Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 1037–1043

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Identification of gentamicin impurities by liquid chromatography tandem mass spectrometry

Rok Grahek[a,*], Lucija Zupančič-Kralj[b]

[a] Lek Pharmaceuticals d.d., Sandoz Development Center Slovenia, Verovškova 57, SI-1526 Ljubljana, Slovenia
[b] University of Ljubljana, Faculty of Chemistry and Chemical Technology, Aškerčeva 5, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 9 February 2009
Received in revised form 3 June 2009
Accepted 3 June 2009
Available online 12 June 2009

Keywords:
Gentamicin
Aminoglycoside antibiotics
Impurity
Related substances
HPLC–MS/MS

ABSTRACT

An HPLC/MS/MS method was developed for identification of impurities in gentamicin. The HPLC was performed on a Synergy Hydro-RP column using 50 mM trifluoroacetic acid (TFA), pH 2 adjusted with ammonium solution and methanol as mobile phase. All impurities in gentamicin were separated from main gentamicin components. Atmospheric pressure chemical ionization (APCI) was used and product mass spectra of protonated molecules were acquired. Seventeen impurities were detected in gentamicin. Reference compounds: gentamicins: C_{2b}, B, B_1, G-418, sisomicin, garamine and gentamines: C_1, C_{1a}, C_2, C_{2a} were used for spectra interpretation and impurities identification. All MS/MS spectra were interpreted and fragmentation transitions for gentamicins and in general for aminoglycoside antibiotics (AG) were proposed. All impurities were identified. More than one isomere were proposed for three impurities.

1. Introduction

Gentamicin is an aminoglycoside antibiotic (AG) produced by *Micromonospora purpurea* [1] widely used for treatment of serious infections caused by both Gram-negative and Gram-positive bacteria [2]. It is a mixture consisting of four major components, designated C_1, C_{1a}, C_2 and C_{2a} together with numerous minor components including gentamicin C_{2b} (sagamicin), A, B, B_1 and X_2 [3,4]. According to Berdy [6] the gentamicin producing *Micromonospora* strains, besides the common A, B and C components, produces a large series from 20 to 35 components with specific bioactivity. Most of them were isolated from gentamicin fermentation broth and identified by the same author [5–7]. Structures and m/z of $[M+H]^+$ of some gentamicins relevant for this study are shown in Fig. 1.

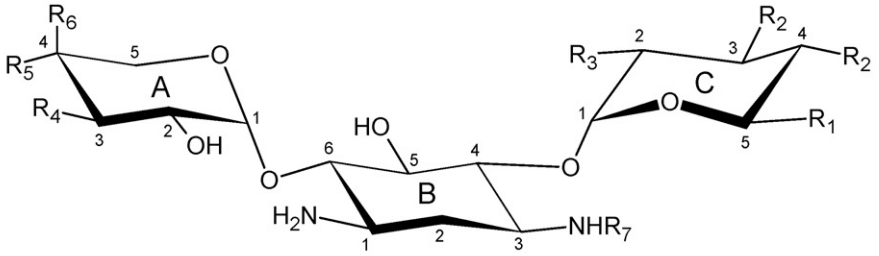
Gentamicins are basic, water soluble, relatively stabile, structurally closely related compounds without UV absorbing chromophores. This makes the HPLC analysis more difficult and challenging. Several HPLC methods have been developed for the analysis of gentamicins using derivatization of amino groups with o-phataladehide [8–10], 2,4,6-trinitrobenzensulfonic acid [11] and 1-fluoro-2,4-dinitrobenzene [12,13]. These improved the chromatographic behavior of gentamicins making them less polar and introducing a chromophore in the molecule to enable fluorescence or UV detection. Additionally to spectrophotometric also some other detections were used for AG. Inchauspe [14] detected aminoglycoside antibiotics in culture broth with refractive index detection. Electrochemical [15–17] and evaporative light scattering detections [18,19] were used in the analysis of AG and gentamicin components with ion pairing chromatography. For detection of gentamicins also mass spectrometry can be used [19–23,26–29].

Perfluorinated carboxylic acids (PA) were used as pairing ions to improve the separation of gentamicins in HPLC analysis. The behavior of trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) as pairing ions for the chromatography of AG antibiotics was studied [24,25] showing that higher concentration of AP increases the retention and the resolution of gentamicin components on non-polar column.

Molecular species are mainly produced with very few fragments and can be hardly used for identification of compounds with soft ionization techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [26]. Ion source fragmentation can be induced with high cone voltage [26] giving insight on the structure of the molecule. Fragmentation and structural characterization of aminoacyl derivatives of kanamycin A with collisionally activated decomposition (CAD) of protonated molecule was investigated by Kotretsou [27]. Glycoside bond cleavage was the main fragmentation mechanism and on the basis of relatively simple mass spectra, fragmentation pathway for two kanamycin A derivatives was proposed. Hu [28] proposed fragmentation mechanism for 10 AG antibiotics systematically investigated with CAD.

