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Comparison of liquid chromatographic methods with direct detection for the analysis of gentamicin

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Abstract

Several liquid chromatographic (LC) methods have been described for the analysis of gentamicin. LC combined with pulsed electrochemical detection (LC-PED) or evaporative light scattering detection (LC-ELSD) was found to be the most suitable technique. A first method, previously developed by Adams et al. used a poly(styrene-divinyl benzene) stationary phase with a mobile phase containing sodium sulphate, sodium-1-octanesulphonate, tetrahydrofuran, 0.2 M phosphate buffer (pH 3) and water. However, the polymer columns show low efficiency, which also leads to poor sensitivity. So, recently the use of newer conventional C18 columns was further investigated. Improved separation was obtained using a Supelcosil LC-18-DB column with an adapted mobile phase. Another method derived from a company method was checked by using a Gemini column and a mobile phase containing an aqueous solution of trifluoroacetic acid (TFA) and pentafluoropropionic acid (PFPA) adjusted to pH 2.6 with sodium hydroxide (NaOH). This method was transferred to ELSD by replacing the non-volatile NaOH with volatile ammonium hydroxide solution. A volatile method, which was originally developed for ELSD using an aqueous solution of 50 mM TFA and gradient elution with methanol, was also combined with PED. In this study, these methods were compared with regard to their selectivity, sensitivity and ease of use.

Keywords: Comparison; LC; PED; ELSD; Gentamicin

1. Introduction

Gentamicin is an aminoglycoside antibiotic obtained from fermentation of a strain of *Micromonospora purpurea* [1]. Gentamicin has a broad spectrum of activity and it is used in the treatment of severe gram-negative infections both in human and in veterinary medicine. A characteristic feature of gentamicin are the two amino sugars glycosidically linked to positions 4 and 6 of 2-deoxystreptamine. Gentamicin is a complex mixture of four major components (C_1, C_{1a}, C_2, C_{2a}) and the minor component C_{2b} (Fig. 1). During fermentation, several related substances like gentamicin B_1, sisomicin, dihydroxygentamicin C_{1a}, JI-20B, degradation products like garamine and 2-deoxystreptamine as well as several other unknown compounds are formed in small amounts [2].

Gentamicin is highly polar, non-volatile and lacks a UV chromophore. This poses a great challenge in the analysis of this drug. Several separation methods like paper and thin layer chromatography [3,4], Craig distribution [5], cation and anion exchange LC [6–8], reversed phase LC [9–20] and capillary electrophoresis (CE) [21,22] have been proposed for the determination of the composition of the main components. Since the detection of gentamicin is also problematic, much effort has been focused on enhancement of detection. Refractive index detection [13] proved to be not sensitive and pre- and post-column derivatization [7,9,10–12,14,15,18,22] were found to be tedious, time consuming and giving problems with quantitation due to reaction incompleteness or instability of the derivatized products. Mass spectrometry can directly detect gentamicin, but its operation costs are relatively high for routine analysis and a volatile mobile phase is required. The most interesting detection techniques for gentamicin seem to be pulsed electrochemical detection (PED) [23,24] and evaporative light scattering detection (ELSD) [25,26]. These detection techniques enable direct detection of gentamicin without derivatization.

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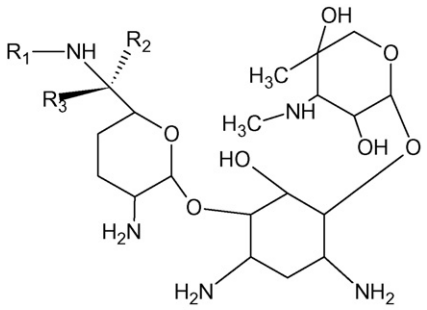
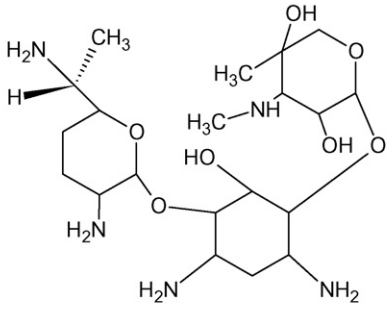
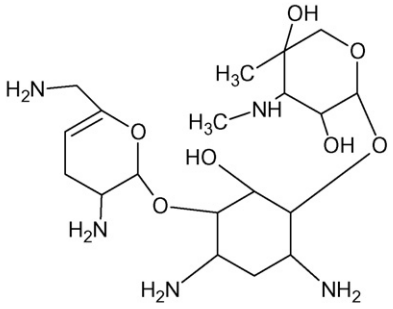


