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Mass spectrometric characterization of gentamicin components separated by the new European Pharmacopoeia method

B. Li, A. Van Schepdael, J. Hoogmartens, E. Adams*

Laboratorium voor Farmaceutische Analyse, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, O&N2, PB 923, Herestraat 49, B-3000 Leuven, Belgium

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ABSTRACT

Liquid chromatography combined with pulsed electrochemical detection (LC–PED) is the method of choice in the European Pharmacopoeia for the determination of gentamicin and its related substances. A recently approved improved LC–PED method, with a reversed-phase C_{18} column and a mobile phase consisting of trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), sodium hydroxide and acetonitrile, showed better separation and more sensitive detection of the gentamicin components than the previous method using a polymer column. More unknown peaks can be separated from the main components and from each other. As the LC–PED method cannot be directly coupled to a mass spectrometer (MS), the unknown substances were collected after the LC column, desalted and analyzed by MS. The structures of the unknown compounds were deduced based on comparison of their fragmentation patterns with those of reference substances investigated by MS^n experiments using an electrospray ion trap mass spectrometer. A comparison was also made with an already previously published LC–MS method using a volatile mobile phase.

1. Introduction

Gentamicin is an aminoglycoside antibiotic obtained by fermentation of a strain of *Micromonospora purpurea* [1]. It has a broad spectrum of activity and it is widely used in the treatment of severe infections caused by aerobic, Gram-negative bacteria both in human and animals. Gentamicin is a mixture mainly consisting of gentamicins C_1, C_{1a}, C_2, C_{2a} and the minor component C_{2b} (sagamicin) [2]. Their chemical structures and m/z of $[M+H]^+$ are shown in Fig. 1. During fermentation, several related substances such as gentamicin B_1, sisomicin, dihydroxygentamicin C_{1a}, JI-20B, degradation products like garamine and 2-deoxystreptamine and some other unknown compounds are also formed in small amounts. None of these compounds contains a strong UV absorbing chromophore.

The purity of the pharmaceutical active substance and identity of impurities have always been considered as essential factors in ensuring drug safety and quality. In general, drug impurities above 0.10% should be identified and quantified by selective methods, which is difficult to comply with for biosynthetic products like gentamicin. So, many attempts have been made to identify the impurities in gentamicin. Berdy et al. isolated impurities from a gentamicin fermentation broth and identified more than 20 components by chemical, proton magnetic resonance (^{1}H NMR) and mass spectroscopic studies [3–5]. In a previous paper from our laboratory, six unknown compounds were completely identified and seven partially identified by interpretation of fragmentation patterns [6]. A volatile mobile phase with TFA at pH 2.4 adjusted with ammonia and a gradient with methanol were used to separate and detect impurities in gentamicin bulk samples with electrospray ionization (ESI) and ion trap mass spectrometry. Recently Grahek and Zupančič-Kralj [7] reported a gradient LC–MS method using a volatile mobile phase containing TFA and methanol. Seventeen impurities were detected and identified in a gentamicin sample with an atmospheric pressure chemical ionization (APCI) probe. Besides structure characterization and elucidation, liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods for the analysis of gentamicin in bovine plasma, urine, milk and tissues have been developed [8–12]. Analysis by LC/MS/MS of gentamicin in hospital waste water has also been reported [13].

Evaporative light scattering detection (ELSD) is another technique to directly analyze compounds without strong UV absorption. ELSD has been applied for developing a LC method with volatile mobile phases that are compatible with MS [6] or for determining gentamicin and its related compounds [14,15]. However, since there is no direct linear relationship between the response and amount of sample, it is less easy to use for quantitative analysis. A wider linear range suitable for analysis could be achieved with a charged aerosol detector as shown in the determination of gentamicin in a pharmaceutical cream [16]. For routine quantitative analysis however, the use of PED following LC has been shown to be the most interesting [17–20]. PED monitors the oxidation of an

* Corresponding author. Tel.: +32 16 32 34 44; fax: +32 16 32 34 48.
E-mail address: Erwin.Adams@pharm.kuleuven.be (E. Adams).

