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Analysis of tobramycin by liquid chromatography with pulsed electrochemical detection

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Abstract

The determination of tobramycin by liquid chromatography using a column packed with poly(styrene-divinylbenzene) and pulsed electrochemical detection on a gold electrode is described. The mobile phase consisted of an aqueous solution containing 52 g/l of sodium sulfate, 1.5 g/l of sodium octanesulfonate and 50 ml/l of a 0.2 M phosphate buffer pH 3.0. The total time of analysis was not more than 30 min. The effects of the different chromatographic parameters on the separation were also investigated. When a number of commercial samples of tobramycin was analyzed using this method, nine different components were separated, five of which are of unknown identity.

Keywords: Tobramycin; Liquid chromatography; Pulsed electrochemical detection

1. Introduction

Tobramycin, or factor 6 of the nebramycin complex produced by *Streptomyces tenebrarius* [1], is a broad spectrum aminoglycoside antibiotic. Like other aminoglycosides, it is potentially oto- and nephrotoxic [2,3]. Its antimicrobial activity and toxicity are similar to those of gentamicin. Beside the main component tobramycin, the presence of minor constituents such as kanamycin B, neamine and the degradation product nebramine, has been reported (Fig. 1). However, no quantitative results for these impurities in commercial samples were given [4–6].

Several methods have been described to determine tobramycin: paper chromatography [4,5], thin-layer chromatography [5,7], gas–liquid chromatography after trimethylsilylation [8], spectrophotometry [9,10], reversed phase liquid chromatography [6,11–19] and chromatography on a high-performance anion-exchange column (CarboPac PA1) [20]. This latter column packing is patented and rather expensive. Most of the methods described focus only on the main component tobramycin, giving no information about the composition of the samples.

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Liquid chromatography (LC) of tobramycin is not straightforward because the drug does not have a significant UV absorbing chromophore. Pre-column derivatization with 2,4-dinitrofluorobenzene [6,11], *o*-phthalaldehyde (OPA) [12,13,19] and 2,4,6-trinitro-benzenesulphonic acid [16,18] as well as post-column derivatization with OPA [14,17] have been described. However, these techniques are time consuming and give problems with quantitation. Polta et al. [15] and Statler [20] used pulsed amperometry to detect tobramycin.

The European Pharmacopoeia (Ph. Eur.) prescribes microbiology as assay method [21] and the United States Pharmacopeia (USP) LC combined with pre-column derivatization with 2,4-dinitrofluorobenzene [22].

In this study 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 is based on that previously used for the analysis of other aminoglycoside antibiotics such as neomycin, kanamycin and amikacin [23–25]. The method has been used to analyze a number of commercial samples. It allows for simultaneous determination of the main component and of the related substances.

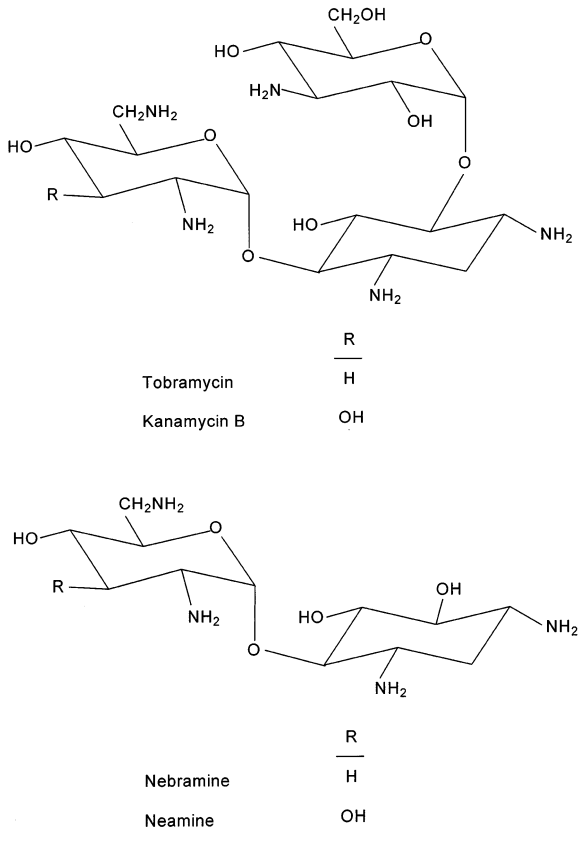

Fig. 1. Structure of some tobramycin components.

