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Studies of carbon-13 n.m.r. spectroscopy in pharmaceutical analysis: the composition of commercial samples of gentamicin sulphate

J. KOUNTOURELLIS, R. T. PARFITT, A. F. CASY*

School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

Two procedures for applying ^{13}C n.m.r. spectroscopy to the quantitative analysis of the C_1, C_{1a}, and C_2 components of gentamicin sulphate samples are described. One is based on the use of calibration plots of peak height ratios of analyte to standard (dioxan) resonance intensities recorded under conditions of full relaxation, the other upon a steady-state experiment and the use of weighting factors. Spectrometer operating conditions are discussed, and the need for measurement of longitudinal relaxation time (T_1) data described. Results for seven commercial samples are given and comparisons made between the two n.m.r. methods and an h.p.l.c. procedure in terms of accuracy, specificity and convenience.

The recent report of h.p.l.c. methods for analysing the three main components of gentamicin sulphate (Kraisintu et al 1981) provides a convenient analytical standard against which to examine the potential of ^{13}C n.m.r. spectroscopy in the quantitative analysis of commercially available mixtures. In general the application of n.m.r. spectroscopy to pharmaceutical analysis has been hampered by problems of low sensitivity and high cost of instrumentation, but in the present example its specificity, direct applicability (no derivatization is required) and non-destructive nature (important for expensive and/or scarce analytes) suggested evaluation of the technique under standard operating conditions.

Proton resonance spectroscopy has been used to control within broad limits the ratios of the main components (C_1, C_{1a} and C_2) of gentamicin; it is, however, unsuitable as a specific assay procedure (Calam et al 1978). ^{13}C n.m.r. spectra of mixtures, even of closely related structures, offer high probability of the identification of resonance signals characteristic of individual components because of the large chemical shift differences in ^{13}C resonances which appear as narrow singlets under conditions of proton-noise decoupling (Levy et al 1980). Fig. 1 illustrates the line separation for a mixture of three gentamicin components in a commercial preparation. All lines arise from carbons of ring C of the gentamicins, and their chemical shifts reflect the structural differences between the three components (Fig. 2). Spectra of base and sulphate forms of

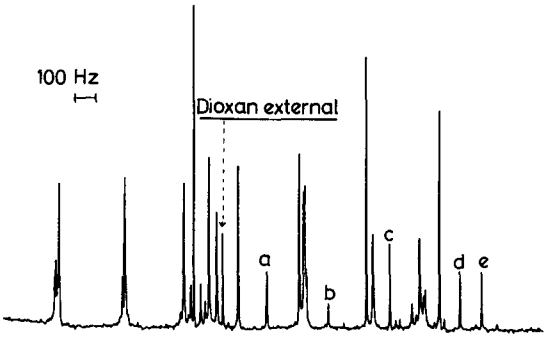

FIG. 1. C.m.r. spectrum of the total base (150 mg) recovered from a commercial sample of gentamicin sulphate (Nicholas Laboratories) in deuterium oxide (1 ml) with external dioxan as standard (operating conditions as in text). Analytical resonances expressed in ppm from TMS, employing 67·4 ppm as the chemical shift of dioxan: C_1 (a) C-6 near 58, (c) C-8 near 34, and (e) C-7 near 15; C_{1a} (b) near 46; C_2 (d) near 19 ppm. Spectral data of the gentamicins as individual components and mixtures correspond closely with those of Morton et al (1973).

gentamicins differ significantly as a result of upfield shifts due to protonation of the many basic centres of the amino-sugar molecules. The decision to perform n.m.r analyses on the free bases—necessitating an ion exchange step—was made because individual components were isolated in this form, and because of the better resolution of the reference dioxan signal in spectra of de-protonated species.

In contrast to ^1H n.m.r. spectra the intensities of ^{13}C resonances usually correlate poorly with spin populations. The reasons are (1) variations in the relaxation times of the carbon atoms of the molecule, (2) differential nuclear Overhauser enhancement (NOE) factors for the different carbon resonances,

* Correspondence.