* Corresponding author. Tel.: +386 1 5803282; fax: +386 1 5681393.
E-mail address: rok.grahek@sandoz.com (R. Grahek).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.06.004



Compound	?M+H?+	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Gentamicin C_1	478	X_1	H	NH_2	$NHCH_3$	CH_3	OH	H
Gentamicin C_{1a}	450	CH_2NH_2	H	NH_2	$NHCH_3$	CH_3	OH	H
Gentamicin C_2	464	X_2	H	NH_2	$NHCH_3$	CH_3	OH	H
Gentamicin C_{2a}	464	X_3	H	NH_2	$NHCH_3$	CH_3	OH	H
Gentamicin C_{2b}	464	CH_2NHCH_3	H	NH_2	$NHCH_3$	CH_3	OH	H
Gentamine C_1	319	X_1	H	NH_2				
Gentamine C_{1a}	291	CH_2NH_2	H	NH_2				
Gentamine C_2	305	X_2	H	NH_2				
Gentamine C_{2a}	305	X_3	H	NH_2				
Garamine	322				NHCH3	CH_3	OH	H
Gentamicin A	469	CH_2OH	OH	NH_2	$NHCH_3$	OH	H	H
Gentamicin A_1	469	CH_2OH	OH	NH_2	$NHCH_3$	H	OH	H
Gentamicin A_3	469	CH_2NH	OH	OH	$NHCH_3$	H	OH	H
Gentamicin B	483	CH_2NH_2	OH	OH	$NHCH_3$	CH_3	OH	H
Gentamicin X_2	483	CH_2OH	OH	NH_2	$NHCH_3$	CH_3	OH	H
II-2	483	X_4	OH	NH_2	$NHCH_3$	OH	H	H
III-1	483	X_4	OH	NH_2	$NHCH_3$	H	OH	H
JI-20A	482	CH_2NH_2	OH	NH_2	$NHCH_3$	CH_3	OH	H
VII-3	436	CH_2NH_2	H	NH_2	$NHCH_3$	H	OH	H
Gentamicin B_1	497	X_2	OH	OH	$NHCH_3$	CH_3	OH	H
G-418	497	X_4	OH	NH_2	$NHCH_3$	CH_3	OH	H
Gentamicin A_4	497	CH_2OH	OH	NH_2	CH_3NCHO	OH	H	H
Sisomicin*	448	CH_2NH_2	H	NH_2	$NHCH_3$	CH_3	OH	H
JI-20B	496	X_3	OH	NH_2	$NHCH_3$	CH_3	OH	H
Y-02077H-?	450	X_2	H	NH_2	NH_2	CH_3	OH	H
VII-2	450	X_2	H	NH_2	$NHCH_3$	H	OH	H
XK-62-5	450	CH_2NHCH_3	H	NH_2	NH_2	CH_3	OH	H
XK-62-7	492	$CH_2N(CH_3)_2$	H	NH_2	$NHCH_3$	CH_3	OH	CH_3
XK-62-8	492	X_1	H	NH_2	$NHCH_3$	CH_3	OH	CH_3
Y-02077H-?	492	X_2	H	NH_2	$NHCH_3$	CH_3	OH	CH_2CH_3
XK-62-3	464	CH_2NH_2	H	NH_2	$NHCH_3$	CH_3	OH	CH_3
Y-02077H-?	464	X_1	H	NH_2	NH_2	CH_3	OH	H
VII-1	464	X_1	H	NH_2	$NHCH_3$	H	OH	H

* Sisomicin has a double bond on ring C between carbons C_4 and C_5

Fig. 1. Chemical structures and m/z $[M+H]^+$ of gentamicins, gentamines and sisomicin.

The major components of gentamicin (C_1, C_{1a}, C_2, C_{2a}, C_{2b}) were identified in bulk preparation of gentamicin sulphate [20]. Graham [21] studied the stability of gentamicin formulation and tentatively identified gentamine C_1 and sisomicin. Minor components or impurities in gentamicin have been rarely identified by HPLC analysis of gentamicin. Using demanding and time consuming analytical techniques, Berdy [6] isolated and identified 25 AG antibiotics from fermentation broth of gentamicin producing *Micromonospora* species. Potentially all this related substances could be present in gentamicin. Clease [9] reported the presence of antibiotic JI 20B and gentamicin C_{2b} in gentamicin sulphate and Clarot [19] detected gentamicin C_{2b} in commercial gentamicin sulphate. Recently Li [29] used a volatile mobile phase with TFA and a gradient with methanol to separate and detect impurities in gentamicin bulk sample with ESI and ion trap mass spectrometry. Six unknown compounds were completely identified and seven partially identified by interpretation of fragmentation patterns. Manyanga [16] used pulsed electrochemical detection and TFA and PFPA in mobile

phase and achieved good separation of gentamicin components and impurities. Deoxystreptamine, JI-20B, gentamicin C_{2b} and sisomicin were identified in commercial gentamicin sample.

The purity of the pharmaceutical active substance and knowing the identity of the impurities has always been considered as an essential factor in ensuring drug safety and quality. In general, drug impurities in excess of 0.1% should be identified and quantified by selective methods, which was not completely true for old biosynthetic product as gentamicin.

