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Development and validation of a simple capillary zone electrophoresis method for the analysis of kanamycin sulfate with UV detection after pre-capillary derivatization

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Abstract

Capillary zone electrophoresis was successfully applied to separate eight related substances of kanamycin and several minor unknowns from the main component. Strategies to enhance derivatization and selectivity and to optimize separation parameters involved the application of experimental designs. This chemometrical approach considers main effects as well as interactions of the influential parameters, thus conducting a more thorough investigation of the method than the common step-by-step approach. Central composite face centered designs established optimal separation conditions: 30 m*M* borax buffer, pH 10.0 containing 16.0% (v/v) methanol and optimal composition of derivatization reagent: 27 mg/ml 1,2-phthalic dicarboxaldehyde and 25 μl/ml mercaptoacetic acid in borate buffer, pH 10.4. The standard curves were linear over the concentration range of 0.007–1.01 mg/ml for the main component and 0.003–0.1 mg/ml for the related substances. The limit of quantitation was 0.14% (m/m) for the related substances and impurities ($S/N=10$). The assay method was used to determine the composition of several commercial samples. Quantitative analysis indicates potential usefulness of capillary electrophoresis as an alternative to the assay method prescribed in the European Pharmacopoeia and the United States Pharmacopeia. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Pharmaceutical analysis; Central composite design; Regression analysis; Derivatization, electrophoresis; Validation; Experimental design; Kanamycin; Antibiotics; Aminoglycosides

1. Introduction

The term kanamycin refers to a group of closely related aminoglycoside antibiotics: kanamycins A, B, C and D (Fig. 1) which are used as sulfate salts. They are highly water-soluble antibiotics, produced by the fermentation of *Streptomyces kanamyceticus* [1]. Kanamycin contains as a main component kanamycin A and kanamycins B, C and D as minor components [2]. Claes et al. [3] described the existence of the minor related substance paromamine and 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl) deoxystreptamine [6-*O*-(3-AG)DS]. The structure and antimicrobial activity of 4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl) deoxystreptamine [4-*O*-(6-AG)DS], a hydrolysis product of kanamycin A have been reported [4]. Adams et al. [5] later reported the presence of 4-*O*-(6-AG)DS and kanamycin D in commercial samples using liquid chromatography with pulsed electrochemical detection (LC–PED). The lack of a UV chromophore or fluorophore made

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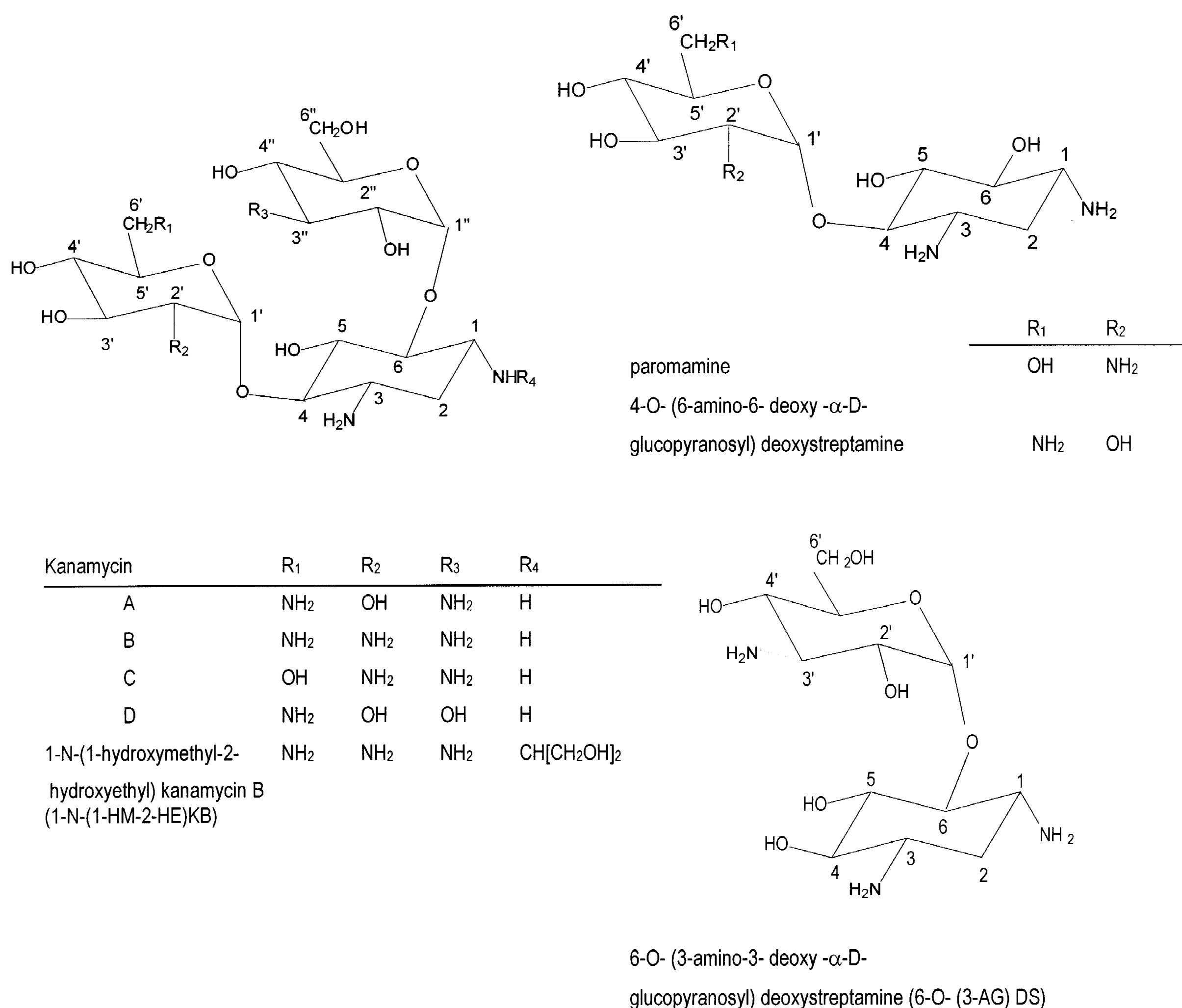

