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Analysis of gentamicin by liquid chromatography with pulsed electrochemical detection

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Abstract

The analysis of gentamicin by liquid chromatography using a column packed with poly(styrene-divinylbenzene) and pulsed electrochemical detection on a gold electrode is described. The mobile phase consists of an aqueous solution containing sodium sulfate, tetrahydrofuran, sodium 1-octanesulfonate and a phosphate buffer of pH 3.0. In contradistinction to methods previously published, this method not only allows a better separation of gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b}, but also the separation of several other, minor components, most of which were not identified. The effects of the different chromatographic parameters on the separation were also investigated. A number of commercial samples was analysed using this method, allowing sensitive detection of gentamicin without derivatization, and the results were compared with the results obtained with the European Pharmacopoeia method, prescribing pre-column derivatization. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Liquid chromatography; Gentamicin; Pulsed electrochemical detection; Sagamicin

1. Introduction

Gentamicin is a broad spectrum water-soluble aminoglycoside antibiotic produced by fermentation of *Micromonospora purpurea* [1]. It has a narrow therapeutic range and is potentially oto- and nephrotoxic like other aminoglycosides. Gentamicin is a complex mixture of four major components (C_1, C_{1a}, C_2 and C_{2a}) (Fig. 1) and several minor components like, e.g. sisomicin [2], gentamicin C_{2b}, also known as sagamicin [3,4], and dihydroxy C_{2a} (Antibiotic JI-20 B), which is a precursor of C_{2a}, C_2 and C_1 [5]. At first only gentamicins C_1, C_{1a} and C_2 were considered as the main components, but it has been shown that gentamicin samples also contain considerable amounts of C_{2a} [6–8]. Since the gentamicin components are closely related, the chromatographic separation is not trivial. Several separation methods have been applied for the determination of the component ratio in commercial samples; paper and thin-layer chromatography [9,10], Craig distribution [11], ion-exchange liquid chromatography (LC) [12–14], reversed phase LC [6–8,15–23] and capillary electrophoresis (CE) [24]. The

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determination of component C_{2b} in commercial gentamicin samples has only been described in a few papers [11,13,23]. Detection of the different components is also problematic because gentamicin has no UV absorbing chromophore. LC methods combined with refractive index detection [15], post-column derivatization with *ortho*-phthaldehyde (OPA) [13,15,20] and pre-column derivatisation with OPA [6–8,16,17] and 2,4,6-trinitro-benzenesulphonic acid [21] were published. In 1983, Getek et al. described a LC system with electrochemical detection, using a glassy carbon electrode [18] and more recently, Kaine and Wolnik used pulsed electrochemical detection (PED) on a gold electrode [14] to detect the gentamicin components. In 1994, a CE method utilizing borate complexation and direct UV detection was reported [24]. Based on the method described by Freeman et al. [16], which was further investigated by Claes et al. [7], the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) prescribe a reversed phase LC method with pre-column derivatization with OPA to determine the composition of gentamicin [25,26]. In this system however the C_{2b} component is located in the ascending part of the C_1 peak and JI-20 B is located in the descending part of the C_1 peak. Due to the insufficient resolution in the C_1 region, the compositions based on the peak heights, in comparison with these based on the peak areas, were in better agreement with the results obtained by ^{13}C NMR [7]. The Ph. Eur. prescribes the use of the peak heights whereas the USP prescribes the use of the peak areas for the calculation of the composition of gentamicin. Both pharmacopoeias limit the amount of C_1 to 25.0–50.0%, C_{1a} to 10.0–35.0% and the sum of C_2 and C_{2a} to 25.0–55.0%. As assay technique in these monographs microbiology is prescribed [25,26].

In this work an ion-pair LC method is described using a column packed with poly(styrene-divinylbenzene). Since pre- and post-column derivatization are time consuming and give problems with quantitation, pulsed electrochemical detection was chosen to detect the gentamicin components. The method has been used to analyze a number of commercial samples.

