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Analysis of amikacin by liquid chromatography with pulsed electrochemical detection

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Abstract

The analysis of amikacin by liquid chromatography using a column packed with poly(styrene–divinylbenzene) and pulsed electrochemical detection on a gold electrode is described. A two-step gradient was necessary to obtain a good separation together with a reasonable analysis time of 60 min. The mobile phases consisted of an aqueous solution of 1 g/l or 60 g/l sodium sulfate, 1.8 g/l sodium octanesulfonate and 50 ml/l 0.2 *M* phosphate buffer, pH 3.0. Sodium hydroxide was added postcolumn. The influence of the different chromatographic parameters on the separation was investigated. When a number of commercial samples of amikacin was analyzed using this method, ten different components were separated.

Keywords: Detection, LC; Pulsed electrochemical detection; Amikacin; Antibiotics

1. Introduction

Amikacin or BB-K8, is a semisynthetic, water soluble, broad spectrum aminoglycoside antibiotic. It is commonly administered parenterally for the treatment of Gram-negative infections resistant to gentamicin, kanamycin or tobramycin because the amikacin molecule has fewer points susceptible to enzymatic attack than have most other aminoglycosides [1]. Its oto- and nephrotoxicity however require careful monitoring of the blood levels [2].

Amikacin is obtained by acylation of the amino group in position 1 of kanamycin A with L-(−)-γ-amino-α-hydroxybutyric acid (L-HABA) (Fig. 1) [3]. Therefore, kanamycin A and L-HABA can be expected to be possible impurities in commercial samples. As the molecule of kanamycin A has four primary amino groups, it is possible during the synthesis to obtain side products that differ only in the position of the acyl group. The three positional isomers of amikacin (BB-K8), which are acylated with L-HABA at the C-3, C-6′, and C-3″ amino groups of kanamycin A are described as BB-K29, BB-K6 and BB-K11, respectively. In contrast to amikacin, these positional isomers are all very weakly active [4]. Kanamycin acylated with L-HABA molecules on the C-1 and C-3 position (di-HABA kanamycin) can also be formed.

Liquid chromatography (LC) of amikacin is not straightforward because the drug does not have a significant UV absorbing chromophore. Pre- and postcolumn derivatization with *o*-phthalaldehyde (OPA) [5–7] and precolumn derivatization with 1-fluoro-2,4-dinitrobenzene [8–11] and trinitrobenzenesulfonic acid [12,13] have been described. However, these techniques are time consuming and give problems with quantitation. It has been mentioned that precolumn derivatization with OPA or 1-fluoro-

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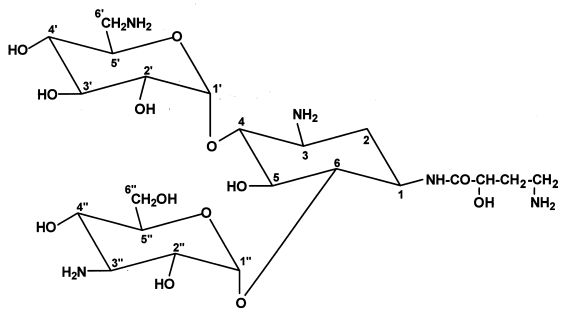

Site of acylation with L-HABA (L(-)-γ-amino-α-hydroxybutyric acid)

BB-K8 = amikacin	C_1
BB-K6	$C_{6'}$
BB-K29	C_3
BB-K11	$C_{3'}$
Kanamycin A	/
di-HABA kanamycin	$C_1 + C_3$

Fig. 1. Structure of some amikacin components.

2,4-dinitrobenzene resulted in unstable derivatives [5–8].

To our knowledge, no paper has been published describing the composition of commercial amikacin samples.

In this work an ion-pair LC method using a column packed with poly(styrene–divinylbenzene) and pulsed electrochemical detection is described. The composition of the mobile phase used in this study is based on that previously used for the analysis of other aminoglycoside antibiotics, neomycin, kanamycin and netilmicin [14–16]. The method has been used to analyze a number of commercial samples.

2. Experimental

2.1. Reagents and reference substances

Water was distilled twice from glass apparatus. The buffer solution was made by mixing a 0.2 *M* solution of phosphoric acid and a 0.2 *M* solution of potassium dihydrogenphosphate until a pH of 3.0 was achieved. These solutions were prepared with phosphoric acid 85% (m/m) and potassium dihydrogen phosphate (Acros Chimica, Geel, Belgium). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany); sodium 1-octanesulfonate, monohydrate 98% from Acros Chimica and helium from Air Liquide (Machelen, Belgium). The 0.5 *M* sodium hydroxide solution was made using sodium hydroxide 50% (m/m) aqueous solution (Baker, Deventer, Netherlands). BB-K11 and BB-K29 were obtained from Bristol-Myers Squibb (Waterloo, Belgium), BB-K6 from Gist-Brocades (Capua, Italy), L-HABA and di-HABA kanamycin from the European Pharmacopoeia laboratory (Ph. Eur., Strasbourg, France) and kanamycin sulfate from Fluka (Buchs, Switzerland). Commercial samples of amikacin base and sulfate were provided by Bristol-Myers Squibb (Syracuse, NJ, USA; Sermoneta, Italy and Paris La Défense, France) and Ph. Eur.

