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An isocratic separation of underivatized gentamicin components, ^{1}H NMR assignment and protonation pattern

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Abstract

A simple method for the separation of the major components of commercial gentamicin sulfate (C-1, C-1a, C-2, C-2a) by high-performance liquid chromatography (HPLC) on an analytical and a semipreparative scale was developed. The method utilized ion-pair reversed-phase chromatography, isocratic elution with an aqueous solution containing 9% trifluroacetic acid and 2.5% acetonitrile as the mobile phase at a flow rate of 2 and 9 mL/min for analytical and semipreparative columns, respectively. Detection was carried out at 213 nm without derivatization. The protonation pattern of the separated gentamicins was determined by potentiometry and ^{15}N and ^{1}H NMR. The full proton NMR assignment for gentamicin C-1 was obtained through the use of ^{1}H 1D and 2D ^{1}H–^{1}H COSY measurements.

Keywords: Aminoglycosides; HPLC; Potentiometery; NMR spectroscopy

1. Introduction

Gentamicin C is a broad-spectrum aminoglycoside antibiotic widely used for the treatment of serious infections caused by both Gram-negative and -positive bacteria.[1] Gentamicin kills bacteria by damaging the plasma membrane and binding to the 16S ribosomal RNA, leading to the inhibition of microbial protein synthesis.[2] In contrast, the mechanisms of aminoglycoside toxicity to the inner ear and possibly the kidney is postulated to result from free-radical formation by redox-active complexes with transition metal ions.[3] Natural and semisynthetic gentamicin-related aminoglycosides share a similar structure containing multiple aminosugar and aminocyclitol rings, with the number of amino functions typically varying between four and six (Fig. 1). These amino groups and the additional hydroxyl groups determine their chemical properties.

The chemistry of aminoglycoside antibiotics was extensively studied in the late 1960s and 1970s through ^{1}H and ^{13}C NMR spectroscopy.[4–6] A major focus of these studies was the delineation of structure and stereochemistry mainly through methanolysis. The development of 2D NMR techniques led to assignments of spin systems of a variety of aminoglycosides including tobramycin,[7] amikacin,[8] neomycin,[9] apramycin,[10] kanamycin A and B and butirosin.[11] The full assignment of the ^{1}H NMR spectrum of gentamicin C-1 components, however, has not been reported to the best of our knowledge. Proton assignments are necessary to determine covalent modifications performed by microbial enzymes (which lead to deactivation of the antibiotics), determination of the solution structures, and interaction of the drug with RNA and DNA targets.

Commercial gentamicin is a mixture of gentamicin C-1, C-1a, C-2 and C-2a. The separation of aminoglyco-

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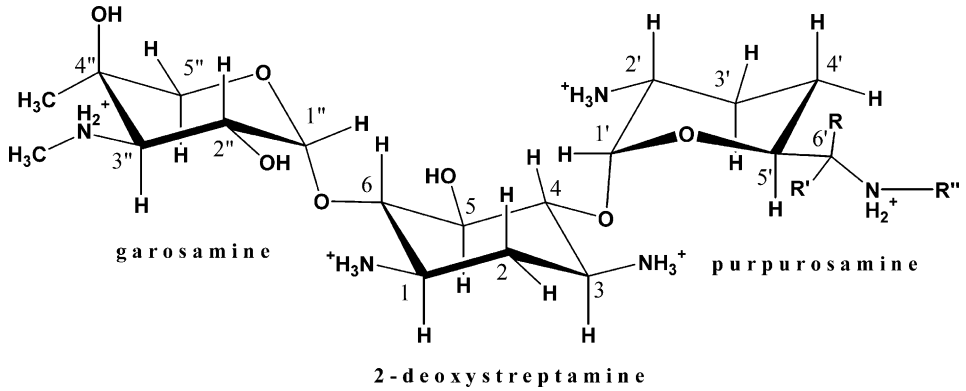

Fig. 1. The structure of the four major components of commercial gentamicin C. Gentamicin C-1, R′ = H, R = R″ = CH_3; C-2, R = CH_3, R′ = R″ = H; C-2a, R′ = CH_3, R = R″ = H; C-1a, R = R′ = R″ = H.

side antibiotics has mainly been accomplished by high-performance liquid chromatography (HPLC). Most of the existing methods require derivatization, which alters their biochemical properties, or the use of refractive index, mass, fluorescence and amperometric detectors.[12–18] The goal of the current study was to develop a simple and effective method for the analytical and semipreparative separation of the aminoglycosides that can avoid their derivatization or the application of specialized detectors. This paper describes the separation of the commercial gentamicin C complex by HPLC using reversed-phase chromatography, isocratic elution and UV detection. In addition, the protonation patterns of gentamicin C-1, C-1a, C-2 obtained from of potentiometry, ^1H and ^{15}N NMR spectrometry are reported. Full proton ^1H NMR assignments of gentamicin C-1 at pH 4 and 11 are also presented. This information will be useful in investigations of aminoglycoside interactions with transition metals, which could lead to a better understanding of the mechanisms underlying their toxicity and possibly result in modification of the drugs to reduce toxicity.