In this work we developed a selective HPLC–MS/MS method for identification of impurities in gentamicin. MS/MS spectra of all impurities in a gentamicin sample were recorded and interpreted. With the use of reference substances and the comprehension of the MS/MS spectra of known gentamicins, which aided the interpretation of mass spectra of impurities, all impurities in gentamicin were identified.

2. Experimental

2.1. Chemicals

Methanol (HPLC grade), trifluoroacetic acid LiChrosolv®, hydrochloric acid 32% p.a., sodium hydroxide p.a. and ammonia solution 25% p.a. were obtained from Merck (Darmstadt, Germany). Ultra pure water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Gentamicin sulphate was from Lek Pharmaceuticals d.d. (Ljubljana, Slovenia) and was dissolved in water at concentration of 5 mg/mL.

2.2. Reference compounds

Sisomicin, gentamicin B, gentamicin B_1 and garamine all as sulphate salts were from Lek Pharmaceuticals d.d. (Ljubljana, Slovenia), geneticin sulphate (G-418) was purchased from Sigma (St. Louis, MO, USA), gentamicin C_{2b} sulphate (sagamicin or micronomicin) was from Jiangxi Pharmaceutical Corp. (Nangchang, China). All reference substances were dissolved in water (sisomicin, gentamicin C_{2b}, gentamicin B, gentamicin B_1, G-418 and garamine) at a concentration 0.6 mg/mL. A mixture of reference compounds was prepared at concentration 0.1 mg/mL.

2.3. Hydrolysis of gentamicin

15 mg of gentamicin was dissolved in 1.5 mL 6 M hydrochloric acid and heated at 80 °C for 3 h. After cooling to room temperature, the solution was neutralized with 6 M sodium hydroxide. For HPLC/MS analysis 20 μL of the neutralized solution was diluted with 2 mL of water.

2.4. HPLC and MS instrumentation and conditions

The HPLC/MS apparatus consisted of a Membrane Degasser mobile phase degasser, SpectraSYSTEM P4000 quaternary pump, SpectraSYSTEM AS3000 auto sampler equipped with a 5 μL loop and Finnigan TSQ7000 mass spectrometer. The system was controlled and the date was collected by Xcalibur software all from Thermo Separation Products (San Jose, CA, USA). The mass spectrometer was equipped with APCI ion source at positive ion mode. The capillary and vaporizer temperature was respectively 220 and 540 °C and the corona discharge current was 4 μA. Nitrogen was used as auxiliary and sheath gas. The scan ranged from m/z 100 to 700 in 0.5 s. In MS/MS mode argon was used as a collision gas and the collision energy was 20 eV.

A time program was used for the product ion scan during the HPLC/MS/MS analysis accordingly to the protonated molecule mass and the retention time of the eluting substance.

The Synergi Hydro-RP, 250 mm × 4.6 mm i.d., 5 μm column from Phenomenex (Torrance, CA, USA) at 30 °C was used and the mobile