Gentamicins	R_1	R_2	R_3
C_1	CH_3	H	CH_3
C_{1a}	H	H	H
C_2	H	H	CH_3
C_{2a}	H	CH_3	H
C_{2b}	CH_3	H	H

GENTAMICIN



JI-20B



SISOMICIN

Fig. 1. Chemical structures of the main gentamicin compounds and some of its impurities.

PED is based on the oxidation of the analyte on the surface of a gold electrode [24]. In practice, a three-electrode system is used, which allows the precise control of the applied potentials. The LC-PED previously developed by Adams et al. [23] and which is the basis of the current official method prescribed in the European Pharmacopoeia [27] for the analysis of gentamicin was selected as starting point of this work. This method utilizes a poly(styrene-divinyl benzene) stationary phase kept at 50 °C and a mobile phase containing 60 g/l of sodium sulphate ($NaSO_4$), 1.75 g/l of sodium octanesulphonate (SOS), 8 ml/l of tetrahydrofuran (THF) and 50 ml/l of a 0.2 M phosphate buffer pH 3. However, the high stability polymer columns showed poor selectivity of the gentamicin components. Silica-based columns are known to have higher efficiency and therefore higher selectivity, but often suffer from stability, limited temperature and pH range. So, recently the use of newer conventional C18 columns was further investigated in-house. Improved separation was obtained using a Supelcosil LC-18-DB column with a slightly adapted mobile phase. However, this method still utilizes a lot of salt and high temperature which is harmful for the stability of silica-based C18 stationary phases. Another method derived from a company method was checked using a Gemini column kept at 35 °C with a mobile phase containing the volatile ion pairing agents, TFA and PFPA adjusted to pH 2.6 with NaOH. This method showed improved selectivity and sensitivity for gentamicin without column life threatening conditions.

ELSD involves nebulisation of the column effluent with nitrogen as a nebulising gas to form an aerosol, followed by solvent evaporation in a heated stainless steel drift tube and detection of the remaining non-volatile particles, which scatter the light emitted by a laser light source. The scattered light is detected by a silicon photodiode, generating a signal that is however not directly linear with the amount injected. ELSD is described as a universal detection mode in LC suitable for non-absorbing analytes [28]. Clarot et al. [25] and Megoulas and Koupparis [26] developed LC methods with ELSD. The former utilized a HighPurity column with a mobile phase containing TFA and methanol while the latter used a Spherisorb column with an aqueous solution of trichloroacetic acid (TCA), TFA and methanol. These methods were tried in-house, the first method allowed the best separation and detection of the gentamicin components. This method was further optimized and combined with MS for the identification of unknown related substances [29]. It was decided to investigate whether methods developed using PED or ELSD can be interchangeable. The LC-PED method which previously showed improved selectivity using the perfluorinated carboxylic acids was transferred to ELSD by simply replacing the non-volatile NaOH with volatile ammonium hydroxide solution. In the same way, the optimized LC-ELSD method was transferred as is to LC-PED. The results showed that it is not straightforward to exchange methods using these two detection techniques.

This study aimed at finding the most suitable method with direct detection for the complete analysis of gentamicin which includes the determination of the composition of the main components as well as the determination of impurities. The currently available LC methods were compared. Special attention was paid to selectivity, sensitivity and ease of use.