2. Experimental

2.1. Materials

Gentamicin C complex, containing C-1, C-1a, C-2 and C-2a was obtained as the sulfate salt from Spectrum Chemicals. MeCN (HPLC grade) and potassium nitrate were purchased from Fisher Scientific. Trifluroacetic acid (TFA), 3-(trimethylsilyl)propionic acid sodium salt (TSP) and the strongly basic anion exchanger Amberlite IRA-400 were obtained from Sigma Chemical Co. Deuterated solvents were purchased from Cambridge Isotope Laboratories. All chemicals with the exception of gentamicin were used without further purification.

2.2. HPLC

The closely related components of the gentamicin C complex C-1a, C-1, C-2 and C-2a, were separated from commercial gentamicin sulfate by HPLC, using a Waters Prep LC 2000 system equipped with Hamilton PRP1 reversed-phase columns (250 × 4.1 mm, particle size 10 μm, or 250 × 21.50 mm, particle size 12–20 μm), and a Waters variable wavelength absorption detector (set at 213 nm) with a flow cell of 3-mm path length and 2.5 μL volume, HP 3395 integrator and Rheodine injector having 50-μL and 2-mL loops for analytical or semipreparative separations. The best separation, using isocratic elution, was achieved with an aq solvent containing 9% TFA and 2.5% MeCN at a flow rate of 2 or 9 mL/min on analytical and semipreparative columns, respectively. The mobile phase was prepared using deionized, doubly distilled water; TFA and MeCN were of HPLC grade. All separations were carried out at rt, and fractions were collected according to the peaks in the spectrum. Each fraction was lyophilized, redissolved in water and passed through a column packed with Amberlite IRA-400 to exchange TFA for chloride. Finally, the samples were lyophilized and stored as solids.

2.3. Electrospray-ionization mass spectrometry (ESIMS)

Mass spectra were obtained with a Micromass VG Platform instrument, equipped with a single quadrupole mass analyzer using the ESI technique, with nitrogen as a nebulizing gas, and a flow of 350 L/h. Samples of 10 μL were injected into a solvent stream of 50% MeCN and 0.1% formic acid. A scan range of 50–2000 *m/z* was used. Samples were prepared by dissolving separated gentamicin C-1a, C-2, C-2a and C-1 hydrochloride salts in water. Then MeCN was added to a final concentration of 50% (v/v) in order to decrease surface tension. The final gentamicin concentration was 0.5 mM.

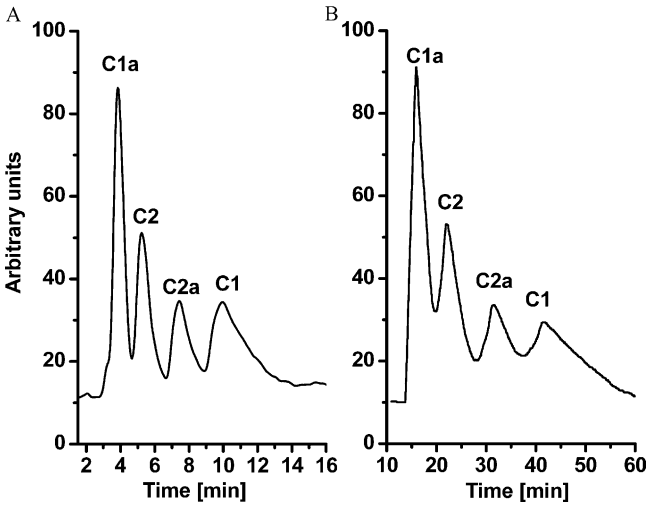

Fig. 2. Ion-pair isocratic chromatograms of commercial gentamicin sulfate. Data were obtained as described in Section 2 using a Hamilton PRP1 reversed-phase column (A) 250 × 4.1 mm, particle size 10 μm and (B) 250 × 21.50 mm, particle size 12–20 μm, respectively. Fractions labeled C-1a, C-2, C-2a and C-1 correspond to gentamicin C-1a, C-2, C-2a and C-1, respectively.

2.4. Potentiometry

Protonation constants for gentamicin C-1a, C-1, and C-2 were determined by potentiometric titration of the purified hydrochloride salts with carbonate-free KOH. The ionic strength of the solutions was maintained at 0.1 M by the addition of KNO_3. Sample volumes of 2 mL were contained under a CO_2-free argon atmosphere in a jacketed cell that was kept at 25 °C by an external circulating water bath. The titrations were controlled by a custom-built autotitrator running within Labwindows v 2.1 on a PC. The autotitrator included a Dosimat Model 655 autoburet equipped with a 1-mL cylinder so that the titrant could be delivered in 1-μL increments. The pH values were measured by a Fisher model 25 pH meter equipped with a Hamilton combination electrode calibrated daily to read the hydrogen-ion concentration by titrations of nitric acid. The autotitrator prompted the addition of an aliquot of titrant, monitored the pH until the drift fell below a preset maximum value, recorded the volume and pH, and then made the next addition of titrant. Typical titrations covered the pH range of 3–12. Data were analyzed using the software program SUPERQUAD.[19]

2.5. NMR spectroscopy

1D and 2D H–H COSY proton and ^{15}N NMR spectra of gentamicin C-1 were collected on a Varian Inova 500 NMR Spectrometer operating at 499.84 MHz. For the ^{1}H NMR studies, samples were prepared by dissolving gentamicin C-1 in 99.9% D_2O to a concentration of 0.001 M. TPS (3-(trimethylsilylpropionic acid sodium salt) was used as an internal standard. ^{15}N NMR measurements were performed as previously described.[20] Solutions of commercial gentamicin C were prepared at higher concentrations (0.6–0.8 M) in order to collect ^{15}N NMR spectra within a reasonable time. An internal deuterium lock was obtained by dissolving gentamicin in a 15% D_2O soln. A satd soln of NH_4Cl was used as an external reference. All measurements were collected at 25 °C. The pH of the samples was adjusted with small amounts of concentrated NaOD and DCl, and the pH-meter reading was not corrected for isotope effects of deuterium on the electrode.