2.2. Apparatus

The chromatographic analysis was carried out using a L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany), a Gilson 234 autoinjector (Villiers-le-Bel, France) with a fixed loop of 20 μl, a laboratory-made pneumatic device, allowing pulse-free postcolumn addition of sodium hydroxide solution and an electronic integrator HP 3393 A (Hewlett-Packard, Avondale, PA, USA). The column (250×4.6 mm I.D.) was packed with poly(styrene–divinylbenzene) PLRP-S 1000 Å, 8 μm (Polymer Laboratories, Shropshire, UK). The temperature of the column was maintained at 40°C by immersion in a water bath with a circulator (Julabo, Seelbach, Germany). The PED-1 pulsed electrochemical detector from Dionex (Sunnyvale, CA, USA) was equipped with a gold working electrode with a diameter of 3 mm, a Ag/AgCl reference electrode and a stainless steel counter electrode. The detector was put in a laboratory-made hot air oven to keep the temperature at 35°C.

2.3. Chromatography

All substances to be analyzed were dissolved in water. Scheme 1 shows an overview of the LC conditions finally chosen. A two-step gradient was

Stationary phase : PLRP-S 1000 Å, 8 μm, 250 mm × 4.6 mm I.D., Polymer Laboratories, Shropshire, UK.

Mobile phase :	A	B
sodium sulfate	1 g/l	60 g/l
sodium 1-octanesulfonate	1.8 g/l	1.8 g/l
phosphate buffer pH 3, 0.2 M	50 ml/l	50 ml/l
water	up to 1 l	up to 1 l

Two-step gradient :	
0 - 3.0 min	100 % A
3.1 - 37.0 min	55 % A - 45 % B
37.1 - 50.0 min	30 % A - 70 % B
50.1 - 60.0 min	100 % A

Flow rate : 1 ml/min
Injection volume : 20 μl
Column temperature : 40 °C

Post-column addition of 0.5 M NaOH : 0.3 ml/min

Pulsed electrochemical detector :
Working electrode : gold
Reference electrode : Ag/AgCl
Counter electrode : stainless steel

Detector settings : t (s)	E (volt)
0 - 0.40	0.05
0.41 - 0.60	0.75
0.61 - 1.00	-0.15

Integration period : 0.20 - 0.40 s
Sensitivity : 1 μC
The detector was kept at 35 °C.

Scheme 1. LC conditions.

necessary to obtain a good separation between the first eluted compounds and to elute the others within a reasonable analysis time. The mobile phases were sonicated before use. Through a mixing tee, 0.5 *M* NaOH was added postcolumn from a helium pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (1.2 m, 500 μl) from Dionex which was linked to the electrochemical cell. The postcolumn addition of the base must be pulse-free and is necessary to raise the pH of the mobile phase to approximately 13 to improve the sensitivity of the detection [17]. The 0.5 M NaOH solution was made starting from a 50% (m/m) aqueous solution which was pipetted into helium degassed water to avoid carbonates that foul the electrodes. It is advisable to pipette the NaOH solution from the center of the bottle and to use only two thirds of the bottle [18].

The time and voltage parameters for the detection are also shown in Scheme 1 and are the same as previously used for neomycin, kanamycin and netilmicin [14–16].

3. Results and discussion

3.1. Chromatographic method

A typical chromatogram of a commercial sample of amikacin base, obtained under the selected chromatographic conditions, is shown in Fig. 2. Three of the ten peaks correspond to components of unknown identity. Poly(styrene–divinylbenzene) was chosen as the stationary phase because of its remarkable stability and batch reproducibility. In order to examine the robustness of the method, the influence of the different chromatographic parameters on the separation of the different amikacin components was evaluated using the capacity factors (k'). Only one parameter was changed while the others were kept constant. Methanol was used to determine t_0. For the calculation of k', the average retention time of two analyses was used.