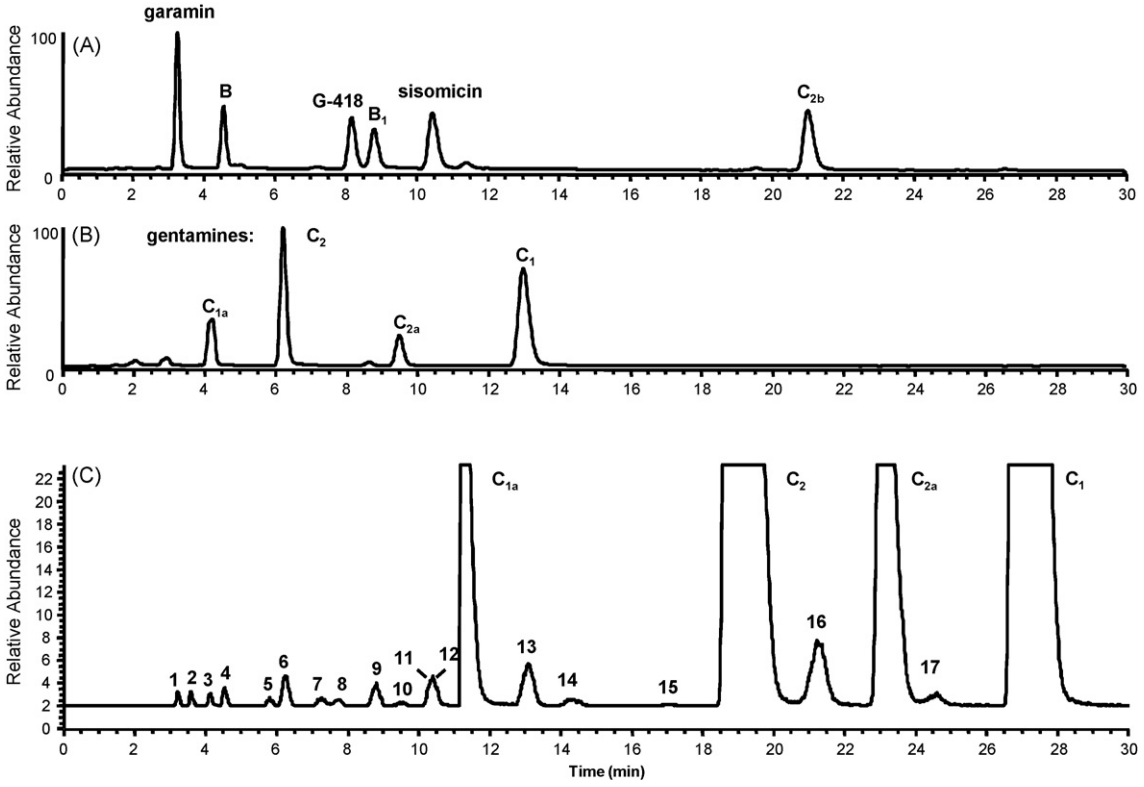


Fig. 2. LC/MS chromatograms of reference compounds (A), hydrolytic products of gentamicin–gentamines (B) and gentamicin sample (C). The same peak numbering is used as in Table 2.

phase was A: 50 mM TFA, pH 2.0 adjusted with ammonium solution 25% and B: methanol. The mobile phase composition was 10 min 100% A and then a linear gradient to 90% A was applied in the next 20 min. A flow rate was 1.5 mL/min.

3. Results and discussion

3.1. Development of the HPLC/MS method

Preliminary HPLC/MS method used 10 mM TFA as mobile phase. A good separation of the main gentamicin components was achieved, but the separation of the impurities was not satisfactory and the retention should be increased. The use of 5 mM pentafluoropropionic acid and 20% of methanol increased the retention but the resolution of the main gentamicin components deteriorated. Gentamicins C_2 and C_{2a} co-eluted in a single peak.

In comparison to Li [29] the separation was improved by adjusting the pH to 2 with ammonium solution and using longer column. Additionally a gradient of methanol was applied to shorten the analysis. At such conditions good separation of impurities and the main components of gentamicin were obtained.

Chromatograms of the mixture of reference compounds (A), hydrolytic products of gentamicin (B) and gentamicin sample (C) are shown on Fig. 2. All components of the mixture of reference compounds were well separated. Hydrolysis of gentamicin produces gentamines as mentioned by Berdy [6]. The elution orders of the main gentamicin components were determined according to the APCI mass spectra and the known component ratio and were the same as reported by Graham [21] and were C_{1a}, C_2, C_{2a} and C_1 respectively. Additionally, 17 minor components were found in the chromatogram of the gentamicin and numbered.

In the APCI mass spectra of the analyzed samples, the protonated molecule was the base peak with very few fragments. Additionally, an adduct of the molecule with trifluoroacetic acid with the relative abundance of 5–30% was observed. Mass spectrum of gentamicin C_{1a} is shown in Fig. 3A as example.

The mass spectrum of the compound eluted just before gentamicin C1a (seen in Fig. 3B) revealed that two components were co eluting with the protonated molecules m/z 448 and 496 and were denoted by 11 and 12 respectively. Adducts with TFA are visible at m/z 562 and 610.

The APCI ionization spray was stable and no post column addition of methanol was needed. Despite relatively high concentration of TFA in mobile phase, the sensitivity of the mass spectrometer was good.

3.2. HPLC/MS/MS analysis

The mass spectra of product ions of gentamicins: C_1, C_{1a}, C_2, C_{2a}, C_{2b}, B, B_1, G-418, sisomicin, garamine and gentamines: C_1, C_{1a}, C_2, C_{2a}, were acquired and are summarized in Table 1.

Letter coding A, B and C for the three aminoglycoside rings was used for better data representation and discussion as shown in Fig. 1 [28]. The center ring B was 2-deoxystreptamine. Ring A garosamine referred to the aminoglycoside linked to the C_6–O of the 2-deoxystreptamine, and ring C purpurosamine referred to the aminoglycoside on the C_4–O of the 2-deoxystreptamine.

The letter coding [28] was also applied for the product ions of the protonated molecule (P). Ions from the individual rings were denoted as (A-18), B and (C-18) because rings A and C were in dehydrated form and ring C appeared as protonated 2-deoxytreptamine. If two rings were found in an ion, two letters were used for coding, such as AB and BC.

Glycosides bond cleavages were the main fragmentation mechanisms of the protonated molecule of gentamicins, sisomicin,

Table 1
Product ions (m/z) and their relative abundance (%) of protonated molecules of known compounds in HPLC/MS/MS of: hydrolytical products of gentamicin–gentamines.