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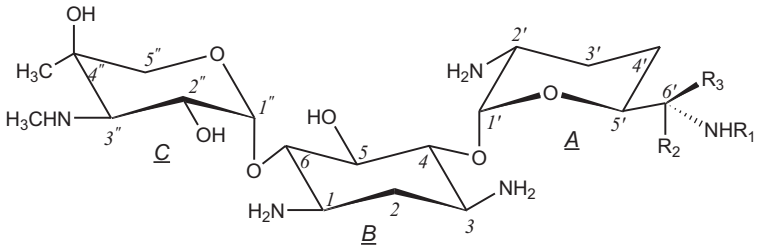


	R_1	R_2	R_3	$[M+H]^+$
gentamicin C_1	CH_3	CH_3	H	478
gentamicin C_{1a}	H	H	H	450
gentamicin C_2	H	CH_3	H	464
gentamicin C_{2a}	H	H	CH_3	464
gentamicin C_{2b}	CH_3	H	H	464

Fig. 1. Chemical structures and *m*/*z* of the main gentamicin components.

analyte on the surface of a gold electrode. A three-electrode system is used, which allows the precise control of the applied potentials. Over the years, the selectivity of the methods was improved by replacing the polymer column [17] with silica-based C18 columns and in the mobile phase perfluorinated carboxylic acids replaced sodium octanesulfonate as ion pairing agent. The pH is adjusted to 2.6 with NaOH [19,20]. The method described in [20] was the basis for the current official method prescribed in the European Pharmacopoeia for the analysis of gentamicin and its related substances [2]. However, only five gentamicin components and two related substances could be identified by comparing the retention times with those of the available reference substances. Other related compounds characterised previously by MS and not available as reference substances could not be attributed to peaks in the pharmacopoeial method as the retention times were obtained with different LC methods. So, in the gentamicin monograph many impurities remain of unknown identity.

It was the intention of this study to identify the impurities separated by this new pharmacopoeial method with MS. However, the LC–PED method cannot be directly coupled to MS because of the presence of sodium ions in the mobile phase. Thus, fractions containing the peaks of interest were collected, desalted and introduced in the MS. The results were also compared with those of previously published LC–MS methods using a volatile mobile phase [6,7].

2. Experimental

2.1. Reagents and samples

All reagents used for LC–PED were of HPLC grade. PFPA (97%), heptafluoropropionic acid (HFBA, 99%) and nonafluoropentanoic acid (NFPA, 97%) were obtained from Acros Organics (Geel, Belgium). Acetonitrile was purchased from Biosolve LTD (Valkenswaard, The Netherlands). Sodium hydroxide solution 50% was from J.T. Baker (Deventer, the Netherlands). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify deionized water. LC–MS grade methanol and trifluoroacetic acid (TFA) were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonia (28%) was from VWR International (Leuven, Belgium). Helium gas was obtained from Messer (Machelen, Belgium) and nitrogen gas was supplied by Air Liquide (Liège, Belgium).

