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Control of the composition of gentamicin sulphate by proton magnetic resonance spectrometry

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The proportions of the main components present in gentamicin sulphate complex, gentamicins C_1, C_{1a} and C_2, can be monitored by ^1H nuclear magnetic resonance (nmr) spectrometry. The method depends on measurement of the peak heights of signals for *N*-methyl and *C*-methyl groups present in all three components and of those present in C_1 and C_2 only, followed by calculation of peak height ratios to control composition within acceptable limits. The precision and reproducibility of the method have been established through two collaborative studies each involving seven laboratories. In the second study, with an improved procedure, the mean variance between laboratories with 10 samples was $3{\cdot}4 \times 10^{-4}$ for the *N*-methyl ratio of the peak at δ 2·75 to that at δ 2·95, and $1{\cdot}25 \times 10^{-3}$ for the *C*-methyl ratio of the peak at δ 1·25 to that at δ 1·35. Within laboratories the mean variance for triplicate determinations was $7{\cdot}4 \times 10^{-5}$ and $8{\cdot}9 \times 10^{-5}$ respectively. The data presented here form the experimental basis for the test controlling the composition of gentamicin sulphate in the British Pharmacopoeia 1973: Addendum 1975, and for the introduction into the British Pharmacopoeia of nmr spectrometry as an analytical technique. The reference standards and all batches of gentamicin sulphate intended for therapeutic use in the United Kingdom examined by this procedure comply with the limits laid down.

Gentamicin, a broad spectrum antibiotic complex from *Micromonospora purpurea* (Weinstein, Luedemann & others, 1963), contains three main aminoglycoside aminocyclitol components (Wagman, Marquez & Weinstein, 1968) designated C_1, C_{1a} and C_2 (Fig. 1) (Cooper, Daniels & others, 1971).

The relative antibacterial activities of the three gentamicins C may differ; their relative toxicities have not been established although the complex is ototoxic and nephrotoxic. In the absence of control over the composition of the complex, specification of a minimum potency is impracticable and adequate control of toxicity is uncertain.

The first British Pharmacopoeia monograph for gentamicin sulphate (British Pharmacopoeia 1968: Addendum 1971a) was based on American specifications (Code of Federal Regulations, 1968) but omitting the test limiting the proportions of gentamicins C_1, C_{1a} and C_2. This omission was deliberate since the method is a paper chromatographic separation followed by bioassay of the individual components; it relies on the use of 'biological constants'. Experience has shown that such constants are satisfactory only in the laboratory in which they have been determined (British Pharmacopoeia 1968: Addendum 1971b).

The proton magnetic resonance (pmr) spectra of the individual gentamicins C_1, C_{1a} and C_2 as free bases in deuterium oxide (Cooper, Marigliano & others, 1969) permit clear distinction between them and suggested to us that pmr might be used to control the composition of the mixture. As a result of collaborative studies in 1973 and 1974, a method was evolved which was subsequently included in the monographs for gentamicin sulphate and gentamicin injection (British Pharmacopoeia 1973: Addendum 1975). The participants in the collaborative studies, apart from the authors were: M. D. Yudis, Schering Corporation; J. F. Chissell, M. Freeman, Roussel Laboratories; R. E. King, R. T. Parfitt, M. Rogers, Nicholas Laboratories; D. W. Mathieson, University of Bradford; J. H. Hunt, Allen and Hanburys; C. A. Johnson and Miss C. M. King, British Pharmacopoeia Commission. The coded samples were prepared by P. J. Campbell (National Institute for Biological Standards and Control).

This paper reports some of the results of these collaborative studies; more detailed information may be obtained from D. H. Calam at the above address.

MATERIALS AND METHODS

Purified samples of gentamicins C_1, C_{1a} and C_2 as sulphates were supplied by Dr M. D. Yudis, Schering Corporation. All other samples of gent-

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amicin sulphate were reference standards or material in current therapeutic use.

Solutions, 10 or 20% (w/v) in deuterium oxide (min 99·7 atom % deuterium), were prepared for pmr examination.

For the collaborative studies aqueous solutions of samples, or gentamicin injection, were coded and then distributed into glass ampoules in 1 ml aliquots and lyophilized. The ampoules were sealed under nitrogen. No participant was aware of the code assignment.