2. Experimental

2.1. Reagents and reference samples

Water was distilled twice from glass apparatus. Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany); sodium 1-octanesulfonate monohydrate 98% and phosphoric acid 85% (m/m) from Acros Organics (Geel, Belgium); potassium dihydrogen phosphate from BDH (Poole, UK) 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, the Netherlands).

Neamine was prepared in the laboratory as described [26]. Kanamycin B was obtained from Bristol Laboratories (Syracuse, NY, USA) and nebramine was kindly provided by Dr K. Kovács-Hadady (Lajos Kossuth University, Debrecen, Hungary). Commercial samples were obtained from Biogal (Debrecen, Hungary), E. Lilly (Windlesham, Surrey, UK and Indianapolis, IND, USA) and Alcon-Couvreur (Puurs, Belgium).

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 post-column 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

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

Mobile phase :

sodium sulfate	52 g/l
sodium 1-octanesulfonate	1.5 g/l
phosphate buffer pH 3, 0.2 M	50 ml/l
water	up to 1 l

Flow rate : 1 ml/min
Injection volume : 20 μl
Column temperature : 55 °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.

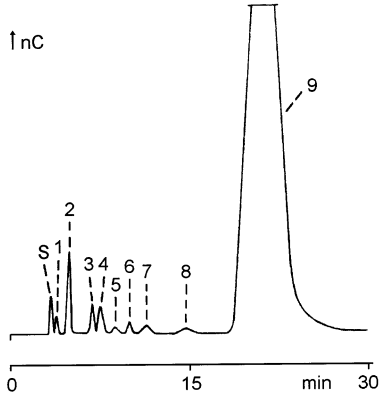

Fig. 2. Typical chromatogram of a commercial sample of tobramycin base (sample 8 of Table 3) (see Scheme 1 for chromatographic conditions) S = solvent, 1 = unknown 1; 2 = unknown 2; 3 = neamine; 4 = unknown 3; 5 = nebramine; 6 = unknown 4; 7 = unknown 5; 8 = kanamycin B; 9 = tobramycin.

μm (Polymer Laboratories, Shropshire, UK). The temperature of the column was maintained at 55°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 cell of 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. The mobile phase was sonicated before use. Through a mixing-tee 0.5 M NaOH was added post-column (0.3 ml/min) from a helium-pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (Dionex, 1.2 m, 500 μl) which was linked to the electrochemical cell. The 0.5 M NaOH solution was made starting from a 50% (m/m) aqueous solution [27].

The time and voltage parameters for the pulsed electrochemical detector are also shown in Scheme 1 and were the same as previously used for other aminoglycosides [23–25]. More information about the post-column addition of the NaOH solution and the cleaning of the electrodes can be found in previously published papers [23–25].

3. Results and discussion

3.1. Chromatographic method

Poly(styrene-divinylbenzene) was chosen as the stationary phase because of its remarkable stability and batch reproducibility. A typical chromatogram of a commercial sample of tobramycin base, obtained under the selected chromatographic conditions, is shown in Fig. 2. Five of the nine peaks correspond to components of unknown identity.

The importance of the individual chromatographic parameters and parameter interactions of this LC method was studied by means of a 4-factorial design. The set-up of the applied factorial design was supported by a statistical graphics software system, Statgraphics version 6 (Manugistics, Rockville, MD, USA). The chromatographic parameters examined as variables were: the concentration of sodium sulfate (Na_2SO_4) and of sodium octanesulfonate (SOS), the pH of the

mobile phase buffer and the column temperature (temp). The values used in the design are shown in Table 1. A 4-factorial design at two levels involves at least $2^4 = 16$ experimental measurements. The central level was repeated three times and was also included in the design. The measured response variables were the retention times of neamine, nebramine, kanamycin B and tobramycin.