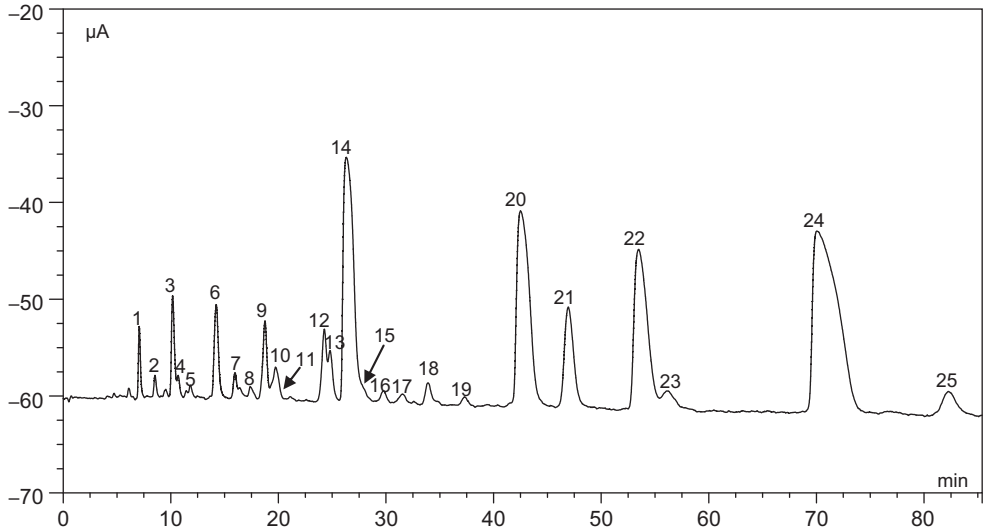


Fig. 2. Typical chromatogram of a gentamicin sample at a concentration of 1 mg/mL obtained by LC–PED. Elution order: 1. *m*/*z* 322; 2. *m*/*z* 469; 3. *m*/*z* 483; 4. *m*/*z* 469; 5. *m*/*z* 483; 6. *m*/*z* 468; 7. *m*/*z* 483; 8. *m*/*z* 305; 9. *m*/*z* 482; 10. *m*/*z* 436; 11. *m*/*z* 497; 12. *m*/*z* 448, sisomicin; 13. *m*/*z* 496; 14. *m*/*z* 450, gentamicin C1a; 15. *m*/*z* 319; 16. *m*/*z* 464; 17. *m*/*z* 450; 18. *m*/*z* 445 and 485; 19. *m*/*z* 462; 20. *m*/*z* 464, gentamicin C2; 21. *m*/*z* 464, gentamicin C2b; 22. *m*/*z* 464, gentamicin C2a; 23. *m*/*z* 478; 24. *m*/*z* 478, gentamicin C1; 25. *m*/*z* 478. The *m*/*z* were obtained by analyzing the individually collected compounds by MS after desalting described in Section 2.3.