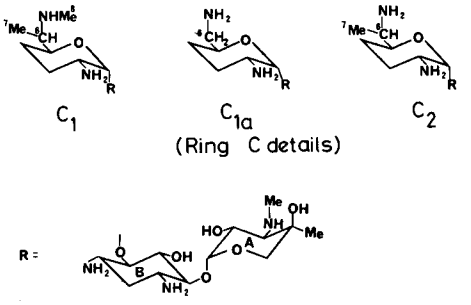

FIG. 2. The structures of gentamicins C_1, C_{1a} and C_2.

and (3) instrumental effects. Allowance was made for (1) by introducing a delay between pulses, long enough to permit complete relaxation of the analytical spin system (at least five times the longest spin-lattice relaxation time of the relevant carbons). Differential NOE effects were avoided by adopting a procedure based on the relationship between peak height ratios of analyte to standard resonance intensities, and analyte concentration (cf Yamasaki & Fujita 1979). Slope differences in calibration graphs confirmed the differential NOE factors; these did not, however, influence the accuracy of the assay.

The inversion recovery method was used to determine T_1 relaxation times of the gentamicin carbons whose resonance signals had been selected for the analyses (details in Materials and Methods section). Four different concentrations of each major component were examined as solutes in deuterium oxide. The results (Table 1) reveal a progressive decrease in each T_1 value with increasing concentration, due to viscosity and intermolecular association changes (Wehrli & Wirthlin 1978). Relaxation times of carbons determined from mixtures of the gentamicin complex were close to those found using the single component, provided total concentrations were similar and hence viscosity differences minimal. At a concentration of 150 mg ml^{-1}, the largest T_1 value of the group is 1·44 s corresponding to C-8 of the C_1 component. Hence the minimum pulse delay is 7·2 s ($5 \times T_1$) and the value of 13 s actually employed is appropriate to the lower concentrations used to construct the calibration plots where larger T_1 values obtain.

Table 1. Longitudinal relaxation times (T_1) of the ring C carbon atoms of gentamicins C_1, C_{1a} and C_2 employed for quantitative analysis.[a]

Concentration mg ml^{-1} in D_2O	50	100	150	350	100[b]	350[b]
T_1 s of C_1 (C-7) 15·0% ppm[c]	0·88	0·37	0·30	0·18	0·29	0·18
T_1 s of C_1 (C-8) 33·8% ppm[c]	2·85	1·53	1·44	0·64	1·20	0·60
T_1 s of C_1 (C-6) 58·6% ppm	2·74	1·01	1·05	0·43	0·98	0·40
T_1 s of C_{1a} (C-6) 45·4% ppm	3·64	3·54	1·15	0·68	3·13	1·01
T_1 s of C_2 (C-7) 18·7% ppm	1·10	0·98	0·91	0·27	1·06	0·37

[a] Measured in deuterium oxide by the inversion recovery method (details in text); values calculated from at least 7 points of an 11 point maximum programme.
[b] From spectra of mixtures of approximate composition C_1 35%, C_{1a} 25%, C_2 35%.
[c] The higher T_1 values of the C_1 methyl C-8 compared with those of C-7 are evidence that the former carbon (terminal atom of the C-5 side chain) undergoes significant relaxation by the spin-rotation mechanism.

Dioxan was used as an *external* standard; this gives a single resonance at 67·4 ppm (from TMS in D_2O) well resolved from signals due to the gentamicins (Fig. 1). Used thus, subsequent recovery (by lyophilization after D_2-H_2 exchange) and re-use of the pure gentamicin components was facilitated. A 5% solution of dioxan in deuterium oxide had a T_1 value of 4·17 s. This value is higher than those of the gentamicin analytical carbons but was lowered to about 2·3 s by mixing four parts of the solution with one part of a solution saturated with oxygen, to ensure full relaxation after a pulse delay of 13 s.