Under the examined conditions, the LC system is principally influenced by the sodium sulfate concentration which has a negative effect on the retention times. This means that the retention time of the four tobramycin components studied will decrease with an increasing amount of sodium sulfate. The second and third most important chromatographic parameter by which the retention times of nebramine and tobramycin are influenced are the column temperature and the sodium octanesulfonate concentration. For the retention times of neamine and kanamycin B, the sodium octanesulfonate concentration is more important than the column temperature. For all components examined, the column temperature has a negative effect on the retention times. The sodium octanesulfonate concentration has a positive effect on the retention times, so that the retention times will increase with an increasing amount of sodium octanesulfonate. This was expected, because sodium octanesulfonate was added as an ion-pairing agent to retain the tobramycin molecules which are positively charged at pH 3.0. The pH of the buffer of the mobile phase has no significant effect on the retention times. In the pH range examined, the amino groups of the tobramycin molecules are protonated and interact with the ion-pairing agent. No important interactions between the different parameters were observed.

Table 1
Factorial analysis: nominal values corresponding to -1, 0 and $+1$

Chromatographic parameter	-1	0	$+1$
Sodium sulfate (g/l)	49	52	55
Sodium octanesulfonate (g/l)	1.2	1.5	1.8
pH of the mobile phase buffer	2	3	4
Column temperature (°C)	50	55	60

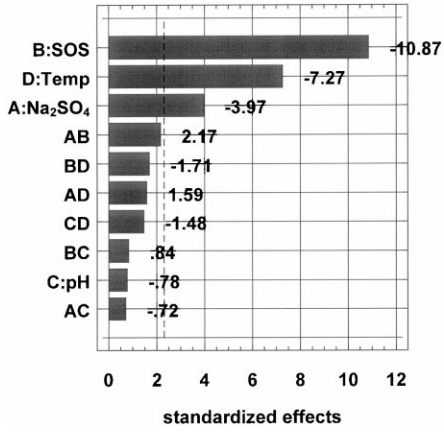

Fig. 3. Standardized pareto chart, representing the estimated effects of the chromatographic parameters and their interactions on the selectivity factor for kanamycin B and tobramycin.

Using the same experimental results, also the separation between kanamycin B and tobramycin was examined. The separation between these two compounds was chosen because they are the most important pair to be separated and because reference components of both substances were available. The selectivity factor for kanamycin B and tobramycin (α_{kt}) was used as response variable. The standardized pareto chart, representing the estimated effects of the four chromatographic parameters and their interactions on α_{kt}, is shown in Fig. 3. As can be seen, α_{kt} is mainly influenced by the sodium octanesulfonate concentration. The column temperature and the sodium sulfate concentration are the second and third most important chromatographic parameters. It is remarkable that these three factors all have a negative effect on the separation between kanamycin B and tobramycin. As expected, the pH of the buffer of the mobile phase has no significant influence on the separation and interactions between the parameters were also found to have no significant influence on α_{kt}.

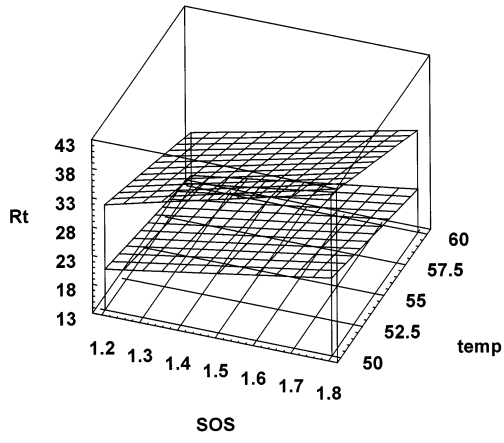

Fig. 4. Estimated response surface plots showing how the retention times (Rt) of tobramycin (upper plane) and kanamycin B (lower plane) vary as a function of the sodium octanesulfonate (SOS) concentration and the column temperature (temp). The sodium sulfate concentration and the pH of the buffer of the mobile phase were kept constant at 52 g/l and pH 3.0, respectively.