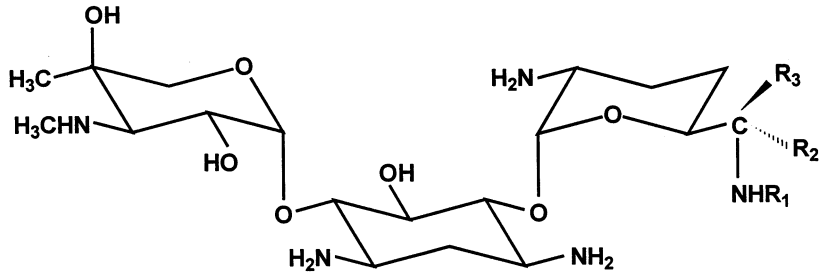
2. Experimental

2.1. Reagents and samples

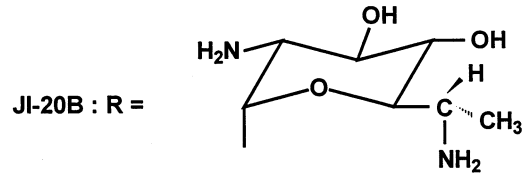
Water was distilled twice from glass apparatus. The buffer solution (pH 3.0) was prepared by mixing 0.2 M phosphoric acid and 0.2 M potassium dihydrogen phosphate, which were prepared with phosphoric acid 85% m/m (Acros Chimica, Geel, Belgium) and potassium dihydrogen phosphate (BDH, Poole, England) respectively. Sodium sulfate anhydrous and tetrahydrofuran (THF), stabilised with 2,6-di-tert-butyl-4-methylphenol were obtained from Merck (Darmstadt, Germany); sodium 1-octanesulfonate, monohydrate 98% and tert-butyl methyl ether from Acros Chimica; acetone from Rathburn (Walkerburn, Scotland), 2-methyl-2-propanol from Vel (Leuven, Belgium) and helium from Air Liquide (Machelen, Belgium). The 0.5 M sodium hydroxide solution was made using 50% (m/m) sodium hydroxide, aqueous solution (Baker, Deventer, the Netherlands). The gentamicin components C_1, C_{1a} and C_2, just as a mixture of the components C_2 and C_{2a} were obtained from Pierrel (Capua, Italy). Gentamicin C_{2b} and JI-20B were provided by Kyowa Hakko Kogyo (Tokyo, Japan) and sisomicin by the Ph. Eur. laboratory (Strasbourg, France). Commercial samples were obtained from Schering (Bloomfield, NJ), Pierrel (Milan, Italy), Chinoin (Budapest, Hungary), Gist-Brocades (Delft, the Netherlands), Lek (Ljubljana, Slovenia), Bufa (Uitgeest, the Netherlands), Bioniche (Inverin, Ireland) and Wuxi Pharmaceuticals (Wuxi, China).

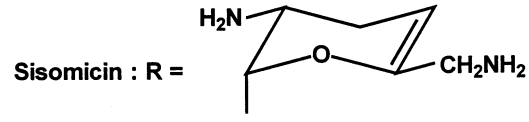
2.2. Apparatus

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 and an electronic integrator HP 3393 A (Hewlett-Packard, Avondale, PA). The column (250 × 4.6 mm I.D.) was packed with poly(styrene-divinylbenzene) PLRP-S (1000 Å, 8 μm, Polymer, Shropshire, UK). The temperature of the column was maintained at 50°C by immersion in a water bath



	R_1	R_2	R_3
Gentamicin C_1	CH_3	H	CH_3
Gentamicin C_2	H	H	CH_3
Gentamicin C_{1a}	H	H	H
Gentamicin C_{2a}	H	CH_3	H
Gentamicin C_{2b}	CH_3	H	H





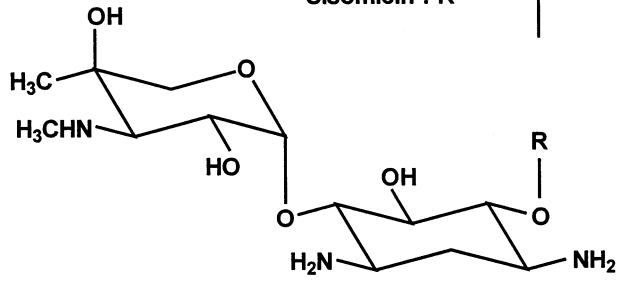


Fig. 1. Structure of gentamicin components.

with a circulator (Julabo, Seelbach, Germany). The C_{18} columns used were: Spherisorb ODS 1, 10 μm (PhaseSep, Queensferry, UK), LiChrosorb RP-18, 10 μm (Merck), RSil C_{18}, 5 μm (Biorad,

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

Mobile phase :

sodium sulfate	60 g/l
sodium 1-octanesulfonate	1.75 g/l
THF	8 ml/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 : 50 °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

Eke, Belgium), Bakerbond C_{18}, 5 μm (Baker, Phillipsburg, NJ) and Chromosphere B C_{18}, 5 μm (Chrompack, Middelburg, the Netherlands). The PED-1 pulsed electrochemical detector from Dionex (Sunnyvale, CA) 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 maintained in a laboratory-made hot air oven to keep the temperature at 35°C. Sodium hydroxide was added post-column, using a laboratory-made pneumatic device.