Kanamycin	R1	R2	R3	R4
A	NH2	OH	NH2	H
B	NH2	NH2	NH2	H
C	OH	NH2	NH2	H
D	NH2	OH	OH	H
1-N-(1-hydroxymethyl-2-hydroxyethyl) kanamycin B (1-N-(1-HM-2-HE)KB)	NH2	NH2	NH2	CH[CH2OH]2

	R1	R2
paromamine	OH	NH2
4-O- (6-amino-6- deoxy -α-D-glucopyranosyl) deoxystreptamine	NH2	OH

Fig. 1. The structures of kanamycin components.

chromatographic detection difficult. Paper chromatography [2], ion-exclusion chromatography [2,3,6], gas–liquid chromatography after silylation [7,8], reversed-phase LC after pre-column derivatization [8], conductivity detection [2], colorimetry after derivatization with ninhydrin [6] and spectrophotometry [9] have been described. Although LC with electrochemical detection has shown good separation performance, stability and operator experience are the major limitations together with longer analysis time per sample, which necessitated a two-step gradient [5]. Capillary electrophoresis (CE) is increasingly being viewed as an alternative and a complement to LC for determination of drug related impurities [10]. Previous attempts have been made to employ CE in the analysis of aminoglycosides with borate complexation [11], indirect methods [12] and capillary zone electrophoresis (CZE) with amperometric detection [13]. However, these methods have disadvantages of being less sensitive and selective for the related substances. CZE with amperometric detection has also been reported for kanamycin and amikacin but could only show selectivity for three components [13].

In this paper, a simple CZE method is presented for kanamycin analysis after pre-capillary derivatiza-

tion with 1,2-phthalic dicarboxaldehyde (OPA) and mercaptoacetic acid (MAA). The background electrolyte (BGE) contains only borate and methanol. A chemometrical approach to enhance selectivity and optimize the derivatization procedure was used. This assay method was used to determine the composition of a number of commercial samples.

2. Experimental

2.1. Reagents, samples and reference standards

Sodium tetraborate decahydrate, 1,2-phthalic dicarboxaldehyde and mercaptoacetic acid were obtained from Acros Organics (Geel, Belgium), sodium hydroxide and methanol (HPLC grade) from BDH (Poole, UK), 2-propanol Chromasolv from Riedel-de Haën (Seelze, Germany), boric acid from Merck Eurolab (Leuven, Belgium), picric acid from UCB (Brussels, Belgium). Kanamycin B was obtained from Bristol Labs. (Syracuse, NY, USA), 1-*N*-(1-hydroxymethyl-2-hydroxyethyl)kanamycin B from Pfizer (NY, USA), kanamycin C from Merck (Rahway, NJ, USA), Paromamine was prepared from commercial neomycin samples [14], 2-deoxystreptamine was obtained from Merck Pharma (Darmstadt, Germany), kanamycin D, kanamycin derivatives 6-*O*-(3-AG)DS and 4-*O*-(6-AG)DS and the kanamycin laboratory standard were prepared as described by Adams et al. [5]. Commercial samples of kanamycin sulfate were obtained from VMD (Arendonk, Belgium), Kela (Hoogstraten, Belgium), Continental Pharma (Mechelen, Belgium) and Fluka (Buchs, Switzerland). All solutions were prepared with ultrapure Milli-Q water (Millipore, Milford, MA, USA) and filtered with a 0.2-μm filter (Euroscientific, Lint, Belgium).