The influence of the pH of the mobile phase on the k' values of the amikacin components was studied in the range from pH 2 to pH 6. Nearly no changes were observed between pH 2 and pH 5. By further increasing the pH, the retention times decreased since less amino groups are protonated and the interaction with octanesulfonate decreases. The influence of the column temperature was examined at 35, 40 and 45°C. As expected, the k' values of the components decrease when the column temperature

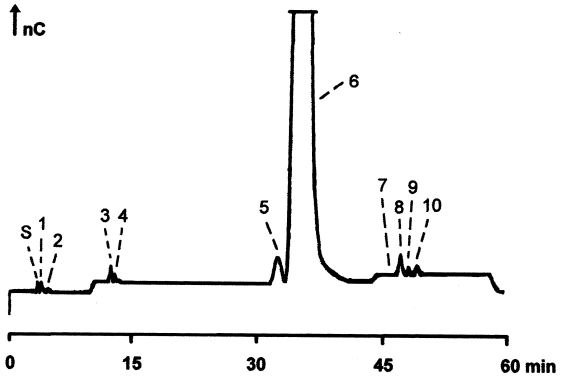

Fig. 2. Typical chromatogram of a commercial amikacin sulfate sample. See Scheme 1 for chromatographic conditions. S=solvent peak; 1=unknown 1; 2=L-HABA; 3=unknown 2; 4=unknown 3; 5=di-HABA kanamycin; 6=amikacin; 7=BB-K11; 8=BB-K29; 9=BB-K6; 10=kanamycin A.

Table 1
Quantitative aspects of the system

Compound	LOD	LOQ	Linearity			
	(μg)	(μg)	Range (μg)	y	r	$S_{y,x}$
BB-K-29	0.005	0.015	0.015–1	$39222x+100$	0.9993	596
Kanamycin A	0.005	0.015	0.015–1	$38617x+155$	0.9993	609
Amikacin			2–12	$36705x+6381$	0.9993	6308

is increased. However, the resolution between the peaks also decreases with higher column temperatures. Sodium octanesulfonate as an ion-pairing agent was added to retain the amikacin molecules, which are positively charged at pH 3.0. When the sodium octanesulfonate concentration of the mobile phase was increased from 1.6 to 2.0 g/l, the capacity factors increased by about 10%. Sodium sulfate has been used in ion-pairing mobile phases to shorten the retention times as the sulfate anions are more hydrophilic than the anions of the ion-pairing agent [19]. The sodium sulfate gradient was chosen so that a good separation between the first eluting components was obtained while the total time of analysis was not too long.

3.2. Quantitative aspects of the LC method

For the determination of the impurities in amikacin a 10-μg sample was used by injecting 20 μl of a 0.5 mg/ml solution. For this quantity the limit of detection (LOD, s/n=3) and limit of quantification (LOQ) for BB-K29 and kanamycin A were determined. The results are shown in Table 1. The linearity of BB-K29 and kanamycin A was examined in the concentration range corresponding to 0.15% to 10% of the sample concentration (0.5 mg/ml). The linearity of amikacin was examined in the concentration range corresponding to 20–120% of the sample concentration (0.5 mg/ml). The results are also shown in Table 1, where y=peak area/1000; x=amount of sample injected (μg); r=coefficient of correlation and $S_{y,x}$=standard error of estimate. The repeatability was checked by analyzing a 0.5 mg/ml solution of amikacin six times. The R.S.D. on the area of the main peak was 1.6%.

3.3. Analysis of commercial samples

Several samples of amikacin base and amikacin sulfate were analyzed using the described method. The obtained composition of the samples is shown in Table 2. All related components are expressed as amikacin, using chromatograms obtained with a 2% (v/v) dilution (0.01 mg/ml) of the examined sample. The most important impurity, di-HABA kanamycin, was never reported to be present in commercial samples. It is also noteworthy that the examined samples contained nearly no kanamycin A.

4. Conclusion

The described method, using poly(styrene–di-

Table 2
Composition of commercial amikacin base and sulfate[a] samples (% (m/m)), relative to amikacin

	Unknown 1	L-HABA	Unknown 2	Unknown 3	di-HABA kan	BB-K11	BB-K29	BB-K6	Kanamycin
1	0.06	0.04	0.13	0.06	1.28	ND	0.52	0.11	0.12
2	0.07	0.04	0.16	0.09	1.15	ND	0.51	0.12	0.13
3	ND	ND	0.19	0.07	1.12	ND	0.45	0.09	0.10
4	0.09	0.04	0.16	0.08	1.09	ND	0.49	0.10	0.14
5	0.21	0.04	0.37	ND	0.43	0.29	0.32	ND	0.12
6	ND	0.04	0.22	0.05	1.19	ND	0.31	±0.05	0.11
7[a]	ND	0.07	0.32	ND	ND	±0.05	±0.06	ND	0.13

ND=not detected.

vinylbenzene) as the stationary phase, allows separation of ten components of amikacin. The total time of analysis is 60 min. This study reports for the first time the presence of di-HABA kanamycin in commercial samples. Pulsed electrochemical detection suffers from some stability problems and some experience is required to obtain a good repeatability. However, compared to the chromatographic methods previously published, this method allows sensitive detection of amikacin without derivatization.

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