2.3. Chromatography

An overview of the LC conditions finally chosen is given in Scheme 1. All substances to be analyzed were dissolved in water. The mobile phase was sonicated before use. Through a mixing tee 0.5 M NaOH was added post-column 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 post-column 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 [27]. 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 [28]. The time and voltage parameters for the detection are

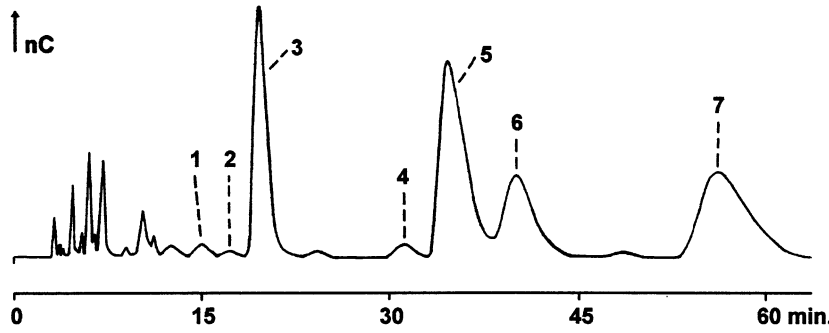


Fig. 2. Typical chromatogram of a commercial sample. 1, JI-20B; 2, sisomicin; 3, gentamicin C_{1a}; 4, gentamicin C_{2b}; 5, gentamicin C_2; 6, gentamicin C_{2a}; 7, gentamicin C_1. Composition, calculated by normalization: 1, 0.9%; 2, 0.4%; 3, 22.0%; 4, 1.2%; 5, 27.6%; 6, 13.3%; 7, 23.3%; other, 11.3%.

also shown in Scheme 1 and are the same as previously used for neomycin, kanamycin, netilmicin and amikacin [29–32]. Although the sequence of the potentials theoretically cleans the electrode surface, it is necessary to polish the gold electrode after about 40 analyses to obtain a good repeatability. After the electrode is cleaned with fine polishing compound it is sonicated in water for 10 min. It takes about 1 h to obtain a stable baseline with a freshly polished electrode. It is also advisable to wipe the counter electrode and the reference electrode at the same time with a wet tissue to remove deposited substances.

3. Results and discussion

3.1. Chromatographic method

A typical chromatogram of a commercial sample, obtained under the selected chromatographic conditions (Scheme 1), is shown in Fig. 2. Poly(styrene-divinylbenzene) was chosen as the stationary phase because of its remarkable stability and batch reproducibility. The composition of the mobile phase previously used for the analysis of netilmicin sulfate [31] was adapted for gentamicin and further optimized using DryLab (LC Resources, Berlin, Germany). The influence of the different chromatographic parameters on the separation of the gentamicin 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 main gentamicin components is illustrated in Fig. 3. As can be seen, there are little changes between pH 2.0 and 6.0. By further increase of the pH, the retention times decrease since less amino groups are protonated and the interaction with octanesulfonate decreases. The influence of the buffer type was also examined. When an acetate buffer of pH 3.0 was used instead of a phosphate buffer of pH 3.0, the separation between the gentamicin components was less good. The influence of the column temperature was examined at 45, 50 and 55°C. As expected the k'-values of the components decrease when the column temperature is increased. Sodium octanesulfonate as an ion-pairing agent is added to the mobile phase to retain the gentamicin components, which are positively charged at pH 3.0. The sodium octanesulfonate concentration of the mobile phase was varied in the range from 1.6 to 1.9 g l^{-1}. As expected the capacity factors decrease by decreasing the sodium octanesulfonate concentration. The influence of the sodium sulfate concentration of the mobile phase on the k'-values of the different gentamicin components was examined in the range from 55 to 65 g l^{-1}. An increase