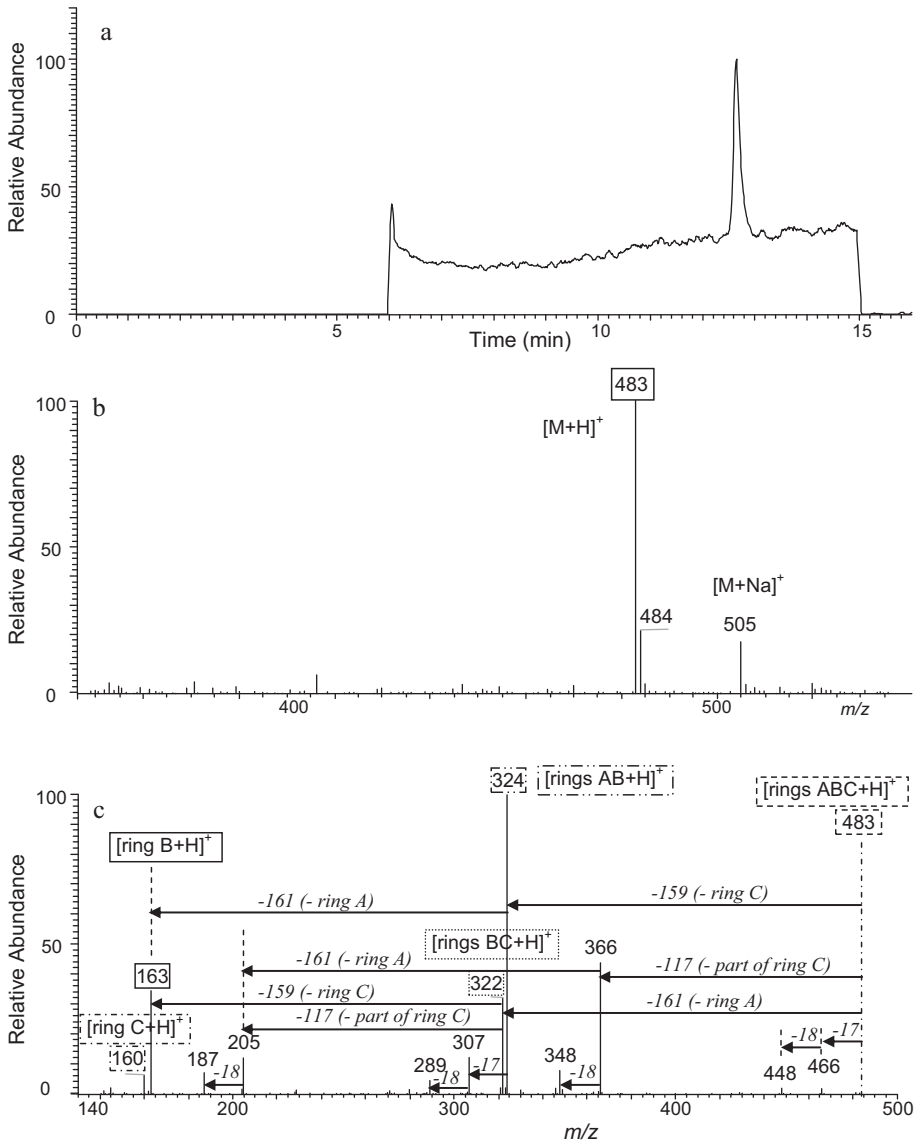


Fig. 3. Characterization of the collected impurity with the $[M+H]^+$ ion at *m/z* 483 in peak 3 by MS following LC–PED: (a) TIC chromatogram, (b) MS spectrum, (c) MS/MS spectrum.

Gentamicin bulk samples of different age and from different origins were available in the laboratory. After screening, the one with most impurities was further studied here. For identification purposes, reference standards of gentamicins C_{1a}, C_2, C_1, C_{2b}, JI-20B, sisomicin, deoxystreptamine and a mixture of C_2 and C_{2a} were available.

2.2. Instrumentation for LC–PED

The LC apparatus consisted of an L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany), a Lachrom Elite L-2200 autosampler (Merck-Hitachi), a Decade II electrochemical detector (Antec, Leyden, the Netherlands) and Chromeleon 6.70 software (Dionex Corporation, Sunnyvale, CA, USA) for data acquisition. The detector cell consisted of a gold working electrode, a HyRef reference electrode and a carbon filled polytetrafluoroethylene (PTFE) counter electrode. HyREF is an alternative for a Ag/AgCl reference electrode. It is principally maintenance free and due to the absence of a salt bridge, it does not require refilling with saturated KCl solution. The detector cell was kept at 35 °C in a hot air oven. The pulse settings were as follows: t_{det} 400 ms with E_{det} +0.05 V, t_{oxd} 200 ms with E_{oxd} +0.75 V and t_{red} 400 ms with E_{red} −0.15 V. The signal, expressed in nA, was measured between 200 and 400 ms.

A Hydrosphere C_{18} column (5 μm, 250 mm × 4.6 mm i.d., YMC, Kyoto, Japan), maintained at 35 °C in a water bath heated by means of a Julabo EC thermostat (Julabo, Seelbach, Germany), was used. The mobile phase consisted of 15 mL/L of acetonitrile and 985 mL/L of an aqueous solution of 7 mL/L TFA, 250 μL/L PFPA and 4 mL/L of 50% sodium hydroxide. The aqueous phase was adjusted to pH 2.6 with 0.5 M NaOH before bringing to volume. The flow rate was 1.0 mL/min. To enhance the detection at the working electrode, 0.5 M NaOH was added post-column at a flow rate of 0.3 mL/min. Since this addition must be pulse free, a helium pressurized device was used. 0.5 M NaOH was prepared by pippeting 25 mL of 50% NaOH in 975 mL of carbon-dioxide free water. Both the mobile phase and 0.5 M NaOH were degassed by sparging helium. The injection volume was 20 μL.

All sample solutions were prepared in the mobile phase. Gentamicin sample solutions at concentrations of 1–5 mg/mL were injected. The higher concentration solution was used to characterize impurities present at lower contents. Following separation in