	Compound	P[a]	(P-18/17)	AB	BC	(BC-18/17)	BCX	(BCX-18)	BX	(A-18)	(A-X)	B	(C-18)	(C-18-18/17)
Rt (min)														
4.2	Gentamine C1a	291	274/3		291	274/3						163/66	129/100	112/17
6.3	Gentamine C2	305	288/5		305	288/5			205/26	160/58	118/11	163/23	143/100	126/13
9.5	Gentamine C2a	305	288/7		305	288/7			205/20	160/29	118/7	163/33	143/100	126/28
13.1	Gentamine C1	319/2	302/8		319/2	302/8			205/13	160/31	118/8	163/1	157/100	139/18
Reference compounds														
3.2	Garamine	322/19		322/19					205/10	160/100	118/13	163/32		
4.5	Gentamicin B	483/27		322/20	324/98		366/20	348/14	205/26	160/58	118/11	163/100	162/30	
8.1	G-418	497/93	480/2	322/60	338/100		380/33	362/5	205/20	160/29	118/7	163/45	176/4	
8,8	Gentamicin B1	497/25		322/13	338/100		380/15	362/9	205/13	160/31	118/8	163/59	176/31	158/5
10.4	Sisomicin	448/35	430/42	322/90		271/100	331/9	313/25	205/12	160/58	118/11	163/28	127/12	109/5
21.2	Gentamicin C2b	464/13	447/16	322/100	305/5	288/8	374/1		205/6	160/35	118/4	163/12	143/26	126/4
Gentamicin sample														
11.2	Gentamicin C1a	450/3	433/2	322/100	291/2	274/4	333/1	316/1	205/14	160/81	118/11	163/37	129/17	112/9
18.8	Gentamicin C2	464/2	447/3	322/100	305/1	288/4	347/1		205/11	160/65	118/8	163/26	143/19	126/3
23.1	Gentamicin C2a	464/3	447/3	322/100	305/2	288/4	347/1		205/12	160/73	118/7	163/31	143/22	126/6
26.9	Gentamicin C1	478/10	461/22	322/82	319/2	302/11	361/1	344/2	205/8	160/52	118/7	163/20	157/100	139/12

[a] P refers to protonated molecule $[M+H]^+$.

Table 2
Product ions (*m/z*) and their relative abundance of protonated molecules of impurities in HPLC/MS/MS analysis of gentamicin substance.

Peak no.	t_R (min)	Proposed stru.	P	(P-18/17)	AB	BC	(BC-18/17)	BCX	(BCX-18)	BX	(A-18)	A-X	B	(C-18)	(C-18-18/17)
1	3,2	Garamine	322/14		322/14					205/12	160/100	118/16	163/42		
2	3,6	Gentamicin A, A1, A3	469/49		308/19	324/100	306/2	366/38	348/32	205/53	146/49	104/12	163/76	162/10	144/3
3	4,2	Gentamine C1a	291	274/3		291	274/3						163/70	129/100	112/25
4	4,5	Gentamicin B	483/31		322/21	324/100		366/25	348/14	205/20	160/59	118/11	163/95	162/26	
5	5,8	II-2, III-1	483/33		308/47	338/100	321/3	380/26	362/12	205/21	146/39	104/3	163/55	176/39	158/9
6	6,3	Gentamine C2	305	288/5		305	288/5						163/27	143/100	126/25
7	7,3	JI-20A	482/48		322/67	323/57		365/16	347/3	205/85	160/100	118/21	163/98	161/38	143/6
8	7,7	VII-2	436/1		308/58	291/1	274/1			205/43	146/8		163/100	129/8	112/3
9	8,8	Gentamicin B1	497/34		322/21	338/100		380/16	362/2	205/12	160/39	118/7	163/61	176/40	158/4
10	9,5	Gentamine C2a	305	288/3		305	288/3						163/38	143/100	126/39
11	10,4	Sisomicin	448/31	430/28	322/71		271/100	331/8	313/16	205/10	160/70	118/16	163/30	127/8	
12	10,4	JI-20B	496/35		322/100	337/83	320/1	379/18	361/4	205/18	160/49	118/11	163/30	175/72	157/1
13	13,1	Gentamine C1	319/1	302/10		391/1	302/10						163/2	157/100	139/29
14	14,4	[a]	450/2	433/4	308/71	305/1	288/5	347/1		205/32	146/11	104/1	163/100	143/29	126/4
15	17	Y-02077H-b	492/6	475/2	350/100		317/2			233/7	160/89	118/13	191/15	143/7	126/2
16	21,2	C2b	464/8	447/19	322/100	305/4	288/17	347/1		205/12	160/78	118/11	163/28	143/46	126/15
17	24,6	VII-1, Y-02077H-g	464/6	447/20	308/27	319/1	302/7		344/3	205/14	146/8	104/1	163/19	157/100	139/16

[a] VII-2, XK-62-5, Y-02077H-δ.

Fig. 3. APCI mass spectra of gentamicin C_{1a} (A) and compounds in a peak eluted just before gentamicin C_{1a} (B). Two compounds (numbered 11 and12) are present with protonated molecule *m/z* 448 and 496.

gentamines and garamine, forming the fragments: AB, BC and B. Following ions: (P-18/17), (A-18), (C-18) and (C-18-18/17), (BCX-18) are produced from the protonated molecules and fragments losing water and/or ammonia. The glycoside residing on the C_6–O of the 2-deoxistreptamine was observed to undergo significant decomposition at the C_2–C_3 and O–C_1 bonds producing fragments: BCX, BX, A-X. Product mass spectra of $[M+H]^+$ of gentamicin B and B_1 are shown in Fig. 4A and B.