3. Results

3.1. HPLC and ESIMS

The optimal conditions for separations of the commercial gentamicin C components (C-1a, C-1, C-2 and C-2a) by HPLC using isocratic elution and UV detection

Table 1
Summary of the observed ESIMS ions (*m*/*z*) from the HPLC fractions shown in Fig. 2 [a]

	Observed *m*/*z*	Assignment
fraction C-1a	450.4	gentamicin C-1a
	322.4	gentamicin–purpurosamine
	160.0	garosamine or 2-deoxystreptamine
	128	purpurozamine
fraction C-2	464.4	gentamicin C-2
	322.3	gentamicin–purpurosamine
	160.0	garosamine or 2-deoxystreptamine
	142.1	purpurosamine
fraction C-2a	464.4	gentamicin C-2a
	322.3	gentamicin–purpurosamine
	160.0	garosamine or 2-deoxystreptamine
	142.1	purpurosamine
fraction C-1	478.5	gentamicin C-1
	322.3	gentamicin–purpurosamine
	160.0	garosamine or 2-deoxystreptamine
	156	purpurosamine

[a] Spectra were collected in the positive-ion mode as described in Section 2. Purpurosamine, garosamine, 2-deoxystreptamine correspond to the prime, double prime and central ring of gentamicin C components as presented in Fig. 1.

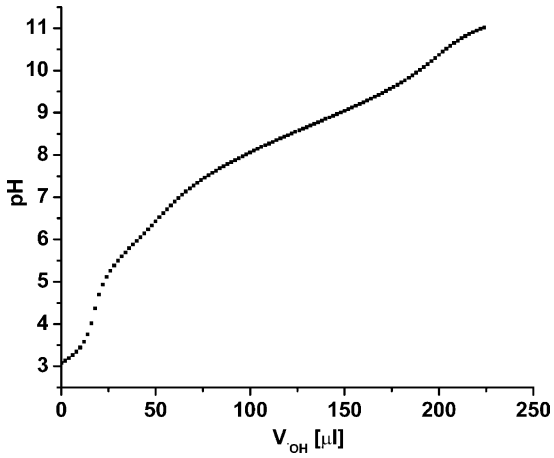

Fig. 3. Potentiometric titration curve of gentamicin C-1. For conditions see Section 2. The inflection points in the curve indicate that deprotonation of gentamicin C-1 starts around pH 4.5 and ends 10. Elaboration of this curve using the software program SUPERQUAD[19] yielded five pK_as (listed in Table 2) that correspond to deprotonation of the five amino groups present in the gentamicin C-1 molecule.

without derivatization were developed using a Hamilton PRP1 reversed-phase analytical column (250 × 4.1 mm, particle size 10 μm). The best baseline separation (presented in Fig. 2(A)) was achieved with eluent containing 9% TFA and 2.5% acetonitrile at a flow rate of 2 mL/min. The total injected amount of gentamicin sulfate was 2 mg (dissolved in eluent at 590 μm/mg), and the limit of detection was observed at 0.2 μg. The presence of TFA in the mobile phase was necessary for satisfactory separation because gentamicin C components were essentially not retained on the column in the absence of counter ions. Omission or decrease of the concentration of acetonitrile resulted in the increase of retention time and broadening of peaks while an increase of the concentration of acetonitrile caused overlapping peaks. Variations of the flow rate within 1 mL/min did not have much influence on the resolution.

The method was the adapted to the separation of the components of gentamicin C on a semipreparative scale using a Hamilton PRP1 reversed-phase column (250 × 21.50 mm, particle size 12–20 μm) as shown on Fig. 2(B) and a flow rate of 9 mL/min. Chromatographic fractions associated with each of the gentamicin components, shown in Fig. 2, were collected, and the purity of each component was confirmed from the characteristic fragmentation patterns in the mass spectra (Table 1).

3.2. Potentiometry and NMR spectroscopy

The protonation patterns of the gentamicin components C-1a, C-1 and C-2 were studied by potentiometery and ^{1}H and ^{15}N NMR spectroscopy at 25 °C over the pH range 3–12. Gentamicin C-2a was not included because it is an isomer of gentamicin C-2 at the 6′-position of the purpurosamine ring. Fig. 3 shows the potentiometric titration curve of gentamicin C-1. Analysis of the titration curves yielded five deprotonation constants for each gentamicin C component corresponding to the five amino groups present in gentamicin C-1a, C-1 and C-2 (Table 2). The species distribution diagram for gentamicin C-1 showing its protonation pattern as a function of pH was then calculated from the protonation constants and the concentrations applied (Fig. 4). pK_a values were also obtained from ^{1}H and ^{15}N resonance spectra of gentamicin C-1 and commercial gentamicin respectively as a function of pH (Table 3(A) and (B)).