2. Experimental

2.1. Reagents and samples

All the reagents used were HPLC grade. Acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Tetrahydrofuran (THF) stabilized with 2,6-di-*tert*-butyl-4-methylphenol and extra pure anhydrous sodium sulphate were obtained from Merck (Darmstadt, Germany). Methanol, trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), potassium dihydrogen phosphate and sodium octanesulphonate (SOS) were purchased from Acros Organics (Geel, Belgium). A 50% sodium hydroxide solution was from J.T. Baker (Deventer, Holland); 85% phosphoric acid was from Sigma Aldrich (Steinheim, Germany) and 28% ammonia was supplied by VWR International (Leuven, Belgium). Water was produced in-house using a MilliQ water purification system (Millipore, Bedford, MA). Helium gas was obtained from Messer (Machelen, Belgium) and nitrogen gas was supplied by Air Liquide (Liège, Belgium). All mobile phases were degassed by sparging helium gas.

An expired gentamicin bulk sample containing a maximum number of secondary peaks was available in our laboratory. The sample concentration was chosen so as to provide adequate response without overloading the detector and degrading the resolution. The injection volume was always 20 μl. For identification purposes, solutions of reference standards of gentamicin C_{1a}, C_2, C_1, C_{2b}, JI-20B, sisomicin and a mixture of C_2 and C_{2a} were injected.

2.2. LC instrumentation and chromatographic conditions

2.2.1. LC-PED method I

The LC apparatus consisted of an L-6200 Intelligent Pump (Merck Hitachi, Darmstadt, Germany), a Hitachi LaChrom Elite L-2200 autosampler (VWR International, Leuven, Belgium), a Decade II electrochemical detector (Antec, Leyden, Netherlands) and Chromeleon 6.70 software (Dionex Corporation, Sunnyvale, CA, USA) for data acquisition. The detector cell was kept at a constant temperature using a hot-air oven. The polymer column; 250 × 4.6 i.d., 8 μm, 1000 Å (Polymer Laboratories, Shropshire, UK) was kept at a constant temperature (50 °C) by using a water bath with a heating immersion circulator (Julabo, Seelbach, Germany). Other experimental conditions are as shown in Table 1 (LC-PED I). 0.2 M phosphate buffer was prepared by mixing 0.2 M potassium dihydrogen phosphate with 0.2 M phosphoric acid until pH 3.0 was reached. For better detection of aminoglycosides with PED, at least a pH 12 is necessary. Since the mobile phase has a lower pH, 0.5 M NaOH was pulselessly added post-column using a helium-pressurized reservoir. The column effluent was mixed with the base in a packed reaction coil from Dionex (1.2 m, 500 μl).

2.2.2. LC-PED method II

The LC apparatus was the same as in Section 2.2.1. Other experimental conditions are as shown in Table 1 (LC-PED II). The columns which were examined included: Hypersil BDS C18, 250 mm × 4.6 mm i.d., 5 μm (ThermoQuest, Runcorn, UK); Supelcosil LC-18-DB, 250 mm × 4.6 mm i.d., 5 μm (Supelco Park, Bellefonte, PA, USA); Zorbax SB C18, 250 mm × 4.6 mm i.d., 5 μm (Agilent Technologies, Inc., CA, USA); YMC-Pack Pro C18, 250 mm × 4.6 mm i.d., 5 μm (YMC, Wilmington, NC, USA); Supelcosil LC-18-DB, 150 mm × 4.6 mm i.d., 5 μm (Supelco); Discovery C18, 250 mm × 4.6 mm i.d., 5 μm (Supelco); Luna C18, 150 mm × 4.6 mm i.d., 5 μm (Phenomenex, Torrance, CA, USA); Astec C18 polymer, 250 mm × 4.6 mm i.d., 5 μm (Resolution Systems, Whippany, NJ, USA) and Gemini C18, 250 mm × 4.6 mm i.d., 110 Å, 5 μm (Phenomenex).