Some instrumental points are now specified: (1) A 90° pulse (20 μs for a 10 mm tube) which yields optimal signal to noise ratios (Abraham & Loftus 1980) was employed as the experiments were run under conditions of full relaxation. The acquisition time was 0·8 s approx. as defined by the spectral width of 5000 Hz and number of data points; (2) The computer resolution (spectral width divided by the number of real data points) is close to 1·25 Hz for a 16K computer with 8K data points on a 5000 Hz sweep width. At this resolution the area of resonance signals with widths at half maximum height ($W_{0.5}$) of 1·25 Hz or more will be sufficiently defined by peak height intensities provided signal broadening is not too pronounced (Shoolery 1977; Shoolery & Jankowski 1973). For most of the resonances recorded in this work $W_{0.5}$ values between 2 and 3 Hz (measured from 10 fold expansions of 5000 Hz spectral width spectra) were found (not sharpened after de-gassing). Peak heights were obtained directly from spectra rather than the computer print-out values to avoid possible dependence upon the method of computation (Martin et al 1980). Errors in setting the base line and threshold level are involved in either procedure. In an attempt to compensate for the small number of data points available per signal, intensity values used for calibration and assays are means of five runs each of 1500 to 2200 scans.

The relationships between concentration of each component (mg ml^{-1}) and the intensity ratio

(analyte:dioxan standard resonance) are linear and are given below.

C_1 (C-6) y = 1·136x − 0·008 (0·997)
(C-7) y = 1·068x − 0·032 (0·995)
(C-8) y = 1·25x − 0·009 (0·997)
C_{1a} (C-6) y = 0·893x − 0·028 (0·998)
C_2 (C-7) y = 1·075x − 0·005 (0·997)
y (intensity ratio), x (concentration)

Analyses of seven gentamicin samples expressed as free bases are compared both with h.p.l.c. results and with methyl proton peak height ratios in Table 2. This shows (1) standard deviations of the ^{13}C n.m.r. analyses are mostly greater than those obtained with the h.p.l.c. procedure; (2) variations found for the C_1 assays were generally lower than those for C_{1a} and C_2 components (although ranges of variation were similar), probably due to the C_1 analyses being based on the intensities of three resonances rather than one; (3) minor components (estimated by difference) are only readily detected by ^{13}C n.m.r. spectroscopy when they give rise to resonances outside the chemical shift range of 14 to 102 ppm because of the spectral complexity of the three main components themselves within this region. One gentamicin sample, for example, showed four clearly defined low intensity resonances near 165 ppm indicative of impurities of an aromatic or unsaturated nature; (4) the *N*-methyl peak height ratio (δ 2·75/2·95 based on proton resonances, B.P. 1980) should provide a measure of C_1 gentamicin content since the δ 2·75 resonance is due to this component alone. Comparative results show that the ratio remains relatively constant over a C_1 concentration range of 19 to 25% with significant rise only where the mixture contains 34% of this base.

MATERIALS AND METHODS

Gentamicin sulphate samples from a variety of manufacturers and geographical sources were converted to corresponding free bases by passing aqueous solutions through an ion-exchange resin column (Amberlite IRA-400 OH form). Sulphate-free eluates were lyophilized and stored in a desiccator. Before analysis the samples were dried at 85 °C to constant weight. Pure standards of the three major components were prepared from a gentamicin sulphate mixture (Nicholas Laboratories) by the method of Cooper et al (1971). The identity and purity of each component was confirmed by field desorption m.s. (Parfitt et al 1976), t.l.c. and h.p.l.c. (Kraisintu et al 1981) in addition to n.m.r. spectroscopy. Proton-noise decoupled ^{13}C spectra were measured on a JEOL FX 90Q n.m.r. spectrometer operating at 22·5 MHz. Samples were examined in 10 mm o.d. tubes at concentrations 50 to 350 mg ml^{-1} in deuterium oxide (not de-gassed except for the T_1 determinations); the deuterium of the solvent provided the lock signal. The sealed capillary containing the standard dioxan (5% solution in D_2O, see text) was placed coaxially in the tube before its insertion in the probe. Spectra were recorded with 8K data points, at a probe temperature of 23 °C and an average spectral width of 5000 Hz. Details of pulse, acquisition time, pulse

Table 2. Analyses of gentamicin samples according to h.p.l.c. and ^{13}C-n.m.r. (calibration plot) procedures together with methyl peak height ratios (^1H-n.m.r.).