The relative abundance of the produced fragments was influenced by the relative basicity of the aminoglycoside rings. The C rings of gentamicin C_1 possess a secondary amino group making it more basic than the C ring of gentamicins: C_{1a}, C_2, C_{2a}, B, B_1, G-418. The fragment (C-18) of gentamicin C_1 was the base peak but the abundance of (C-18) ions for the other gentamicins were below 35%.

In Fig. 5 the fragmentation transitions of gentamicin B_1 are shown. The simplicity of the MS/MS spectra of gentamicins, sisomicin, gentamines and garamine summarized in Table 1 shows that the same fragmentation scheme can be applied to all gentamicins and in general to all AG antibiotics. The main fragments can be predicted and compared with acquired MS/MS spectrum of unknown compound and used for positive identification for a known AG structure. The fragmentation behavior were in accordance with the work of Hu et al. [28]. MS/MS spectra of all denoted impurities in HPLC/MS/MS analysis of gentamicin substance are summarized and the predicted structures are in Table 2.

The general procedure for the unknown peaks identification was as follows: the protonated molecule of the unknown impurity and the gentamicin with the same molecular mass (listed in Fig. 1) were selected and their predicted fragments were compared with the acquired MS/MS spectrum. Structure was based on complete match of the main fragments. In the case where more than one isomer

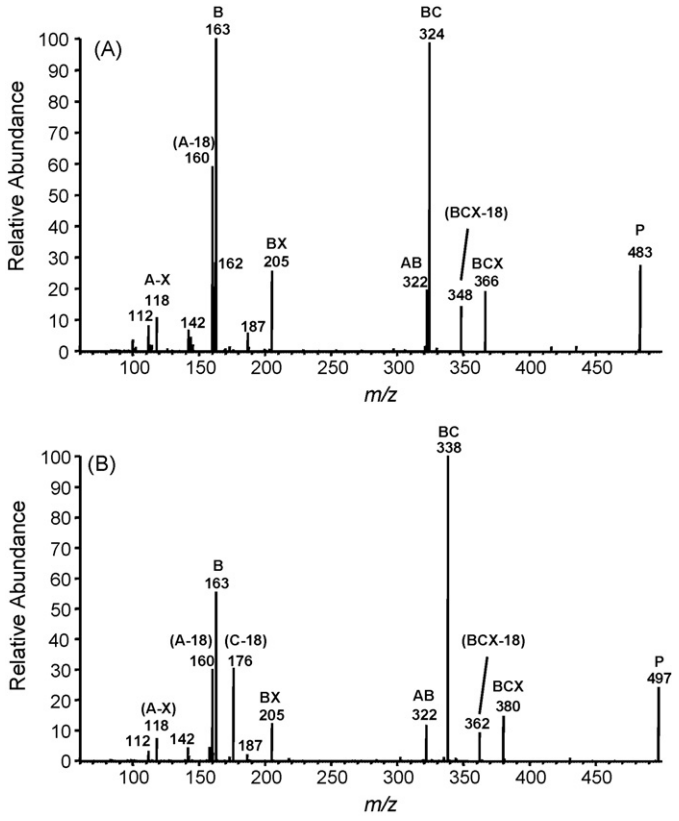


Fig. 4. Product mass spectra of reference compounds gentamicin B (A) and gentamicin B_1 (B). The main fragments are denoted by letter coding.

has the same predicted fragments, all of them were proposed as the possible match. Retention time and MS/MS spectrum of some reference compounds and gentamines were also used for unknown peak identification.

Peak 1 was identified as garamine with the same retention time as reference substance and practically identical MS/MS spectrum. The protonated molecule of peak 2 was m/z 469 and corresponds to the molecular weight of gentamicins A, A_1 and A_3 isomers on the C ring. The fragmentation pattern corresponds to all three isomers and therefore is impossible to differentiate among them. Gentamine C_{1a} was peak 3 related to the retention time and MS/MS spectrum of the reference substance. Four possible structures for peak 4 with protonated molecule m/z 483 were found: gentamicins II-2 and III-3, isomers on ring A and gentamicins X_2 and B, isomers on ring C, respectively. The predicted MS/MS spectra of II-2 and III-3 do not corresponded with the peak spectrum having (A-18) and (C-18) fragments m/z 146 and 176 where the fragments in the peak spectrum were m/z 160 and 163, respectively. The reference substance gentamicin B has the same retention time and the MS/MS spectrum as peak 4 and is not probable that gentamicin X_2 would have the same retention time as gentamicin B. The predicted MS/MS spectra of gentamicins II-2 and III-3 were in accordance with peak 5 spectrum. Gentamine C_2 was peak 6, which was confirmed with the gentamines substance. The only known gentamicin from literature [5–7] with protonated molecule m/z 482 is JI-20A and the predicted fragments were in accordance with the MS/MS spectrum of peak 7. Gentamicin VII-3 has the same molecular mass as the substance in peak 8 and the predicted MS/MS spectrum was in accordance with the measured spectrum. The identities of peaks 9, 10 and 11 were confirmed with the reference substance gentamicin B_1, gentamine C_{2a} and sisomicin and the MS/MS spectra of unknowns were in accordance with reference spectra. Gentamicin JI-20B has the same molecular weight as the substance in peak 12 and the predicted fragments were the same as in the MS/MS spectrum of the peak. The reference substance gentamine C_1 confirmed the identity