Table 1
Chromatographic conditions for the analysis of gentamicin using LC-PED

	LC-PED I	LC-PED II	LC-PED III	LC-PED IV
Stationary phase	PRPL-S, 8 μm, 1000 Å, 250 mm × 4.6 mm i.d.	Supelcosil LC-18-DB, 3 μm, 150 mm × 4.6 mm i.d.	Gemini, 5 μm, 110 Å, 250 mm × 4.6 mm i.d.	Hydrosphere, 5 μm, 150 mm × 4.6 mm i.d.
Mobile phase	$NaSO_4$ 60 g/l, SOS 1.75 g/l, THF 8 ml/l, phosphate buffer 0.2 M, pH 3 50 ml/l, water up to 1 l	$NaSO_4$ 60 g/l, SOS 1.75 g/l, THF 6 ml/l, phosphate buffer 0.2 M, pH 3 50 ml/l, water up to 1 l	ACN 15 ml/l, TFA 7 ml/l, PFPA 250 μl/l, 0.5 M NaOH till pH 2.6, water up to 1 l	50 Mm TFA ammonia till pH 2.4, water up to 1 l
Flow rate	1 ml/min	1 ml/min	1 ml/min	1 ml/min
Injection volume (concentration in mobile phase)	20 μl (0.5 mg/ml)	20 μl (0.5 mg/ml)	20 μl (0.3 mg/ml)	20 μl (0.5 mg/ml)
Column temperature	50 °C	50 °C	35 °C	25 °C
Post-column addition of 0.5 M NaOH	0.3 ml/min	0.3 ml/min	0.3 ml/min	0.3 ml/min
PED cell				
Working electrode	Gold			
Reference electrode	Hy-Ref			
Auxiliary electrode	Carbon filled with polytetrafluoroethylene			
PED settings	*t*(s): 0.1–0.4, *E*(V): +0.05; *t*(s): 0.4–0.6, *E*(V): +0.75; *t*(s): 0.6–1.0, *E*(V): −0.15			
Integration period	0.20–0.40 s			
Sensitivity	5 μA			
Detector cell was kept at	35 °C			

SOS: sodium octane sulphonate; *E*(V): potential in volts; THF: tetrahydrofuran; *t*(s): time in seconds; TFA: trifluoroacetic acid; PFPA: pentafluoropropionic acid.

2.2.3. LC-PED method III

The LC instrumentation was the same as in Section 2.2.1. Other experimental conditions are as shown in Table 1 (LC-PED III). The column used was Gemini C18, 250 mm × 4.6 mm i.d., 110 Å, 5 μm (Phenomenex, USA). The aqueous phase was adjusted to pH 2.6 using 0.5 M sodium hydroxide.

2.2.4. LC-ELSD method I

The LC apparatus consisted of a Merck Hitachi, L-6200 Intelligent pump, a Gilson 234 autoinjector (Villiers-le-Bel, France), an Alltech ELSD 2000 (Deerfield, IL, USA) and Chrom Perfect software (Justice Laboratories, Fife, UK) for data acquisition. A Hydrosphere RP C-18, 150 × 4.6 i.d., 5 μm (YMC) was kept at a constant temperature of 25 °C by using a water bath with a heating immersion circulator (Julabo, Seelbach, Germany). The following mobile phases were used for separation: (A) 50 mM TFA solution pH 2.4, (B) 50 mM TFA solution pH 2.4/methanol (80:20, v/v). The TFA solution was adjusted to the required pH by adding ammonia. The one step-linear gradient was performed as follows: 0–20 min, 0–30% B at a flow rate of 1.0 ml/min. A 2 mg/ml solution in water was prepared. The following parameter settings were used for ELSD: N_2 gas flow rate: 1.5 l/min; drift tube temperature: 82 °C; impactor: on; gain: 4.

2.2.5. LC-ELSD method II

The LC instrumentation was the same as in Section 2.2.4. The mobile phase was derived from LC-PED method III and consisted of 15 ml/l acetonitrile and 985 ml/l of an aqueous solution of 7 ml/l TFA, 250 μl/l PFPA, adjusted to pH 2.6 using ammonium hydroxide solution. The flow rate was 1.0 ml/min. The column used was Gemini C18, 250 mm × 4.6 mm i.d., 5 μm (Phenomenex, USA). A 2 mg/ml solution in mobile phase was prepared. The following parameter settings were used for ELSD: N_2 gas flow rate: 2.5 l/min; drift tube temperature: 100 °C; impactor: on; gain: 4.