Pmr spectra were recorded mainly at 60 MHz on Perkin Elmer R12A and R12B spectrometers. A Perkin Elmer 24A, Varian A60, EM360 and HA100D were also used. Sweep width was 10 ppm and scan speed 10 or 11 min.

Procedure for 1st study. The resolution of the instrument was optimized as described by the manufacturer. The H_1 level used was such that saturation of the measured signals did not occur (checked by ensuring that peak height ratios did not alter significantly when the spectrum was re-run at a lower H_1 setting). The sensitivity was adjusted so that the signals at about δ 1·35 and about δ 2·95 occupied between 60 and 95% of the available chart height. The base-line variation measured immediately on either side of the largest peak was required to be not more than 2% of the chart height (to confirm correct setting of the phase control). The entire contents of each ampoule were dissolved in 0·5 ml deuterium oxide. Use of internal or external standards was allowed. For each solution the absorption spectrum only was recorded three times on separate charts. The peak height ratios δ 2·75 : δ 2·95 and δ 1·25 : δ 1·35 were determined, all heights being measured with respect to the baseline between δ 0·5 and δ 1·00.

Procedure for 2nd study. Precision pmr tubes (Wilmad 505 or equivalent) were used throughout. Sealed tubes containing 0·5 ml of a solution of one of the reference standards (20% w/v) in deuterium oxide (Sample T) were also distributed for analysis. All solutions were filtered, then freed from oxygen by slow bubbling of nitrogen. The methods for recording three spectra and measurement were unchanged. Spectrometers were adjusted to give a resolution of better than 0·35 Hz (acetaldehyde quartet); a sweep width of 8 or 10 ppm, a scan speed of about 1 Hz s^{-1} and a filter setting which gave a baseline noise level between δ 0 and δ 0·5 of 1–3% were used. The phase control was adjusted to the pure absorption mode using the water signal from 10% (w/v) magnesium sulphate heptahydrate, analytical grade, in D_2O. Adjustment of H_1 power was achieved by scanning one sample of gentamicin sulphate at successive settings then selecting the highest setting at which the height of the peak at δ 2·95 exceeded that at δ 1·35. The sensitivity was adjusted on each sample so these two peaks occupied as near 90–95% of the available chart height as the controls would permit.

RESULTS AND DISCUSSION

In the ^1H spectrum of a typical sample of gentamicin sulphate in D_2O at 60 MHz (Fig. 2), the peaks of interest and their assignments are: δ 2·95: $N-CH_3$ present in all three components (Fig. 1), δ 2·75: the second $N-CH_3$ present in C_1, δ 1·35: $C-CH_3$ present in all three components, δ 1·25: one peak of the methyl doublet for $CH-CH_3$ present in C_1 and C_2; the other, buried in the side of the δ 1·35 peak, is partially separated in the spectrum of the sulphate at 100 MHz.

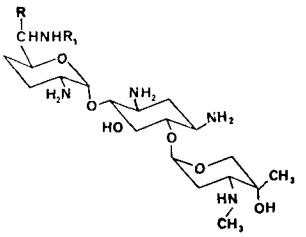

FIG. 1. The structures of gentamicins C_1, C_{1a} and C_2. C_1: R = R_1 = CH_3; C_2: R = CH_3; R_1 = H; C_{1a}: R = R_1 = H.

The type of instrument used in this work was limited to those operating at 60 MHz since these are more generally available, and changes in resolution, especially of the *C*-methyl resonances, at other operating frequencies prevented direct comparison of results. Peak heights were selected as basis for measurement of resonances in view of the highly subjective nature of integration curve measurement.

One factor which has to be taken into account is the limited reproducibility of recording a spectrum on a single instrument. This problem was minimized in the collaborative studies by recording three spectra, the means of which allowed peak height ratios to be determined with confidence limits of approximately ±5% for *N*-methyl and ±10% for *C*-methyl. Three is probably the minimum number of spectra which should be used in a test of this type.