Sample[a]	Composition by h.p.l.c.[b,c]				Composition by c.m.r.[c,d]				Methyl peak height ratios[f]	
	C_1	C_{1a}	C_2	Minor[e]	C_1^g	C_{1a}^h	C_2^i	Minor[e]	1·2/1·35	2·75/2·95
1	34·1	24·4	34·1	7·4	34·6 (7)	23·8 (9)	34·5 (10)	7·0	0·23	0·37
2	19·6	20·0	49·5	10·8	19·1 (11)	23·1 (10)	49·4 (12)	8·4	0·25	0·28
3	20·3	19·6	47·8	12·2	21·1 (6)	21·4 (5)	48·1 (9)	9·4	0·27	0·29
4	20·9	19·5	46·8	12·7	19·5 (4)	21·1 (9)	46·8 (4)	12·6	0·28	0·28
5	21·8	19·3	49·1	9·9	21·8 (8)	19·4 (11)	49·4 (8)	9·4	0·27	0·29
6	19·7	20·5	50·2	9·7	19·2 (3)	21·2 (8)	50·2 (12)	9·4	0·27	0·29
7	25·4	18·6	50·1	5·9	25·3 (4)	18·6 (9)	50·7 (13)	5·4	0·28	0·29

[a] Sample 1 is from an American (USA) source and the rest are of Italian manufacture.
[b] Kraisintu et al 1981.
[c] mg free base in 100 mg free base mixture; values from c.m.r. data are means of percentages calculated from each of five runs. Percentage standard deviations are shown in parentheses for c.m.r. results (standard deviations for the h.p.l.c. data were not more than ±5%).
[d] Details of spectrometer conditions and external standard (dioxan) are given in the text; analyte concentrations were 125 to 150 mg free base in 1 ml deuterium oxide.
[e] Minor components obtained by difference.
[f] Calam et al 1978; chemical shifts (δ ppm) of resonances employed are shown below.
[g] Mean of values based on resonances at 15, 33·7 and 58·2 ppm.
[h] Based on resonance at 46·3 ppm.
[i] Based on resonance at 18·8 ppm.

delay and number of scans are given in footnotes to the Tables. The sequence 180° pulse - delay τ - 90° pulse was used in the inversion recovery method of determining spin-lattice relaxation times (T_1), each cycle being interrupted by a pulse delay that allowed full relaxation (Vold et al 1968). The program was designed to take a maximum of 11 pulse spacings (τ) in the range $\tau << T_1$ to $\tau < T_1$, and values reported (Table 1) were calculated from the data of at least seven of such sequences.

Steady-state conditions

A second quantitative procedure was examined which was based on intensity data from spectra run under conditions of steady-state rather than fully relaxed magnetization, and achieved by reducing the pulse delay from 13 s to 1 s (cf Blunt & Munro 1976). Under such conditions intensity variation amongst individual resonances will occur due to non-identical T_1 times and NOE factors. These may be reduced to a minimum by (a) running spectra under conditions of closely similar concentrations and temperatures, and the use of identical pulse widths and delay times, and (b) the use of a small pulse width whereby perturbation of spin populations is not great (a 25° rather than 90° pulse was used).

In moderately large molecules, such as the gentamicins, relaxation occurs chiefly by a dipole-dipole mechanism characterized by T_1 relaxation times and is very sensitive to the viscosity of the medium through its influence on rates of molecular tumbling. Variations in T_1 rates (and the attendant NOE factors which operate only via dipole-dipole interactions) may therefore be kept small if the total solute concentration is held within narrow limits. Since solutes concerned here are all of similar molecular nature and size, viscosity variations will then also be minor. When a small pulse is used and peak intensities involve comparison amongst carbon nuclei that do not differ unduly in T_1 values, then as concentration changes the steady-state magnetizations of all carbons will alter in similar degree leaving the ratios of intensities little changed; the procedure thus permits some flexibility in sample concentration. Choice of a small pulse necessitates longer accumulation times than those required after a 90° pulse in order to recover an adequate signal to noise ratio, and scans were accordingly increased to 4000. However, the method obviates the need for lengthy calibration data collection and requires only knowledge of weighting factors* which relate individual peak intensities and the standard peak intensity, to the amount of each component present, and may be obtained from single samples of known composition. Dioxan was again used to provide the standard peak but used *internally* as a 0·3% solution in deuterium oxide. Its relaxation time was assumed to fall below the value of 4·17 s recorded for a 5% solution in the same solvent, as a result of the high viscosity of solutions used in the steady-state procedure. In addition, application of the small pulse ensured little variation in the degree of steady-state magnetization for carbons exhibiting T_1 values 0·3 to 5 s (cf Blunt & Munro 1976).