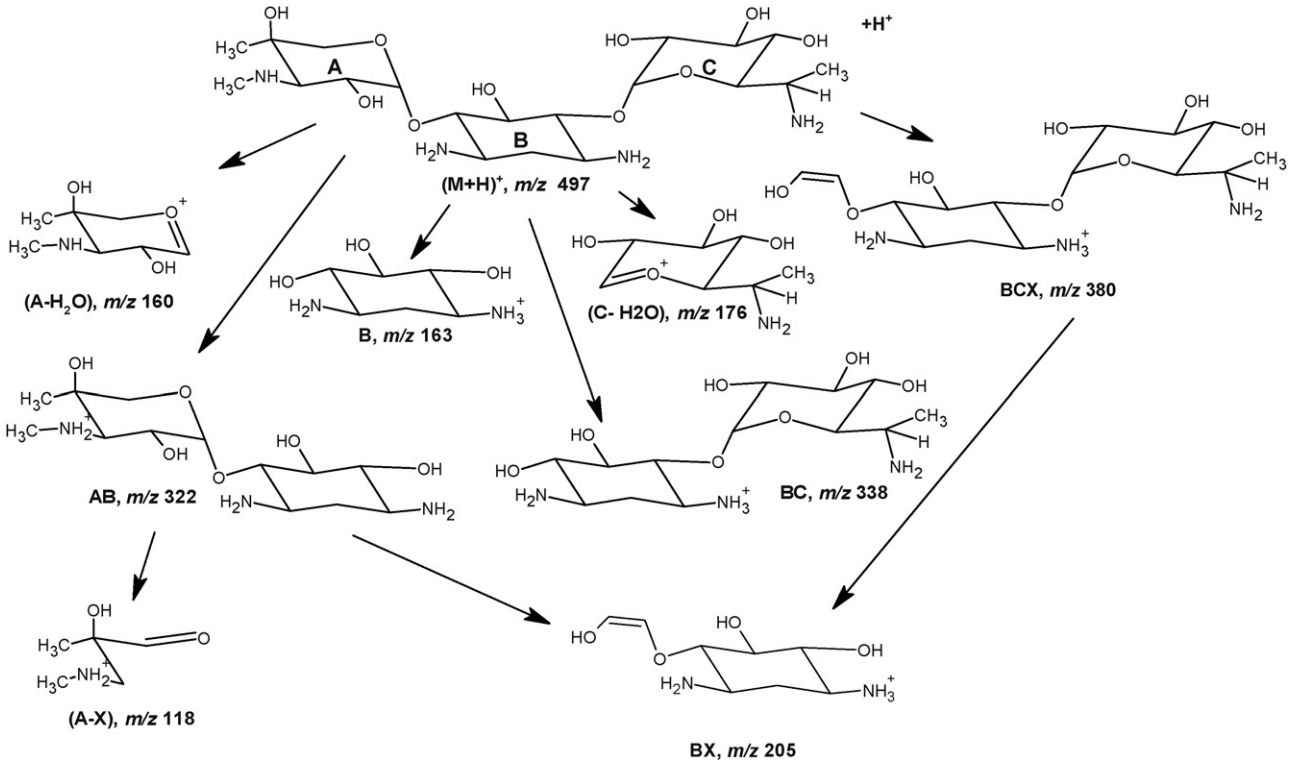


Fig. 5. Predicted fragmentation transitions of gentamicin B_1.

of peak 13. Gentamicins Y-02077H-δ, VII-2 and XK-62-5 are isomers and have the same molecular mass as peak 14. The predicted fragments corresponded to the MS/MS spectrum of the peak 14 so were proposed as three possible matches. Regarding the molecular mass, the peak 15 probably represents gentamicins: XK-62-7, XK-62-8 and Y-02077H-β. The predicted fragments of gentamicin Y-02077H-β were the same as in the peak. Interestingly a different structure of ring B was observed showing an additional ethyl group. Peak 16 was confirmed as gentamicin C_{2b} with the reference substance. Regarding the molecular mass, the last unknown peak with the protonated molecule of m/z 464 has three possible gentamicin structures: VII-1, XK-62-3 and Y-02077H-γ. The predicted fragments of VII-1 and Y-02077H-γ, isomers on ring A, were in accordance with the MS/MS spectrum of the peak 17.

The impurities: garamine, gentamicin A or A_1 or A_3, gentamicin B, gentamicin B_1, JI-20A, sisomicin, JI-20B and gentamine C_1 were also found in bulk gentamicin by Li [29]. In contrast the same author reports for the presence of other impurities which are not present in our sample and we have additionally detected gentamine C_{1a}, gentamine C_2, gentamine C_{2a}, VII-2 (or isomers), Y-02077H-β and VII-1 (or isomers) which were not mentioned by Li [29].