Table 2
Linearity of neamine, nebramine, kanamycin B and tobramycin

	Range (μg)	y	r	$S_{y,x}$
Neamine	0.005–0.4	$59220x+170$	0.9994	336
Nebramine	0.008–0.4	$73524x-30$	0.9998	219
Kanamycin B	0.012–0.4	$76522x-153$	0.9996	360
Tobramycin	1.6–9.6	$51878x+4017$	0.9996	5476
	0.012–0.4	$52325x-102$	0.9996	293

In Fig. 4, estimated response surface plots show how the retention times (Rt) of tobramycin and kanamycin B vary as a function of the sodium octanesulfonate concentration and the column temperature. The sodium sulfate concentration and the pH of the buffer of the mobile phase were kept constant at 52 g/l and pH 3.0, respectively. Under the conditions examined both components where always separated from each other, since the planes do not overlap.

3.2. Quantitative aspects of the LC method

For the determination of the impurities in tobramycin an amount of 8 μg was used by injecting 20 μl of a 0.4 mg/ml solution. For this quantity the limit of detection (LOD) for neamine was 0.02% (m/m) (1.6 ng), for nebramine 0.03% (m/m) (2.4 ng) and for kanamycin B 0.05% (m/m) (4 ng). The limit of quantification (LOQ) for neamine was 0.06% (m/m) (R.S.D. = 9.8%, $n=4$), for nebramine 0.10% (m/m) (R.S.D. = 8.1%, $n=4$) and for kanamycin B 0.15% (m/m) (R.S.D. = 9.2%, $n=4$). The linearity of neamine was examined in the concentration range corresponding to 0.06–5% of the sample concentration (0.4 mg/ml). For nebramine, the linearity was examined in the range 0.10–5% and for kanamycin B in the range 0.15–5%. The results are shown in Table 2. The linearity of tobramycin was examined in the concentration range corresponding to 20–120% and 0.15–5% of the sample concentration (0.4 mg/ml). The results are also shown in Table 2, 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 0.4 mg/ml solution of tobramycin five times. The R.S.D. on the area of the main peak was 1%.

3.3. Analysis of commercial samples

Nine commercial samples of tobramycin were analyzed using the described method. The obtained composition is shown in Table 3. All minor components are expressed as the relative amounts of tobramycin, using chromatograms obtained with a 5% (v/v) dilution (0.02 mg/ml) of the examined sample. As can be seen, the total amount of related components found in the commercial samples is rather low for a natural antibiotic. The amount reported for impurities of unknown identity is more important than that of the known ones. This may be due to the fact that the unknowns have a higher reponse in the detection system. To eliminate this possibility, leading to a quantitation error, isolation and identification work has to be carried out.

Table 3
Composition of tobramycin samples (%), relative to tobramycin[a]

Sample	Unk. 1	Unk. 2	Neamine	Unk. 3	Nebramine	Unk. 4	Unk. 5	Kanamycin B
1	±0.02	0.06	±0.02	0.90	ND	±0.04	0.22	0.20
2	ND	0.37	±0.05	0.22	0.14	±0.06	0.11	0.25
3	ND	0.27	±0.03	1.29	±0.07	0.10	0.45	±0.08
4	ND	0.18	0.11	0.50	±0.05	±0.06	0.24	ND
5	ND	0.25	0.08	0.46	±0.08	±0.06	0.18	±0.05
6	±0.02	0.17	±0.04	0.50	±0.03	±0.05	0.20	±0.05
7	±0.02	±0.03	0.11	0.09	ND	ND	±0.04	±0.05
8	0.29	0.77	0.33	0.44	0.14	0.19	0.31	0.31
0	±0.02	0.05	±0.04	0.76	ND	±0.04	±0.11	0.22

[a] ND, not detected; ± is used for values below LOQ; Unk., unknown.

4. Conclusion

The described method, using poly(styrene-divinylbenzene) as the stationary phase, allows the separation of nine components of tobramycin. The total time of analysis is 30 min. It is the first time quantitative results are given for the minor components of tobramycin. Pulsed electrochemical detection suffers from some stability problems and experience is required to obtain a good repeatability, but it allows sensitive detection of tobramycin without derivatization.

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