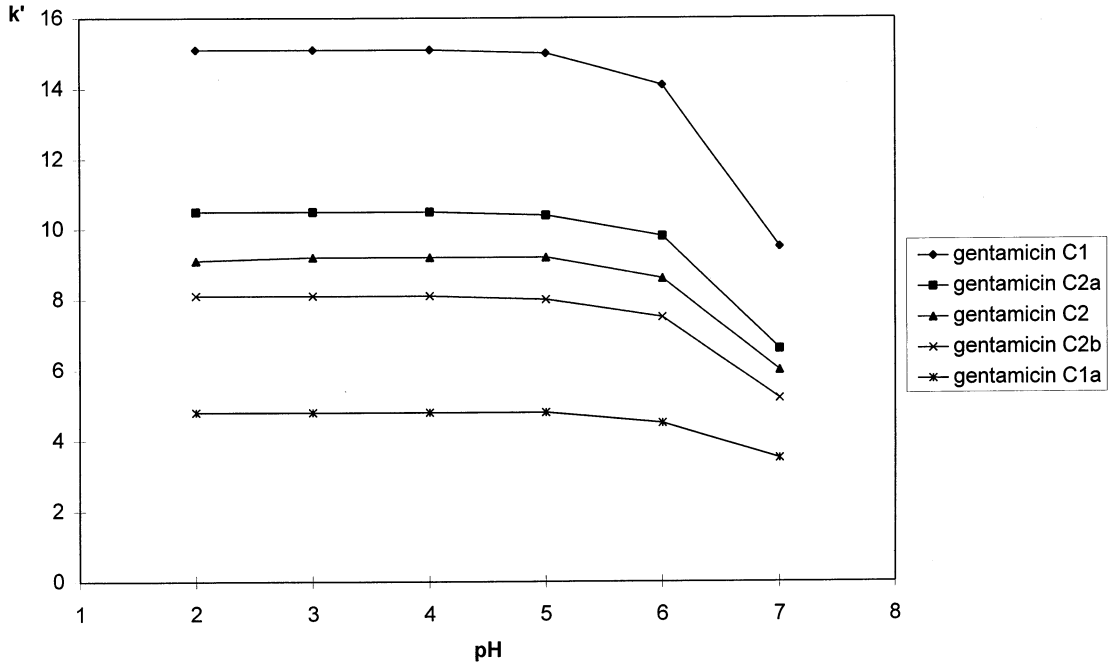


Fig. 3. Influence of the pH of the mobile phase on the capacity factors.

of the sodium sulfate concentration results in a decrease of the k'-values. THF was added to the mobile phase to improve the peak symmetry and to improve the separation between gentamicins C_{2b} and C_2. The influence of THF was examined at 7, 8 and 9 ml l^{-1}. Increasing the THF concentration improves the separation between gentamicins C_{2b} and C_2, but reduces the separation between the other components. Other organic modifiers were also investigated: methanol and ethanol can not be used because they are not compatible with pulsed electrochemical detection; 2-methyl-2-propanol and acetone caused an unstable baseline and a poor peak symmetry and tert-butyl methyl ether decreased the resolution between gentamicins C_2 and C_{2a}.

Using the mobile phase thus developed (Scheme 1), the chromatography was also performed using C_{18} columns (5 and 10 μm) of different manufactures. On these reversed-phase materials the separation between gentamicins C_{2b} and C_2 was poor and no separation between the isomers C_2 and C_{2a} could be obtained.

3.2. Robustness

By means of a half-fraction 5-factorial design, the importance of the individual chromatographic parameters and parameter interactions of this LC method was studied. The set-up of the applied factorial design was supported by the statistical graphics software system, Statgraphics version 6 (Manugistics, Rockville, MD). The chromatographic parameters examined as variables were: the concentration of sodium sulfate (Na_2SO_4), the sodium octanesulfonate (SOS) concentration, the amount of THF, the pH of the mobile phase buffer and the column temperature (temp). The values used in the design are shown in Table 1. In order to reduce the number of experiments, a half-fraction factorial design at two levels was chosen. This involves at least 2^5: $2 = 16$ 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 gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b}. The results showed that, under the examined conditions, the LC system was principally

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

Chromatographic parameter	Low value (−1)	Central value (0)	High value (+1)
Sodium sulfate (g l^{-1})	55	60	65
Sodium octanesulfonate (g l^{-1})	1.60	1.75	1.90
THF (ml l^{-1})	7	8	9
pH of the mobile phase buffer	2	3	4
Column temperature (°C)	45	50	55

influenced by the sodium sulfate concentration and the amount of THF. The column temperature and the sodium octanesulfonate concentration were the third and fourth most important factors. In the examined range, the pH had no significant effect on the retention times and no important interactions between the parameters were observed.