4. Conclusion

This paper describes an HLPC/MS/MS method for impurities identification in gentamicin. APCI was successfully applied for the ionization of polar aminoglycosides and no ionization suppression was observed at high (50 mM) TFA concentration. All impurities were separated from gentamicin components and were identified on the basis of MS/MS spectra and reference substances. MS/MS spectra of reference compound were interpreted and a general fragmentation transitions for gentamicins were proposed. A total of 17 impurities were identified in gentamicin. More than one isomer was proposed for three impurities. Six of them were different as reported by Li [29].

References

[1] M.J. Weinstein, G.M. Luedemann, E.M. Oden, G.H. Wagman, Antimicrob. Agents Chemother. 1 (1963) 1–13.
[2] R.S. Edson, C.L. Terrell, Mayo Clin. Proc. 74 (1999) 519–529.
[3] G.H. Wagman, J.A. Marquez, J.V. Balely, D. Cooper, J. Weinstein, R. Tkach, P. Daniels, J. Chromatogr. 70 (1972) 171–173.
[4] B.K. Lee, R.G. Condon, G.H. Wagman, E. Katz, Antimicrob. Agents Chemother. 9 (1976) 151–159.
[5] J. Berdy, J.K. Pauncz, Zs.M. Vanja, Gy. Horvath, J. Gyimesi, I. Koczka, J. Antibiot. 30 (1977) 945–954.
[6] J. Berdy, M. Jarai, Process Biochem. 6 (1986) 93–100.
[7] J. Berdy, M. Jarai, Process Biochem. 8 (1986) 103–106.
[8] M. Freeman, P.A. Hawkins, J.S. Loran, J.A. Stead, J. Chromatogr. 2 (1979) 1305–1317.
[9] P.J. Claes, R. Busson, H. Vanderhaeghe, J. Chromatogr. 298 (1984) 445–457.
[10] A.I. Al-Amoud, B.J. Clark, H. Chrystyn, J. Chromatogr. B 769 (2002) 89–95.
[11] P. Gambardella, R. Punziano, M. Gionti, C. Guadalupi, G. Mancini, A. Mangia, J. Chromatogr. 348 (1985) 229–240.
[12] D.M. Barends, C.L. Zwaan, A. Hulshott, J. Chromatogr. B 222 (1981) 316–323.
[13] D.M. Barends, J.S.F. Van Der Sandt, A. Hulshoff, J. Chromatogr. B 182 (1980) 201–210.
[14] G. Inchauspe, C. Deshayes, D. Samain, J. Antibiot. 38 (1985) 1526–1535.
[15] Y. Cai, Y. Cai, J. Cheng, S. Mou, L. Yiqiang, J. Chromatogr. A 1085 (2005) 124–130.
[16] V. Manyanga, K. Kreft, B. Divjak, J. Hoogmartens, E. Adams, J. Chromatogr. A 1189 (2008) 347–354.
[17] V. Manyanga, O. Grishina, Z. Yun, J. Hoogmartens, E. Adams, J. Pharm. Biomed. Anal. 45 (2007) 257–262.
[18] N.C. Megoulas, M.A. Koupparis, J. Pharm. Biomed. Anal. 36 (2004) 73–79.
[19] I. Clarot, P. Chaimbault, F. Hasdenteufel, P. Netter, A. Nicholas, J. Chromatogr. A 1031 (2004) 281–287.
[20] T.A. Getek, M.L. Vestal, T.G. Alexander, J. Chromatogr. 554 (1991) 191–201.
[21] A.E. Graham, E. Speicher, B. Williamson, J. Pharm. Biomed. Anal. 15 (1997) 537–543.
[22] L.G. McLaughlin, J.D. Henion, P.J. Kijak, Biol. Mass Spectrom. 23 (1994) 417–429.
[23] D. Löffler, T.A. Ternes, J. Chromatogr. A 1000 (2003) 583–588.
[24] G. Inchauspe, P. Delrieu, P. Dupin, M. Laurent, D. Samain, J. Chromatogr. 404 (1987) 53–66.
[25] D. Samain, P. Dupin, P. Delrieu, G. Inchauspe, Chromatographia 24 (1987) 748–752.
[26] O. Curcuruto, G. Kennedy, M. Hamdan, Org. Mass Spectrom. 23 (1994) 417–429.
[27] S.I. Kotretsou, V. Constantinou-Kokotou, Carbohydr. Res. 310 (1998) 121–127.
[28] P. Hu, E.K. Chess, S. Brynjelsen, G. Jakubowski, J. Melchert, R.B. Hammond, T.D. Wilson, J. Am. Soc. Mass Spectrom. 11 (2000) 200–209.
[29] B. Li, E. Adams, A. Van Schepdael, J. Hoogmartens, Rapid Commun. Mass Spectrom. 20 (2006) 393–402.