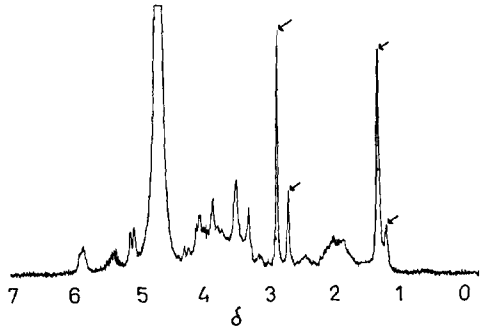

FIG. 2. The ^1H nuclear magnetic resonance spectrum of 20% (w/v) gentamicin sulphate in deuterium oxide, recorded at 60 MHz. The peaks used to obtain the ratios limiting composition are indicated with arrows.

The first collaborative study indicated the need to improve the signal to noise ratio; this was achieved by using 20% solutions. It further revealed the differential effects of the H_1 level on the peaks of interest. The order of peak saturation with increasing H_1 power was δ 2·95, δ 2·75, δ 1·35 and δ 1·25; thus both *N*-methyl and *C*-methyl ratios passed through a minimum value then increased. Phase adjustment and sweep rate were also shown to influence the results. These parameters were adjusted as described earlier under *procedure for second study*.

The resolution of the spectrometers employed in the second study varied between 0·27 and 0·33 Hz, with the signal : noise ratio (for ethylbenzene) of between 21 : 1 and 37 : 1. With some instruments, the phase control setting for the magnesium sulphate reference differed slightly from that for gentamicin sulphate.

To overcome the difficulties of defining the adjustments necessary on a wide variety of instruments produced by several manufacturers, the use of a reference sample was investigated. However, a set of sealed tubes containing the same solution of gentamicin sulphate were examined by four laboratories with acceptable agreement and reproducibility, showing that the revised directions were adequate without use of a reference. These sealed tubes (sample T) were distributed with the other samples in the second study, and the scatter of results obtained using them did not differ from that for the solid samples.

Two samples common to both studies provided a means for assessing the improvement in results. The ranges of ratios obtained during the second study were less than half those obtained during the first study (Table 1). Statistical evaluation by

Table 1. *Ranges of peak height ratios (mean values in brackets) determined in the seven laboratories for the two samples included in both collaborative studies.*

	1st study	2nd study
Sample A		
NMe	0·056 (0·310)	0·024 (0·306)
CMe	0·052 (0·221)	0·023 (0·224)
Sample B		
NMe	0·066 (0·427)	0·030 (0·412)
CMe	0·045 (0·234)	0·018 (0·232)

analysis of variance (Table 2) showed that variation between laboratories is significantly greater than the variation within laboratories.

During both studies, a number of artificial mixtures containing weighed amounts of the individual gentamicin C_1, C_{1a} and C_2 sulphates were prepared. The *N*-methyl ratio should give a good indication of the amount of gentamicin C_1 in a mixture of the gentamicins since the second $N-CH_3$ group giving rise to a signal at δ 2·75 is present only in C_1. This prediction was borne out; to a close approximation, the ratio found × 100 gives the percentage of C_1 directly and estimates made in this way agree well with expectation and with those made by a colorimetric method based on paper chromatography (M. D. Yudis, personal communication). The *C*-methyl ratio indicates the proportion of $C_1 + C_2$ in a mixture of the three components, and thus by difference the amount of C_{1a}. The relationship is less direct than for the *N*-methyl ratio since the $CH-CH_3$ group in C_1 and C_2 gives rise to a doublet, which would be expected to be no more than half the height of the peak at δ 1·35, so that the ratio will be less responsive to overall composition. Linear least squares analysis

Table 2. (*a*) *Variances* (× 10^3) *between the seven laboratories of mean value of peak heights and* (*b*) *means of the variance within laboratories of triplicate determinations of peak height ratios.* Data obtained in the second collaborative study.