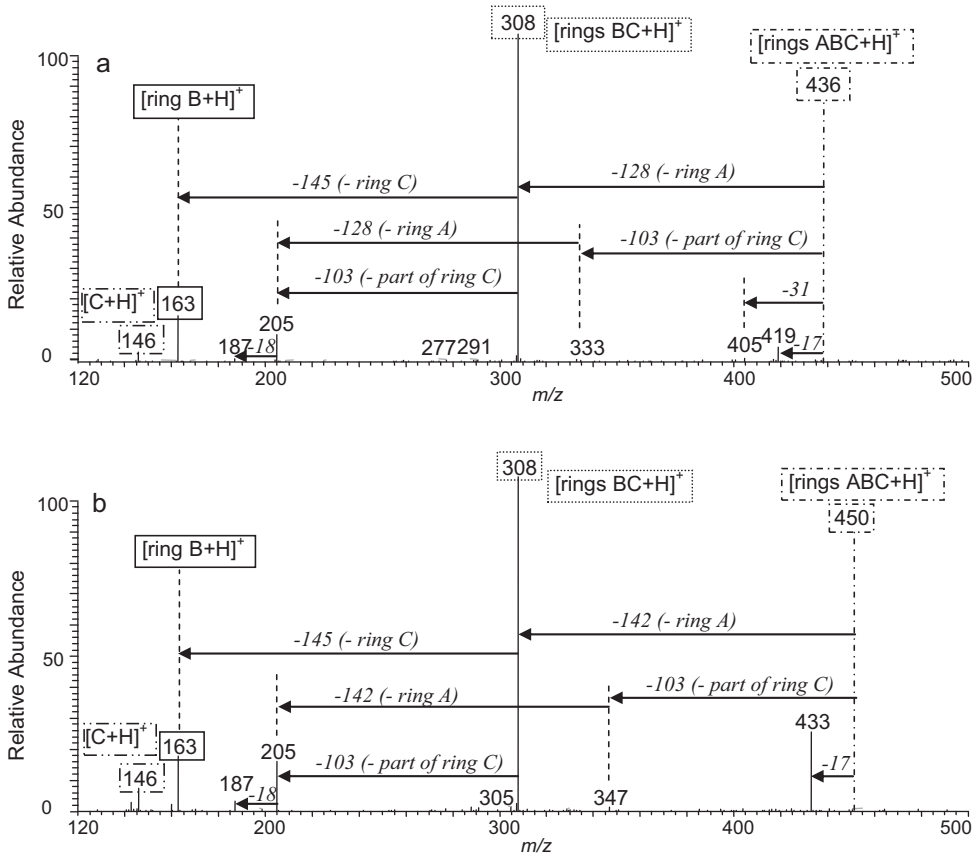


Fig. 4. LC–MS/MS spectra of new impurities found in the gentamicin sample, (a) $[M+H]^+$ ion at *m/z* 436 (peak 10) and (b) $[M+H]^+$ ion at *m/z* 450 (peak 17).

the LC column, the flow was split using a T-piece. The set-up was so that 0.5 mL/min of the LC flow was sent to the PED (necessary to follow the separation) while the other half was collected in vials according to the peaks detected by PED and shown by the software on the computer screen.

2.3. Instrumentation for LC/MS

The LC apparatus consisted of a P680 HPLC pump (Dionex, Sunnyvale, CA, USA), a SpectraSERIES AS100 autosampler (TSP, Fremont, CA, USA) equipped with a 20 μL loop. MS data were acquired on a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) operated in the positive ion mode. Nitrogen was used as sheath and auxiliary gas. Helium was used as collision and damping gas in the ion trap at a pressure of 0.1 Pa. The Xcalibur 1.2 software from Thermo Finnigan was used for instrument control, data acquisition and processing. Mass Frontier software version 2.0 from Thermo Finnigan was used to simulate and study the fragmentation behavior of the described compounds.

A Hypersil BDS C_{18} column (3 μm, 100 mm × 2.1 mm i.d., Thermo Quest, Belgium) was kept at a constant temperature of 25 °C using a water bath with a heating immersion circulator. Mobile phase A was 0.1% HFBA in a mixture of water and acetonitrile (90:10, v/v) and mobile phase B 0.1% HFBA in water/acetonitrile (10:90, v/v). A gradient elution (0–15 min: 15–100% for mobile phase B) was used to separate the compounds from the salt. The flow rate was 0.2 mL/min. 500 μL solutions collected from the LC–PED system were injected to the column of the desalting system by manual injection.

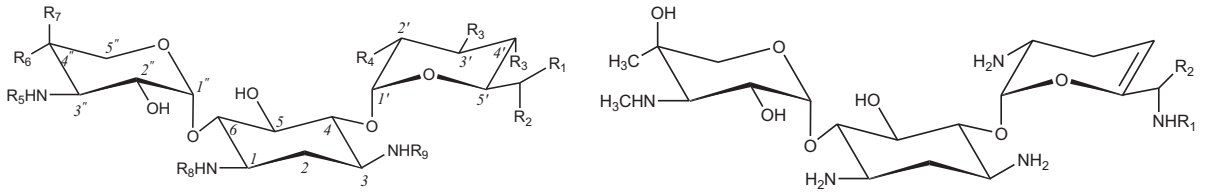
2.4. Tuning and mass spectrometric investigation of the reference substances

The LCQ equipped with the ESI interface was tuned by direct infusion of a 10 μg/mL sisomicin solution using the built-in syringe pump at a flow rate of 10 μL/min. The solution was mixed with mobile phase A at 200 μL/min through a T-piece. Sisomicin was used to tune the MS instrument because it consists of one main compound, contrary to gentamicin which consists of several main components.