Using the same experimental results, also the separation between gentamicins C_{2b}, C_2 and C_{2a} was examined. The selectivity factors for gentamicins C_{2b} and C_2 (α_{C2b-C2}) and for C_2 and C_{2a} (α_{C2-C2a}) were used as response variables. The standardized pareto charts, representing the estimated effects of the five chromatographic parameters and their interactions on α_{C2b-C2} and α_{C2-C2a} are shown in Figs. 4 and 5. As can be seen, the α-factors are principally influenced by the amount of THF. However, THF has a positive effect on the separation between C_{2b} and C_2, whereas it has a negative effect on the separation between C_2

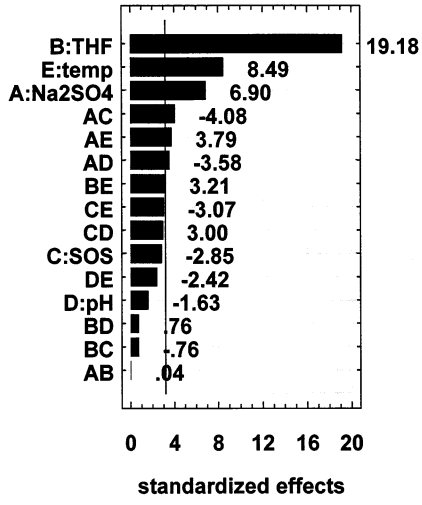


Fig. 4. Standardized pareto chart, representing the estimated effects of the chromatographic parameters and their interactions on the selectivity factor for gentamicins C_{2b} and C_2.

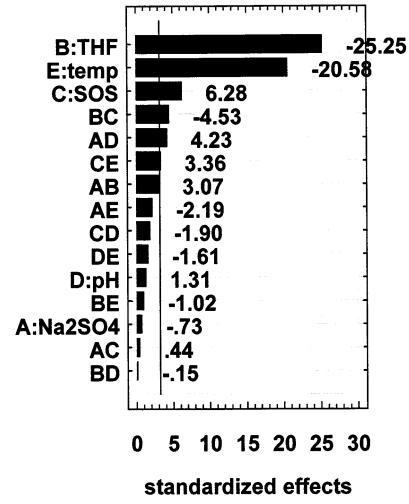


Fig. 5. Standardized pareto chart, representing the estimated effects of the chromatographic parameters and their interactions on the selectivity factor for gentamicins C_2 and C_{2a}.

Table 2
Linearity of gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b}

	Range (μg)	y	r^2	$S_{y,x}$
Gentamicin C_1	1–12	16 735x+581	0.9997	1197
Gentamicin C_{1a}	0.8–8	16 577x+1421	0.9996	1009
Gentamicin C_2	1–10	16 325x+1007	0.9998	993
Gentamicin C_{2a}	0.6–6	16 205x+1113	0.9992	1024
Gentamicin C_{2b}	0.06–1	16 539x+85	0.9993	181

Table 3
Composition of commercial samples of gentamicin, calculated by normalization

Samples	%JI-20B	%sisom.	$\%C_{1a}$	$\%C_{2b}$	$\%C_2$	$\%C_{2a}$	$\%C_1$	%other
1	0.9	0.4	22.0	1.2	27.6	13.3	23.3	11.3
2	1.4	0.5	18.1	1.4	22.4	11.9	25.8	18.5
3	0.7	0.4	21.7	1.0	32.5	12.8	25.1	5.9
4	2.3	0.3	18.8	1.6	23.4	17.6	22.4	13.4
5	1.7	0.3	21.2	2.8	18.0	15.8	29.6	10.8
6	0.5	0.6	15.6	0.5	36.1	13.5	27.0	6.3
7	0.6	6.8	23.0	2.3	15.0	16.0	19.8	16.4
GST/679	0.5	0.8	14.8	0.5	34.0	12.3	28.0	8.9
073/R	0.6	0.9	16.3	0.8	32.9	11.1	24.8	12.6
058/R	0.5	0.7	16.3	0.8	34.8	10.6	28.1	8.1
USQ-3-GMF-N-6020	0.3	±0.1	16.8	0.9	30.1	13.4	35.6	2.9
SZ-6MC-2-L-1006	0.7	0.4	21.0	1.5	28.1	12.6	30.2	5.6
GMC-7-M-9012	0.9	0.6	24.2	2.0	22.2	7.6	29.4	13.1
GMC-5-M-4-1	0.6	0.5	24.3	2.3	20.1	8.2	25.8	18.3
GMC-7-M-6103	1.1	0.5	16.3	2.4	25.1	6.4	35.7	12.7
83-06-147	0.4	±0.1	21.8	1.3	29.1	12.9	32.2	2.1
83-06-148	0.5	0.2	19.2	1.2	30.0	13.1	33.4	2.5
83-06-145	0.4	0.4	21.0	1.0	28.5	12.9	32.9	3.1