2.2.6. LC-PED method IV

The LC instrumentation was the same as in Section 2.2.1. Other experimental conditions are as shown in Table 1 (LC-PED IV). The column used was Hydrosphere RP C-18, 150 × 4.6 i.d., 5 μm (YMC). The mobile phase was derived from LC-ELSD method I. The mobile pH was adjusted to 2.4 by adding ammonium hydroxide solution.

3. Results and discussions

3.1. LC-PED method I

This LC-PED method, which was previously developed by Adams et al. [23], is the basis of the current method prescribed in the European Pharmacopoeia [27]. It was selected as the starting point for this work. Using poly(stryrene-divinylbenzene) as a stationary phase, the efficiency, as a consequence the sensitivity and also the selectivity are rather poor, especially for the peak pairs C_{2b}–C_2 and C_2–C_{2a}. A typical chromatogram is shown in Fig. 2. Peaks with longer retention times become also more asymmetric, which hampers proper integration. On the other hand, polymer columns show a good stability and reproducibility. However, due to recent improvement in silica-based column manufacturing, their stabilities improved a lot. So, it was decided to apply this method using RP C-18 columns, giving better efficiency.

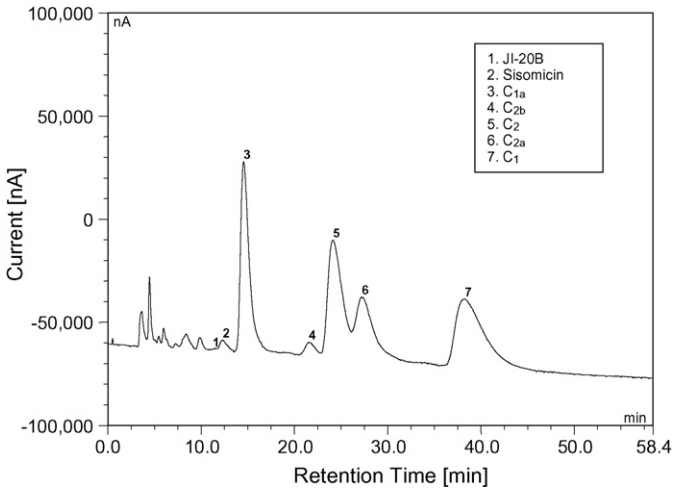


Fig. 2. Typical chromatogram obtained by using a polymer column. Chromatographic conditions are mentioned in Table 1 (LC-PED I).

3.2. LC-PED method II

The above-mentioned method was adapted by replacing the polymer stationary phase by silica-based stationary phases. In total nine different C18 silica-based stationary phases were investigated. Among them, seven columns were selected to behave similarly based on the previously developed column classification system in our laboratory [30]. Astec, a C18 derivatized polyvinyl alcohol column, 250 mm × 4.6 mm i.d., 5 μm, was also included in the study since this stationary phase is described to combine the separation efficiency of silica-based reversed phase columns and the stability of polymer columns. A Gemini column C18 (250 mm × 4.6 mm i.d.), made by Twin technology, was also included in the study since it has been reported to withstand extreme conditions. These columns were evaluated for their selectivity, especially of the critical peak pairs. Improved selectivity was achieved for most of the columns, but also a longer analysis time was observed, up to about 200 min. The selection of the most suitable column was done using the chromatographic response function (CRF) [30]. CRF values always lie between 0 (two or more peaks are not separated from each other) and 1 (all peaks are completely separated from each other). The use of these values has been described in thin layer chromatography (TLC), but they can be used in LC as well [31]. The CRF values obtained for the different columns examined are given in Table 2. From the columns examined, only one column, Supelcosil C18 (150 mm × 4.6 mm i.d.) gave a CRF value of 1, which means that the peaks are completely separated. Moreover, this shorter column also gave a shorter analysis time compared to the other investigated C18 columns. However, the relatively high column temperature and the amount of salt used in the mobile phase constrain the life time of silica-based