The assays of seven samples of gentamicin by this method are set out in Table 3: they agree generally within a few per cent with those established by the calibration procedure but with standard deviations of somewhat greater range. The minor gain in precision following use of calibration data does not, however, offset the labour and time-saving advantages of the steady-state method.

Table 3. Analyses of commercial samples of gentamicin by c.m.r. spectroscopy under steady-state conditions.[a]

	Composition[c]		
Sample[b]	C_1	C_{1a}	C_2
1	35·9 (10)	20·4 (9)	34·7 (8)
2	18·8 (13)	25·3 (13)	46·3 (12)
3	20·4 (9)	23·4 (11)	46·4 (10)
4	18·5 (9)	22·7 (13)	47·7 (11)
5	20·9 (8)	21·4 (12)	47·4 (13)
6	18·4 (8)	23·1 (14)	46·9 (12)
7	23·5 (8)	20·7 (14)	48·7 (13)

[a] Spectra run using solutions containing approximately 150 mg antibiotic in 1 ml 0·3% dioxan in deuterium oxide, with 25° pulse, acquisition time 0·8192 s, pulse delay 1 s, and spectral width 5000 Hz. Weighting factors and analytical determinations are based on five runs each of 4000 scans. The weighting factors of each gentamicin component were determined in the presence of the other two in 50:25:25 mixtures, calculations being applied to the major constituent.

[b] Numbered samples correspond with those of Table 2.

[c] Footnotes c,g-i of Table 2 apply.

The order of accuracy achieved by ^{13}C n.m.r. methods is low compared to the h.p.l.c. procedure and probably arises from errors in recording resonance intensities. With a computer resolution of about 1·25 Hz (see earlier in this paper), errors in recording the full intensity of a resonance signal are compounded if absorption signals are very narrow ($W_{0.5} \leq 1$ Hz). In this study $W_{0.5}$ values of all analyte resonances fell between 2 and 3 Hz while the dioxan signal was distinctly narrower (1 to 1·5 Hz), a discrepancy which could well lead to a difference in

* Weighting factor = standard peak intensity × weight of analyte divided by peak intensity of analyte.

the relative accuracies of analyte and standard resonance intensities. It is likely, therefore, that the accuracies of the ^{13}C n.m.r. procedures described could be improved by use of more efficient methods of integration. Artificial broadening of each peak (by applying an exponential weighting factor to the free induction decay before it is Fourier-transformed) (Mareci & Scott 1977) is not acceptable in the analysis of gentamicins because of attendant loss of resolution. The degree of computer resolution may be improved by using spectral widths of 2500 Hz or less (gentamicin analyte and standard resonances fall within 1300 Hz) but such narrower spectra require acquisition times (number of data points divided by twice the spectral width) considerably greater than those used in the present procedures.

If orders of accuracy superior to those of the methods here described are not critical, then the advantages of ^{13}C n.m.r. procedures over other methods are clear. Both calibration and steady-state variants are simple to apply once parameters on reference compounds have been established (calibration plots or weighting factors) and entail no loss of material. Spectral data recorded during the assays provide precise characterization of the three main components of gentamicin mixtures and may yield evidence of minor components and contaminants. Although the hardware and theory of n.m.r. spectroscopy (especially when ^{13}C nuclei are involved) are complex, technological advances in the field permit operations, once established, to be carried out in a routine manner reproducible and amenable to automation. It may be concluded from this exploratory study that the potential of ^{13}C n.m.r. spectroscopy for quantitative analysis is great and that studies of its application to pharmaceutical analysis (especially when distinctions between analytes of closely related structure are involved) are well worth undertaking.

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