Table 4
Amounts of gentamicins C_1, C_{1a}, C_2 and C_{2a} (%), relative to the sum of these four components

Batch number of samples	LC-PED				Ph. Eur.			
	$\%C_1$	$\%C_{1a}$	$\%C_2$	$\%C_{2a}$	$\%C_1$	$\%C_{1a}$	$\%C_2$	$\%C_{2a}$
GST/679	31.4	16.6	38.1	13.8	33.8	15.9	38.9	11.4
073/R	29.2	19.2	38.6	13.0	30.3	19.0	40.1	10.6
058/R	31.3	18.2	38.7	11.8	33.5	17.9	39.0	9.6
USQ-3-GMF-N-6020	37.1	17.6	31.4	14.0	38.5	17.3	32.1	12.1
SZ-6MC-2-L-1006	32.9	22.9	30.6	13.7	35.2	22.6	31.1	11.1
GMC-7-M-9012	35.3	29.0	26.6	9.1	37.1	29.1	26.6	7.2
GMC-7-M-6103	32.9	31.0	25.6	10.5	35.5	30.8	25.2	8.4
GMC-5-M-4-1	42.8	19.5	30.1	7.7	44.3	19.3	30.2	6.2
83-06-147	33.5	22.7	30.3	13.4	34.4	22.5	30.8	12.3
83-06-148	34.9	20.0	31.4	13.7	36.4	19.5	32.1	12.0
83-06-145	34.5	22.1	30.0	13.5	35.9	21.0	31.1	12.1

and C_{2a}. This means that higher amounts of THF improve the separation between C_{2b} and C_2, but make the separation between C_2 and C_{2a} worse. The second most important factor is the column temperature, which has a similar effect as the amount of THF. The sodium sulfate concentration has only a significant effect on α_{C2b-C2} and the sodium octanesulfonate concentration has only a small effect on α_{C2-C2a}. The pH has, as expected, no influence.

3.3. Quantitative aspects of the LC method

For the analysis of gentamicin an amount of 20 μg was used by injecting 20 μl of a 1 mg ml^{-1} solution. For this quantity the limit of detection for gentamicin C_{2b} was 0.1% (m/m) (20 ng), determined at a signal-to-noise ratio of 3. The limit of quantification was 0.3% (m/m) (60 ng) (R.S.D. = 9.6%, $n = 4$). The results found for the linearity of gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b} are shown in

Table 2, where y, peak area/1000; x, amount of sample injected (μg); r^2, coefficient of determination and $S_{y,x}$, standard error of estimate. The repeatability was checked by analyzing six times a 1 mg ml^{-1} solution of gentamicin. The R.S.D. values on the areas of the main gentamicin peaks were less than 2%.

The response factors for gentamicins C_{1a}, C_2 and C_{2b} relative to C_1 were all about one. The response factor of gentamicin C_{2a} could not be determined, because a sufficiently pure reference substance was not available, but since it is an isomer of gentamicin C_2, the response factor was also assumed to be one.

3.4. Analysis of commercial samples

Several commercial samples of gentamicin were analyzed using the described method. The obtained percentages, calculated by normalization, are shown in Table 3. As can be seen, the composition of commercial gentamicins is quite variable. Beside gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b}, several samples contain considerable amounts of other components, most of which were not identified. In order to compare our results with the figures reported previously using the Ph. Eur. method [7], for the relevant samples, the component ratio for the four main components was also calculated by normalization, so that the sum of the four main components equals 100%. The results are shown in Table 4. The most important differences between the figures obtained by both methods were observed for gentamicins C_1 and C_{2a}. Compared with the Ph. Eur. method, higher amounts for gentamicin C_{2a} and lower amounts for gentamicin C_1 were found using the LC-PED method. A possible explanation for the latter was already mentioned above: insufficient resolution by the Ph. Eur. method in the gentamicin C_1 area. For this reason, the use of peak heights instead of peak areas was preferred [7].

4. Conclusion

The described method, using poly(styrene-divinylbenzene) as the stationary phase, allows to separate gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b}, as well as several other minor components. It is the first time the separation of JI-20B and sisomicin by LC is described. The total time of analysis is 65 min. 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 gentamicin without derivatization.

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