To correlate the macroconstants derived from pH titrations with the deprotonation of a specific amino group, one-dimensional ^{1}H and ^{15}N NMR spectra were obtained for gentamicin C-1 and commercial gentamicin in unbuffered D_2O at various pH between 4 and12 (Fig. 5(A) and (B)). The data for gentamicin C-1 on the dependence of the ^{1}H chemical shift on pH and the 2D

Table 2
Protonation constants (log β and pK_a values) of gentamicin C-1a, C-2, C-1 [a]

	Gentamicin C*1a		Gentamicin C-2		Gentamicin C-1	
Species	log β	pK_a	log β	pK_a	log β	pK_a
H_5L	39.689(1)	5.768	39.85(1)	5.83	39.801(1)	5.686
H_4L	33.921(1)	7.389	34.021(1)	7.421	34.115(1)	7.317
H_3L	26.532(1)	8.181	26.600(1)	8.211	26.798(1)	8.121
H_2L	18.351(1)	8.86	18.389(1)	8.793	18.677(1)	8.817
HL	9.491(1)	9.491	9.596(1)	9.596	9.86(1)	9.86

[a] Potentiometric titrations were performed on the purified hydrochloride salts with carbonate-free KOH. The ionic strength of the solutions was maintained at 0.1 M by the addition of KNO_3. Sample volumes of 2 mL were contained under a CO_2-free argon atmosphere in a jacketed cell that was maintained at 25 °C by an external circulating water bath as described in Section 2. Values for log β were calculated from β $(H_nL) = (H_nL)/(L)(H^+)^n$ using the software program SUPERQUAD[19]; standard deviations of the last digit are given in parentheses.

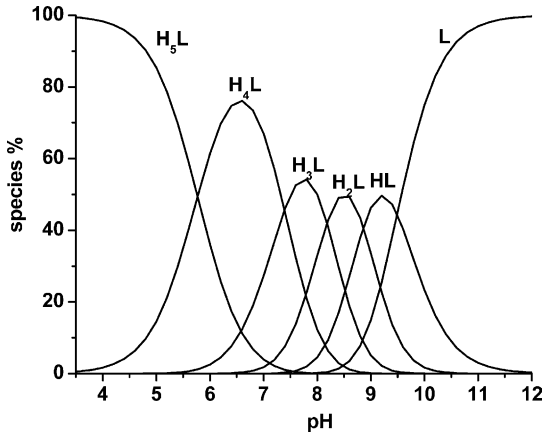

Fig. 4. Species distribution diagram of gentamicin C-1 showing its protonation pattern as function of pH. The diagram was constructed using protonation constants obtained from potentiometry (Table 2) and the concentration applied in this method (1 mM). LH_5, LH_4, LH_3, LH_2, LH, L correspond to gentamicin C-1 molecule carrying the following charge: +5 (fully protonated), +4, +3, +2, +1 and 0 (fully deprotonated), respectively.

H–H COSY spectra recorded at pH 4 (Fig. 6) and 11 allowed for assignment of its spin system (Table 4). The values of J_{HH} essentially did not change by more than 0.5 Hz upon variation of pH between 4 and 11. The protonation constants (pK_a^*) were calculated from the titration curves for each proton and nitrogen signal (Fig. 7) using the Hill equation. The measured pH values were not corrected for deuterium isotopic effects on the electrode because the interaction does not influence the magnitude of the pK_as.[21]

4. Discussion

Although aminoglycosides do not contain strongly absorbing chromophores, they can be detected in the UV region of the spectrum at 213 nm. With an appropriate mobile phase, as demonstrated here, the four forms of commercial gentamicin can be separated and detected without derivatization. Three analytical methods, ESIMS spectrometry, potentiometry and ^1H NMR spectroscopy confirm that pure gentamicin components (C-1, C-1a, C-2, C-2a) could be obtained. Principal fragments in the mass spectra of each separated gentamicin indicate the formation of the garosamine, purpurosamine, and 2-deoxystreptamine units during the ionization process, confirming that glycosidic bonds of aminoglycosides are the most susceptible to cleavage.[22]

The inflection points in the titration curve of gentamicin C-1 (Fig. 3) demonstrate that a stepwise deprotonation of this compound takes place in the pH range between 4.5 and 10. Analysis of this curve yielded five protonation constants ranging from 5.586 to 9.86 (Table 2). Further inspection of the data revealed a close agreement of the constants between the gentamicin C-1a, C-2, and C-1 components and suggested that they share the same protonation pattern. In view of this similarity and the structural relationships of gentamicin C-1a, C-2, and C-1, which differ only in the degree of methylation of the purpurosamine unit, their ^1H NMR spectra can also be expected to be similar. Therefore, ^1H NMR measurements were only conducted on gentamicin C-1.