For the study of the fragmentation behavior, solutions of available reference standards were separately infused to the LCQ using the syringe pump. The base peaks in the positive ion mode were $[M+H]^+$. First-order mass spectra and MS/MS spectra were obtained with 10 μg/mL solutions and MS^n ($n = 3$, 4, 5) with 50 μg/mL. Precursor ions were isolated with an isolation width of 3 *m/z* units. In the MS/MS and MS^n experiments, the collision energy level was set at 40%, a percentage of the 5 V a.c. voltage applied to the end-caps at the resonance frequency of the selected ion.

3. Results and discussion

A characteristic feature of gentamicin is the two amino sugars glycosidically linked to positions 4 and 6 of 2-deoxystreptamine (Fig. 1). The major fragmentation pathways of gentamicin and other aminoglycosides with collisionally activated decomposition (CAD) of protonated molecules are glycoside bond cleavages [6,7,21,22]. The low abundance product ions formed by a cross-ring cleavage involving the amino sugar linked to C-6 of 2-deoxystreptamine could also be observed from the $[M+H]^+$ ions and the product ion consisting of rings A and B.



(a) (b) sisomicin

LC–PED/MS (Fig. 2)			Proposed structures (all refer to (a) except sisomicin)										LC–MS (Li et al. [6])	LC–MS (Grahek et al. [7])	
Peak No.	$[M+H]^+$	Content (%)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	compound	Peak No.	Peak No.	Proposed structure
1	322	0.45	-	-	-	-	CH_3	CH_3	OH	H	H	garamine (rings B+C)	1	1	
2	469	0.12	NH_2	H	OH	OH	H	CH_3	OH	H	H	gentamicin A_3	2	2	gentamicin A, A_1, A_3
			OH	H	OH	NH_2	CH_3	H	OH	H	H	gentamicin A_1			
			OH	H	OH	NH_2	CH_3	OH	H	H	H	gentamicin A			
			NH_2	H	OH	OH	CH_3	H	OH	H	H				
			NH_2	H	OH	OH	CH_3	OH	H	H	H				
			OH	H	OH	NH_2	H	CH_3	OH	H	H				
3	483	0.67	NH_2	H	OH	OH	CH_3	CH_3	OH	H	H	gentamicin B	3	4	
4	469	0.11										isomer of the compound in peak 2	4		
5	483	0.13	OH	H	OH	NH_2	CH_3	CH_3	OH	H	H	gentamicin X_2	4		
6	468	0.75	NH_2	H	OH	NH_2	H	CH_3	OH	H	H	3"–demethyl–JI–20A	U_1 [a]		
			NH_2	H	OH	NH_2	CH_3	H	OH	H	H	4"–demethyl–JI–20A			
			NH_2	H	OH	NH_2	CH_3	OH	H	H	H				
7	483	0.24	NH_2	CH_3	OH	OH	H	CH_3	OH	H	H		5		
			NH_2	CH_3	OH	OH	CH_3	H	OH	H	H				
			NH_2	CH_3	OH	OH	CH_3	OH	H	H	H				
			OH	CH_3	OH	NH_2	H	CH_3	OH	H	H				
			OH	CH_3	OH	NH_2	CH_3	H	OH	H	H	III–1		5	III–1, II–2
			OH	CH_3	OH	NH_2	CH_3	OH	H	H	H	II–2			
8	305	0.08										rings A-B of gentamicin $C_2/C_{2a}/C_{2b}$		6	Gentamine C_2
9	482	0.75	NH_2	CH_3	OH	NH_2	H	CH_3	OH	H	H		6		
			NH_2	CH_3	OH	NH_2	CH_3	H	OH	H	H				
			NH_2	CH_3	OH	NH_2	CH_3	OH	H	H	H				
			NH_2	H	OH	NH_2	CH_3	CH_3	OH	H	H	antibiotic JI–20A		7	
10	436	0.10										3" or 4"–demethyl–gentamicin C_{1a} (VII–3)		8	VII–3 [b]
11	497	0.07	NH_2	CH_3	OH	OH	CH_3	CH_3	OH	H	H	gentamicin B_1	7	9	gentamicin B_1
			OH	CH_3	OH	NH_2	CH_3	CH_3	OH	H	H	antibiotic G–418	8		
12	448	0.67	H	H	(b)							sisomicin	9	11	
13	496	0.50	CH_3	H	OH	NH_2	CH_3	CH_3	OH	H	H	antibiotic JI–20B	7	12	
14	450											gentamicin C_{1a}			
15	319	NS	$NHCH_3$	CH_3	H	NH_2	-	-	-	H	H	purpuramine	10	13	Gentamin C_1
16	464	0.07	NH_2	H	H	NH_2	CH_3	CH_3	OH	H	CH_3	XK–62–3	11		
			NH_2	H	H	NH_2	CH_3	CH_3	OH	CH_3	H				
17	450	0.10										3" or 4"–demethyl–gentamicin $C_2/C_{2a}/C_{2b}$		14	Y-02077H-δ, VII–2, XK-62-5 [c]
18	445 + 485	0.24										Unknown	13		
19	462	0.06	CH_3	H	(b)							G52	12		
			H	CH_3	(b)							verdamicin			
20	464											gentamicin C_2			
21	464											gentamicin C_{2b}		16	
22	464	0.21										gentamicin C_{2a}			
23	478											isomer of the compound in peak 24	U_2 [a]		
24	478											gentamicin C_1			
25	478	0.31	NH_2	CH_3	H	NH_2	CH_3	H	CH_3	H	H		16		
			NH_2	CH_3	H	NH_2	CH_3	CH_3	H	H	H				