Table 2
Chromatographic response functions (CRF) and the total analysis time for gentamicin sample obtained on different columns

Column	CRF values	Analysis time (min)
Hypersil BDS, 250 mm × 4.6 mm, 5 μm	0.80	140
Supelcosil LC-18-DB, 250 mm × 4.6 mm, 5 μm	0.76	191
Zorbax SB-C18, 250 mm × 4.6 mm, 5 μm	0.54	111
YMC-Pack Pro C18, 250 mm × 4.6 mm, 5 μm	0	65
Supelcosil LC-18-DB, 150 mm × 4.6 mm, 3 μm	1.00	80
Discovery C18, 250 mm × 4.6 mm, 5 μm	0.94	113
Luna C18, 150 mm × 4.6 mm, 5 μm	0.90	180
Astec C18 Polymer, 250 mm × 4.6 mm, 5 μm	0.67	50
Gemini C18, 250 mm × 4.6 mm, 5 μm	0	80

columns. The Gemini column showed a longer analysis time and co-elution of the peak pair C_2–C_{2a}. Typical chromatograms obtained with a Supelcosil and a Gemini column are shown in Fig. 3. The chromatographic conditions as well as the sample were the same. Efforts to improve the chromatographic conditions on the Supelcosil column by lowering the amount of salt in the mobile phase or decreasing the column temperature resulted in either tremendous baseline instability or very long analysis times (up to 300 min).

3.3. LC-PED method III

This method was derived from that obtained from a company (Lek, Ljubljana, Slovenia) which prescribed the use of a Gemini column and a mobile phase containing an aqueous solution of 7 ml/l TFA, 250 μl/l PFPA and 4 ml/l NaOH adjusted to pH 2.6 using dilute NaOH. Using these conditions, it was observed that the analysis time was rather long (82 min) and there was co-elution of gentamicins C_2 and C_{2b}. Efforts to improve the separation within a reasonable run time resulted in addition of an organic modifier to the mobile phase. The choice of an organic modifier in combination with PED is not straightforward [24]. Tetrahydrofuran is reported to improve the peak symmetry and

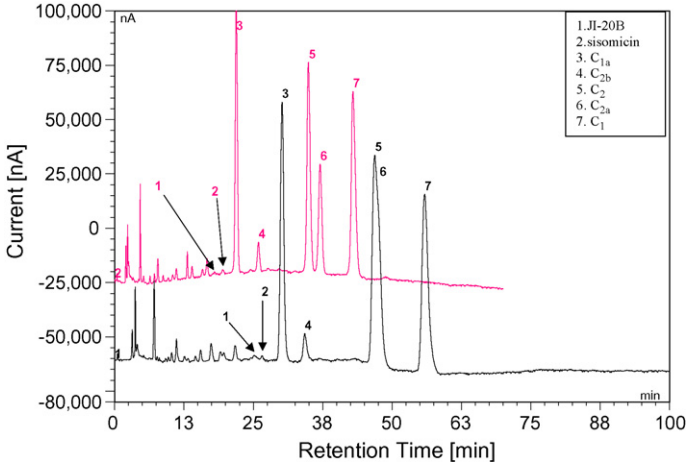


Fig. 3. Typical chromatograms obtained using C18 columns: Gemini (1) and Supelcosil LC-18-DB (2). Chromatographic conditions are mentioned in Table 1 (LC-PED II).