The advantage of gentamicin C-1 for ^1H NMR is that it has two secondary amino groups on garosamine (3″-N) and purpurosamine (6′-N), and expected singlets of the methyl moieties in the ^1H NMR spectra can conveniently be used to monitor deprotonation of these

Table 3
(A) Protonation constants (pK_a^*) of gentamicin C-1 obtained using ^1H NMR titration [a]

Gentamicin C-1		
Group	pK_a^*	N
3	6.19 ± 0.4^a	0.4 ± 0.1^a
2′	7.40 ± 0.2^b	0.7 ± 0.1^a
1	7.67 ± 0.4^c	0.5 ± 0.1
3″	8.78 ± 0.03^d	0.9 ± 0.1
6′	9.86 ± 0.1^e	0.9 ± 0.1

(B) pK_a^{**} values and ^{15}N chemical shifts (relative to NH_4Cl saturated in water as external standard) of the amino groups of the commercial gentamicin C components [b]

Amino group nitrogen	pK_a^{**}	Most downfield shift (δ ppm)	Most upfield shift (δ ppm)
3-N	7.22 ± 0.2	15.03	8.2
2′-N	8.17 ± 0.2	13.60	7.095
1-N	8.91 ± 0.2	14.78	8.07
3″-N	9.25 ± 0.2	8.81	−2.85
6′-N	9.99 ± 0.2 [c]	2.99 [c]	−7.21 [c]

[a] Spectra were obtained at 25 °C and samples were prepared by dissolving gentamicin C-1 in 99.9% D_2O to a concentration of 0.001 M. Protonation constants were calculated from the dependence on pH of the chemical shift of 4-H (a), 2′-H (b), 6-H (c), 3″-CH_3 (d), 6′-CH_3 (e) using the Hill equation; N, cooperativity coefficient; pK_a^* values are given with standard deviations.

[b] ^{15}N NMR measurements were performed as previously described.[23] Solutions of commercial gentamicin C (0.6–0.8 M) were prepared in 15% D_2O. pK_a^{**} values are given with standard deviations.

[c] Most likely corresponds to the resonance ^{15}N of gentamicin C-1a (the compound with the highest concentration in commercial gentamicin) due to the chemical shift of the signal, as discussed in Section 4.

groups. The chemical shifts of the protons of gentamicin C-1 are sensitive to pH because of the change in protonation state of the amino groups (Fig. 5(A) and Table 4). This phenomenon was used to calculate the pK_a^* values for each of the protonation sites in gentamicin C-1. Singlets of the protons of the CH_3 groups of both 3″- and 6′-secondary amino groups, as well as the multiplet of 2′-H, yielded uniform titration curves, described by the cooperativity coefficients of 0.9 ± 0.1, 0.9 ± 0.1 and 0.7 ± 0.1, respectively (Fig. 7(A) and Table 3(A)). pK_a^* values obtained in these calculations are numerically similar to the corresponding macroconstants derived from potentiometry (Table 2).