[a] U_1 and U_2 are additional impurities found in the second gentamicin sample [6] ;
[b] VII–3, 4"–demethyl–gentamicin C_{1a};
[c] Y-02077H-δ, 3"–demethyl–gentamicin C_2; VII–2, 4"–demethyl–gentamicin C_2; XK-62-5, 3"–demethyl–gentamicin C_{2b}.
NS : not well separated

Fig. 5. Proposed structures of unknown impurities in a gentamicin sample by collecting fractions eluted from the LC–PED system followed by MS analysis after desalting and comparison with impurities found with two reported LC–MS methods using volatile mobile phases.

A typical chromatogram of a gentamicin sample at a concentration of 1 mg/mL obtained by LC–PED is shown in Fig. 2. The known compounds were identified with the available reference substances by comparing the retention times. The non-volatile mobile phase used for this method was not compatible with MS. The salt may cause blockage to the heated capillary and the precipitate inside the instrument may cause electrical distortion and deteriorate the detection of the compounds investigated. Also, the sodium adducts for the compounds investigated are much more dominant than the protonated molecules and in some cases, they are the only ions observed by the mass spectrometer. This makes it difficult to elucidate the structure of the unknown compounds with tandem MS.

The most direct approach to adapt this mobile phase to be compatible with the mass spectrometer was by replacing sodium hydroxide with ammonia, because the sodium hydroxide was the only non-volatile additive in the mobile phase for LC–PED and its function was mainly the adjustment of the pH of the mobile phase. However, the LC method using volatile ammonia instead of sodium hydroxide in the mobile phase did not yield the same selectivity. Therefore, a second approach was tried out where the flow after the LC column was split in a way that a part of the LC flow was collected in vials while the other part was sent to the PED to follow up the separation. The collected fractions were injected in a second LC system where the compounds of interest were retained by an ion-pairing agent (HFBA) while the non-volatile salts were flushed out of the column without any retention. To retain the basic and polar analytes, several ion-pairing agents were investigated: TFA, PFPA, HFBA and NFPA. HFBA was chosen because it provided sufficient retention at the lowest concentration (compared to TFA and PFPA), which ensures high sensitivity for the MS detection. NFPA tested contained an unknown compound, producing a very strong background signal and therefore it was not suitable for this study.