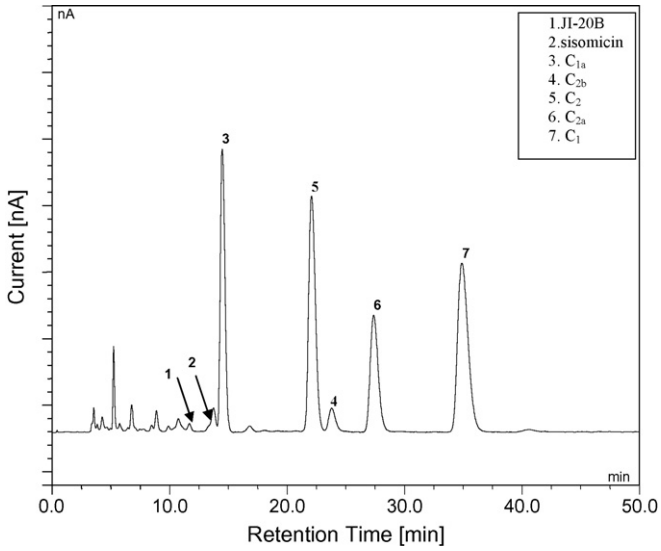


Fig. 4. Typical chromatogram obtained according to the conditions mentioned in Table 1 (LC-PED III). The same peak numbering was used as in Fig. 3.

the separation between C_{2b} and C_2 [23]. Here, it resulted in severe baseline instability which made peak detection very difficult. Methanol did not give a nice separation. Acetonitrile gave the better separation at a concentration of 1.5% (v/v) in the final mobile phase. Although acetonitrile has been reported to give stability problems in combination with PED [24], it was found here to give the better results. This is probably related to the quality used. A typical chromatogram obtained under these conditions by analyzing the same commercial gentamicin sample as used in LC-PED methods I and II is shown in Fig. 4. As can be seen, the main gentamicin components are well separated. Several impurities, both of known and unknown identity are well separated. Since the peaks are narrower, sensitivity improved considerably compared to LC-PED method I. C_{2b}, which was eluted before C_2 in the previously discussed methods, is now eluted after this peak. It is important that this method utilizes more column friendly chromatographic conditions. The column temperature is lower (35 °C) than in the previous methods and the mobile phase contains less salt.

3.4. LC-ELSD method I

Megoulas and Koupparis [26] successively separated the main gentamicin components. However, this method was not successful in our hands. The mobile phase was difficult to vaporize inside our ELSD, which might be accounted to the use of a different brand of ELS detector. On investigating the LC–ELSD method from Clarot et al. [25], it was found that the main gentamicin components were well separated. This method was further adapted by raising the mobile phase pH to 2.4 in order to protect the stationary phase and developing a gradient elution to shorten the run time (Section 2.2.4). From the results obtained, it was shown that the main gentamicin components are separated as well as their impurities. Some of these could be characterized by coupling this method to MS [29]. ELSD is a useful tool for pharmaceutical analysis because it has the ability to detect all

semi- and non-volatile solutes regardless of their optical properties, however it has no direct linear relationship between the response and amount of sample injected. It was observed that ELSD is less sensitive compared to PED and therefore it is only suitable to analyze the composition of the main components, but not for the analysis of impurities.

3.5. LC-ELSD method II

Since LC-PED method III resulted in improved selectivity and sensitivity of gentamicin and its related substances, the method was also transferred to LC-ELSD by just replacing the non-volatile NaOH with ammonia. The sample concentration was increased to 2 mg/ml to improve sensitivity. The elution was unexpectedly faster than observed with LC-PED method III and the chromatogram showed only the main peaks. Omitting ACN in the mobile phase resulted in a longer retention time and further decreased sensitivity.

3.6. LC-PED method IV

The LC-ELSD method I was also transferred to PED. Methanol was omitted from the mobile phase as it did not give good results in LC-PED method III as reported above. The mobile phase consisted now of an aqueous solution of 50 mM TFA adjusted to pH 2.4 using ammonia. Poor sensitivity for the gentamicin components was observed. This is probably due to the fact that the ammonium adsorbs on the surface of the working electrode and so obstructs oxidation of the analytes of interest.

4. Conclusion

For decades, researchers have tried to solve the problems related to the analysis of polar, non-chromophoric aminoglycoside antibiotics. In this work several LC methods combined with PED and ELSD were compared for the complete analysis of gentamicin. Among the methods examined, LC-PED method III using perfluorinated carboxylic acids as ion pairing agents proved to be more sensitive and selective at an acceptable run time. This method needs to be further validated so that it can eventually replace the currently available official method. It was also shown that method transfer between PED and ELSD is not straightforward.

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