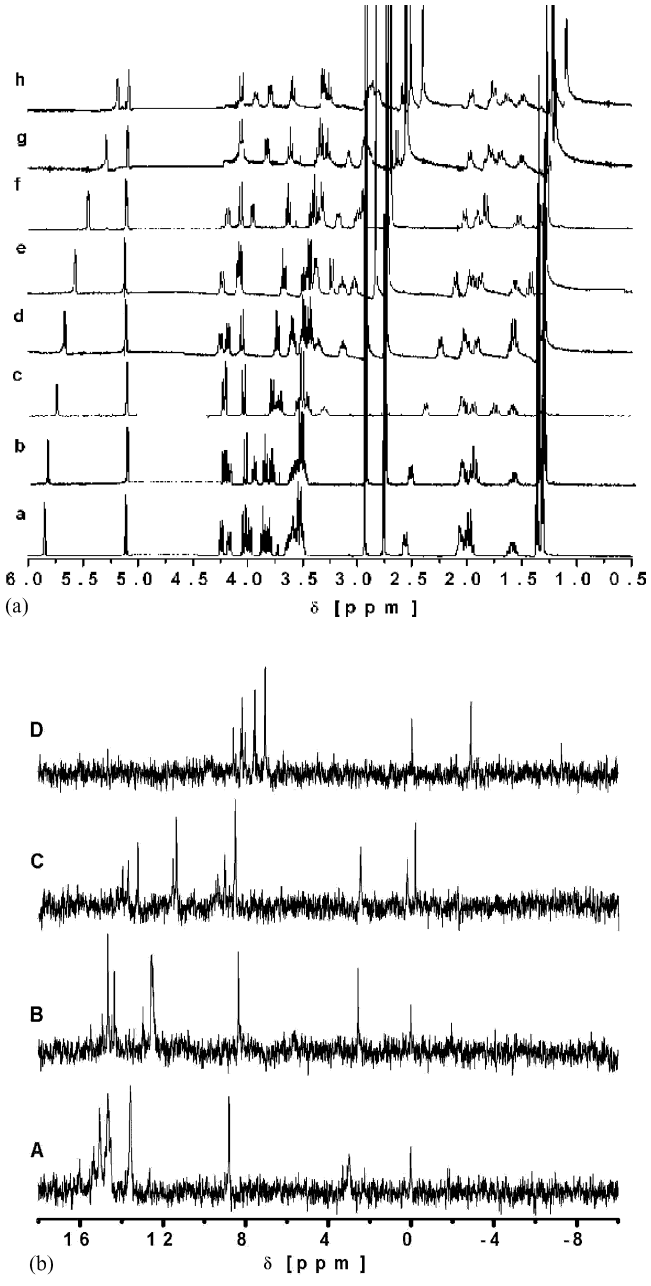

Fig. 5. NMR spectra of gentamicins. (A) ^1H NMR spectra of gentamicin C-1 (1 mM in D_2O) recorded at pH 4 (a), 5.2 (b), 6 (c), 7.2 (d), 8.12 (e), 8.9 (f), 10.2 (g), and 10.9 (h). To simplify the figure the proton signal from water was manually removed. (B) ^{15}N NMR spectra of commercial gentamicin C (0.6–0.8 M in 15% D_2O) recorded at pH 4.8 (a), 7.1 (b), 9.1 (c), and 11.2 (d). For the conditions of mesurements see Section 2.

In contrast, the determination of the pK_a^* for the 1- and 3-amino groups was more complicated since the chemical shifts for all protons belonging to the 2-deoxystreptamine ring were affected by the deprotonation of either group. Additionally, ^1H signals overlapped extensively. The chemical shift of 1-H, 2-H, (axial and equatorial) and 3-H had the appearance of two titration shifts due to deprotonation of the first amino group to form an H_4L corresponding the molecule carrying $+4$ charge (the first titration step) followed later by deprotonation the next the group belonging to the same moiety (the third titration shift). Thus, this chemical shift data cannot be used to assign the particular protonation sites. However, the signals of the 4-H and 6-H protons were satisfactorily resolved at 500 MHz, and their chemical shift titration curves were used to determine the pK_a^* of these groups. These calculations yielded lower values for the cooperativity coefficients and higher deviations from the values obtained from potentiometry. Lower values of the cooperativity coefficients suggest interactions between the 1- and 3-amino groups.

Another commonly used method for determining pK_a values of amino groups of the aminoglycoside antibiotics[23–27] is monitoring of ^{15}N resonances as a function of pH (Fig. 7(B)). For this procedure, a separation of commercial gentamicin is not essential because gentamicin C-1a, C-2, C-2a and C-1 have four chemically identical amino groups (1, 3, 2′ and 3″) with very close pK_a values, so that they deprotonate in the same pH range. Consequently, the ^{15}N signals of each of these nitrogens will be observed at the same chemical shift and with a higher intensity compared to those nitrogens modified by methylation of the 6′ group of purpurosamine ring. Indeed, four signals of higher intensity were detected in the ^{15}N NMR spectra of gentamicin C (Fig. 5(B)). An assignment of these ^{15}N resonances could be made based on previously published assignments of other aminoglycosides with similarly located amino groups and similarities in the magnitudes of the pK_a values.[20,23–27] The signal at 2.99 ppm obtained at pH 4.8 corresponds to the ^{15}N resonance of the 6′-amino group of gentamicin C-1a. The methylated amino group (present in gentamicin C-1) would be shifted downfield by ~ 3 ppm, and the amino group of gentamicin C-2 and C-2a (isomers differing only by orientation of the 6′-methyl group) would be seen between 3 and 6 ppm.[20] The pH profiles

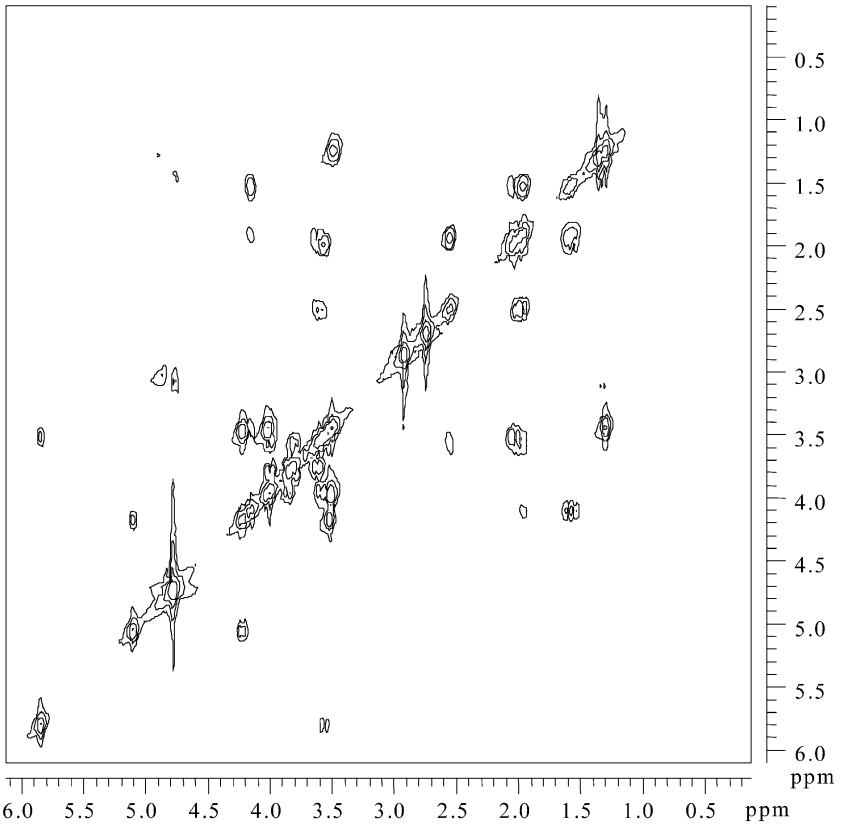

Fig. 6. The correlation (COSY) spectrum of gentamicin C-1 (0.001 M in D_2O) recorded at pH 4. The spectrum was collected in the absolute value mode on a Varian Inova 500 NMR spectrometer operating at 499.84 MHz and processed using Varian software as described in Section 2.

