–283.

United States Pharmacopoeia, 20th Edn., 1980, p. 1332, Mack Publishing, Easton, PA.

Weinstein, M.J., Wagman, G.H., Oden, E.M. and Marquez, J.A., Biological activity of the antibiotic components of the gentamicin complex. J. Bacteriol., 94 (1967) 789–790.

Williams, W.J., Handbook of Anion Determination, Butterworths, London, 1979, pp. 541–543.

Wilson, W.L., Richard, G. and Hughes, D.W. Chemical determination of component ratio and potency of the gentamicin complex. J. Pharm. Sci., 62 (1972) 282–284.

Wilson, W.L., Richard, G. and Hughes, D.W., Thin layer chromatographic identification of the gentamicin complex. J. Chromatogr., 78 (1973) 442–444.