	NMe		CMe	
Sample	(a)	(b)	(a)	(b)
1	0·305	0·026	0·375	0·033
2	0·329	0·030	0·688	0·051
3	0·309	0·099	0·423	0·086
4	0·381	0·069	1·905	0·047
5	0·459	0·057	0·740	0·058
6	0·090	0·034	1·365	0·130
7	0·108	0·053	0·817	0·087
8	0·719	0·169	3·396	0·241
9	0·464	0·102	0·798	0·050
T	0·243	0·076	2·045	0·106
Mean	0·341	0·072	1·255	0·089

of the results derived from the artificial mixtures gave the following characteristics, demonstrating the better fit of the *N*-methyl data:

$$Y = 9{\cdot}0434 \times 10^{-3}X + 3{\cdot}4055 \times 10^{-2}$$
$$\text{Sum of squares} = 3{\cdot}3917 \times 10^{-3} \text{ (12 points)}$$

where X is the % C_1 and Y is the *N*-methyl peak height ratio.

$$Y = 1{\cdot}7077 \times 10^{-3}X + 1{\cdot}0815 \times 10^{-1}$$
$$\text{Sum of squares} = 6{\cdot}1998 \times 10^{-4} \text{ (11 points)}$$

where X is the % $C_1 + C_2$ and Y is the *C*-methyl peak height ratio.

The lines do not pass through the origin because of contributions from neighbouring peaks to the measured peak of interest.

Determination of the percentage of each of C_2 and C_{1a} would involve accumulation of the errors in determination of C_1 as well as in the *C*-methyl ratio measurement. Because of this, no attempt was made in the collaborative studies, or in the test as finally adopted, to determine percentage composition but only to determine limits within which all material satisfactory in other respects should fall.

Since the test was designed with the limited objective, in the first instance, of controlling composition within defined limits, data were accumulated on as many as possible of the batches of gentamicin sulphate which had been used or were intended for therapeutic use in the U.K. From the results it was possible to set limits of 0·26–0·44 for the *N*-methyl ratio and 0·20–0·26 for the *C*-methyl ratio. These would include all material which has been shown to be clinically acceptable.

This pmr method is very rapid, simple in execution and applicable both to the bulk antibiotic and to the injection formulation. However, it is capable of providing control only over the relative proportions of the gentamicin C components. It provides no control over other gentamicins nor over the proportion of gentamicin C complex in the material. It was recognized at the outset that traces of gentamicin A cannot be measured by pmr but since the gentamicin monograph contains a chromatographic test limiting impurities including gentamicin A to those present in the reference and at a lower level, adequate control is ensured. Attempts were made unsuccessfully to exert some control over related compounds, containing either *C*-methyl or *N*-methyl groups, by setting limits for the ratio of the heights of the peaks at δ 1·35 and δ 2·95.

A new British Pharmacopoeia Chemical Reference Substance of gentamicin sulphate, more representative of material in current use, was circulated to four of the laboratories which had participated in the collaborative studies. It was examined by them under the usual conditions; the mean results and ranges of the means were: *N*-methyl ratio 0·369 (0·352–0·388), *C*-methyl ratio 0·223 (0·221–0·237).

To our knowledge, the present paper is the first in which a putative pmr method has been submitted to critical appraisal and experimental assessment at the hands of several laboratories in a collaborative study, thus allowing the necessary statistical evaluation of the method and effects of instrumental variation on which the widespread acceptance of pmr as a viable analytical technique for quantitative analysis will ultimately depend. The results are reassuring in many respects. It is clearly possible with appropriate definition of experimental conditions to apply pmr to a relatively complex problem with an acceptable level of agreement between different laboratories using different instruments. We hope that our results may encourage others to explore the use of pmr in pharmaceutical analysis.

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REFERENCES

British Pharmacopoeia 1968: Addendum (1971a), 38–41.

British Pharmacopoeia 1968: Addendum (1971b), xii.

British Pharmacopoeia 1973, Addendum (1975), 22.

Code of Federal Regulations (1968), Title 21, Food and Drugs, Part 148q. 1.b.8.

Cooper, D. J., Daniels, P. J. L., Yudis, M. D., Marigliano, H. M., Guthrie, R. D. & Bukhari, S. T. K. (1971), *J. chem. Soc. (C)*, 3126–3129.

Cooper, D. J., Marigliano, H. M., Yudis, M. D. & Traubel, T. (1969), *J. infect. Dis.*, **119**, 342–344.

Wagman, G. H., Marquez, J. A. & Weinstein, M. J. (1968), *J. Chromat.*, **34**, 210–215.

Weinstein, M. J., Luedemann, G. M., Oden, E. M. & Wagman, G. H. (1963), *Antimicrob. Ag. Chemother.*, 1–7.