(Fig. 7(B)) and the pK_a values (Table 3) are in good agreement with previously published results obtained under similar conditions.[24–27]

Significant differences in pK_a values emerge when comparing results obtained from ^{15}N NMR with the data from potentiometry and ^{1}H NMR. However, the much higher concentration of gentamicin sulfate utilized in the ^{15}N NMR titration (0.6–0.8 M) caused a much higher ionic strength and viscosity of the samples as compared to potentiometry and ^{1}H NMR (0.001 M). Both factors influence protonation constants. This may also explain differences to values reported by Botto and Coxon.[23] These authors studied gentamicin-related aminoglycosides using ^{15}N NMR at considerably lower concentrations (0.05–0.1 M) than we did. In any case, the protonation pattern of the gentamicin C components obtained from ^{1}H and ^{15}N NMR studies is the same, suggesting that deprotonation of the amino groups proceeds in the following manner: 3, 2′, 1, 3″ and 6′.

The protonation patterns of gentamicin C-1a, C-1 and C-2 are similar to those previously reported for other gentamicin-related aminoglycosides.[28] Within each compound, the pK_a value of the 3-amino group of the 2-deoxystreptamine ring is significantly lower than that of the other amino groups, suggesting that deprotonation of the 3-amino group increases the basicity of the 1-NH_2 sufficiently so that its deprotonation takes place at a higher pH. The most likely reasons for this behavior is the high overall positive charge of the fully protonated molecules and electrostatic repulsion of the two amino groups belonging to the 2-deoxystreptamine moiety. The other protonation constants range from 7 to 10, which is typical for simple amino sugars with similarly located amino groups.[29,30] Methylation of the purpurosamine moiety of gentamincin C components leads to the increase of the pK_a value of the 6′-amino group, due to the electron-releasing ability of the methyl moiety.

The values of J_{HH} coupling constants indicate that all three rings of gentamicin C-1 exist in chair conformations, which remain unchanged throughout the investigated pH range, despite the charge change from +5 to 0.

Table 4
Chemical shifts δ and coupling constants J_{HH} (in parentheses) of gentamicin C-1 at pH 4 and 11 [a].

Proton	δ and J_{HH} pH 4	δ and J_{HH} pH 11	Protonation shift
1-H	3.601 [b] (11.230, 3.906) [a]	2.922 [b]	0.679
2-H_{eg}	2.558 (12.451, 4.395)	1.973 (12.845, 3.950) [c]	0.585
2-H_{ax}	1.974 [b] (12.695)	1.247 [b]	0.727
3-H	3.580 [b] (11.230, 3.906)	2.812 [b]	0.768
4-H	3.989 (9.277)	3.602 (9.521)	0.296
5-H	3.859 (9.033)	3.308 [b]	0.551
6-H	3.801 (10.01)	3.267 (9.666)	0.534
1′-H	5.848 (3.418)	5.198 (3.385)	0.65
2′-H	3.455 [b] (3.906, 11.230) [d]	2.875 [b]	0.58
3′-H_{ax}	4.167 (2.441 12.207)	3.934 (2.521, 11.963)	0.233
3′-H_{eg}	3.553 [b]	3.265 (9.863)	0.288
4′-H_{eg}	1.985 [b]	1.785 [b]	0.2
4′-H_{ax}	1.594 (4.395, 12.207)	1.503 (4.395, 12.207)	0.091
5′-H	1.981 [b] (12.695, 3.906)	1.174	0.807
6′-H	3.496 [b]	2.925 [b]	0.571
6′-CH_3	1.314 (6.836)	1.110(6.592)	0.204
6′-N–CH_3	2.754	2.409	0.345
1″-H	5.110 (3.662)	5.098 (4.150)	0.012
2″-H	4.235 (3.906, 10.254)	3.804 (3.960, 10.254)	0.431
3″-H	3.522 [b] (10.742)	2.591 (10.742)	0.931
3″-N–CH_3	2.930	2.525	0.405
4″-CH_3	1.360	1.223	0.137
5″-H_{eg}	4.036 (12.695)	4.064 (12.695)	0.028
5″H_{ax}	3.531 0[b] (12.695)	3.320 [b] (12.695)	0.211

[a] For notation see Fig. 1. TPS (3-(trimethylsilylpropionic acid sodium salt) was used as an internal standard. Spectra used to determine δ and J_{HH} were collected as described in Section 2.
[b] Denoted overlapping lines; if a coupling constant is not listed it was impossible to obtain it due the extensive overlapping of the signals.
[c] Coupling constant obtained at pH 8.89.
[d] Coupling constant obtained at pH 7.12.