The known compounds were identified using the available reference substances and confirmation was done using the MS^n fragmentation pathways. Unknown compounds were characterized by comparing their fragmentation patterns with those of the reference substances. The characterization of the impurity in peak 3 of Fig. 2 is shown as an example. The LC flow was diverted to the waste for the first 6 min and sent to the MS for analysis until 15 min. The gradient elution started with 15% and stopped with 100% of mobile phase B, which contained 90% of acetonitrile. This ensured the good wash out of the non-volatile salt and next the elution of the compound of interest for characterisation by MS. The TIC chromatogram and the LC/MS and LC/MS/MS spectra are shown in Fig. 3. The protonated molecule $[M+H]^+$ at m/z 483 was the most abundant ion in the LC/MS spectrum (Fig. 3b), while the less abundant sodium adduct ion $[M+Na]^+$ at m/z 505 was also present. Fragmentation of the ion $[M+H]^+$ at m/z 483 resulted in ions at m/z 324, 322 and 366 by the loss of ring C, ring A and part of ring C by the cross-ring cleavage, respectively. Further fragmentation of the product ion at m/z 324 yielded the main product ion at m/z 163, which corresponds to the protonated ring B, formed by the loss of ring A. Further fragmentation of the ion at m/z 322 resulted in the ions at m/z 163 and 205 by the loss of the entire ring C or a part of it. The product ion at m/z 205 could also be found from the fragmentation of the ion at m/z 366 by the loss of ring A. The product ions formed by the losses of NH_3 and H_2O molecules either from the protonated molecule or its product ions were also present in the tandem MS spectra. The fragmentation pattern of this ion was the same as that of gentamicin B [6] and thus this compound was deduced as gentamicin B.

Except for the compounds we previously reported [6], three additional impurities were found to be present in the sample. These three impurities were deduced (i) as rings A-B of gentamicins $C_{2a}/C_2/C_{2b}$ for the compound with the $[M+H]^+$ ion at m/z 305 in peak 8, (ii) as 3″-demethyl- or 4″-demethyl-gentamicin C_{1a} for the compound with the $[M+H]^+$ ion at m/z 436 in peak 10 and (iii) as 3″-demethyl- or 4″-demethyl-gentamicin $C_2/C_{2a}/C_{2b}$ for the compound with the $[M+H]^+$ ion at m/z 450 in peak 17, respectively. The last two unknown compounds showed comparable fragmentation pathways with those of gentamicin C_{1a} and gentamicin $C_2/C_{2a}/C_{2b}$ respectively, with a difference in the neutral losses corresponding to ring C of the gentamicins. A down shift of 14 m/z units indicated that there was one methyl group less in ring C of these two unknown compounds than the corresponding gentamicin components (Fig. 4). Two impurities previously reported [6], $[M+H]^+$ ion at m/z 461 in peak 14 and $[M+H]^+$ ion at m/z 463 in peak 15, were not found in the sample analyzed by LC–PED.

Grahek and Zupančič-Kralj reported another LC/MS-APCI method for the characterization of gentamicin components [7], in which gentamicin main components C_1, C_{1a}, C_2, C_{2a} and 17 impurities were separated and characterized. Thirteen of the reported impurities were also found by the LC–PED and LC/MS-ESI methods described in this paper. Four extra impurities were identified as gentamine C_{1a} (m/z 291), gentamine C_{2a} (m/z 305), Y-02077H-b (m/z 492) and VII-1 or Y-02077H-g (m/z 464).

All the components in the gentamicin sample identified by the LC–PED method are shown in Fig. 5. It also shows a comparison with previously published methods. The concentrations of the impurities mentioned were obtained by the LC–PED system and calculated versus a sisomicin reference solution of 10 μg/mL. The separation between peaks 15 and 14 was not sufficient to determine the area of peak 15. The impurities characterized by two LC/MS methods, using volatile mobile phases, are also included with the peak numbers from their corresponding figures [6,7]. Different names used in the literature are also listed.

4. Conclusions

LC–PED is still the method of choice for the routine analysis of gentamicin and its related substances. With the improvement of the LC method using a reversed-phase C_{18} column and TFA and PFPA as ion-pairing agents, a better separation could be achieved. In total, 21 impurities (most of them unknown) were detected together with the five main gentamicin components. Two impurities co-eluted in one peak could be separated by LC/MS because of different m/z. Since the mobile phase used for LC–PED was not compatible with MS, the characterization of unknown impurities was not straightforward. Thus, the peaks of interest were collected from the LC–PED system, desalted and introduced into the MS. Six impurities were fully characterized by MS^n. For 13 impurities, isomers were proposed. Two impurities could not be identified due to the lack of characteristic product ions of gentamicins.

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