5. Conclusion

The components of commercial gentamicin C have sufficiently different chromatographic mobility to be separated in a single HPLC run using an ion-pair eluent on a reversed-phase column. UV detection at 213 nm is sensitive enough to monitor the elution of the separated components without derivatization.

The three gentamicins studied share similar protonation patterns with a low pK_a value for the 3-amino group of the 2-deoxystreptamine moiety, while the other amino groups undergo deprotonation in the typical pH range for simple amino sugars. Both ^{1}H and ^{15}N NMR titrations can be applied to determine protonation constants of particular amino groups and the concentration of the aminoglycoside has a significant influence on the values. It is also worth noting that ^{1}H NMR and potentiometery measurements, while being much less time consuming and requiring much lower gentamicin concentrations than ^{15}N NMR determination, more closely approximate physiological conditions.

^{1}H NMR studies confirmed that all three rings of gentamicin C-1 have chair conformations, which are stable in both acidic and basic environments.

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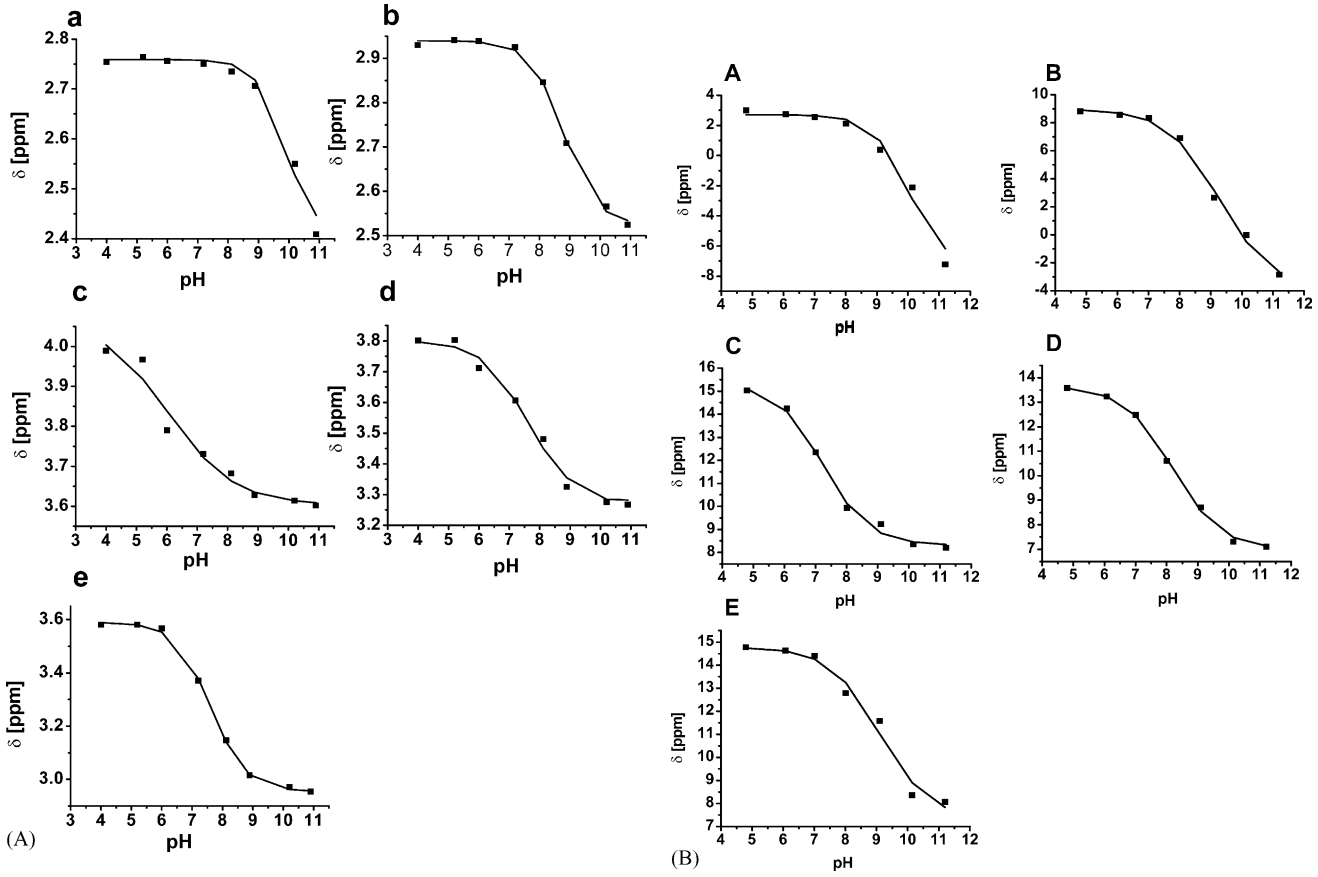

Fig. 7. (A) Dependence of chemical shift of the following selected gentamicin C-1 protons on pH: (a) 6′-CH_3; (b) 3″-CH_3; (c) 4-H; (d) 6-H; and (e) 2′-H. (B) ^{15}N NMR titration curves constructed using dependence of chemicals shift of the following amino groups: (a) 6′-NH_2; (b)3″-NH_2; (c) 3-NH_2; (d)1-NH_2; (e) 2′-NH_2. Spectra in both ^{1}H and ^{15}N NMR titration were collected as described in Section 2.

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