2.2. Preparation of derivatization reagent, background electrolyte and samples

The reagent buffer was freshly prepared by dissolving 540 mg of OPA in 2 ml of methanol and about 15 ml of 30 m*M* boric acid previously adjusted with 8 *M* potassium hydroxide to give a pH of 10.4, and 500 μl of MAA was added. The resulting solution was adjusted to pH 10.4 using 8 *M* potassium hydroxide solution. The volume was made to 20 ml with 30 m*M* boric acid previously adjusted to pH 10.4.

In CZE the pH of the BGE has an important influence on the selectivity. Thus to ensure consistent results the pH meter Consort C831 was calibrated before each measurement with buffers prescribed by the Ph. Eur. [15]. To prepare the electrolyte 1.15 g of sodium tetraborate decahydrate was dissolved in about 80 ml of water and the pH was adjusted to 10.0 with 1.0 *M* sodium hydroxide. To this solution 16.0 ml of methanol was added and the volume was made up to 100.0 ml with water.

A sample solution containing 0.7 mg/ml kanamycin sulfate and 1.0 mg/ml picric acid used as internal standard in water was stored at room temperature. To 10.0 ml of this solution, 5.0 ml of 2-propanol and 4.0 ml of the derivatization reagent were added and mixed thoroughly. The volume was made to 25.0 ml with 2-propanol. The mixture was heated in a thermostated water bath at 40°C for 5 min and cooled prior to injection.

2.3. Instrumentation

Method development was performed on a SpectraPhoresis 1000 CE instrument controlled by PC 1000 software version 3.0.1 (Thermoseparation Products, Fremont, CA, USA). The pH measurements were performed on a Consort C831 multichannel analyzer (Turnhout, Belgium). The uncoated fused-silica capillary was obtained from Composite Metal Services (Hallow, UK).

2.4. Electrophoretic conditions

The capillary was conditioned every morning and whenever the buffer system was changed. This was performed with 0.1 *M* sodium hydroxide for 5 min, water for 5 min both at 60°C and running electrolyte for 5 min at 20°C. During analysis the capillary temperature was kept at 20°C. The derivatized samples were hydrodynamically introduced at a pressure of 0.75 p.s.i. (1 p.s.i.=6894.76 Pa) for 4 s injection time. An uncoated fused-silica capillary of 40 cm (effective length 33.7 cm)×50 μm I.D.×375 μm O.D. was used, with UV detection at 335 nm and a separation voltage of 23.5 kV. Peak identification

was carried out by spiking commercial samples with standard material.

2.5. Experimental design

Strategies to enhance selectivity and optimize the derivatization protocol resorted in the use of a chemometrical approach. Screening and response surface modeling were performed by experimental design and multivariate analysis using Modde 4.0 software (Umetri, Umeå, Sweden). Important factors were determined by a screening experiment and optimized by a response surface modeling (RSM). Detailed mathematical manipulation plus interpretations are described elsewhere [16,17].

3. Results and discussion

3.1. Method development and optimization

The influence of different electrophoretic parameters known to influence selectivity was investigated. The buffer system developed previously for gentamicin was employed but co-elution was a main drawback of this system [17]. Different cyclodextrins, inclusion complex forming agents, were studied with no success. The selectivity was even better if CZE alone was applied without any cyclodextrin additives. With β-cyclodextrin co-elution was critical for some peaks. Probably this was due to lack of differential selectivity among kanamycin components towards cyclodextrin hence identical migration behavior. This is opposite with what we found with derivatives of gentamicin [17].

Micellar electrokinetic capillary chromatography (MECC) with sodium dodecyl sulfate (SDS), cetyltrimetylammonium bromide (CTAB) and Brij 35 each was investigated by adding them to the borate buffer, but peak tailing and poor selectivity were serious limitations. The possible reason for this is that derivatized kanamycins are highly negatively charged at pH 10. These findings are in agreement with a study by Oguri and Miki using MECC for aminocyclitol antibiotics where the derivatives of amikacin, arbekacin, dibekacin, kanamycin had almost identical migration times in MECC implying poor selectivity of these substances in a micellar system [18].

In CZE, organic solvents are more often employed as buffer modifier to fine-tune the selectivity by enhancing the polarity and viscosity of the BGE. The most frequently encountered organic modifiers are methanol, 2-propanol and acetonitrile. These were added to the borate at a concentration range between 2.5 and 20% (v/v) each. It was methanol at around 15% (v/v), which separated many peaks. To further enhance selectivity, a multivariate optimization of the system was considered. A chemometrical approach was appropriate to achieve simultaneous alteration of all influential parameters within a predefined matrix as shown in Table 1 [17]. The screening experiment was carried out as a two-level full factorial design with four variables with three center points. The influence of buffer concentration was insignificant thus, further optimization by a central composite response surface modeling experiment considered only methanol, temperature and pH. The target set in this optimization study is the number of peaks separated. Fig. 2A summarizes the effect of the variables, interactions and their significance. Methanol concentration and temperature are the only influential parameters. Increasing methanol and temperature from their lower level to 16% and 20°C, respectively increases the number of peaks to 22 as shown in Fig. 2B. The number remained constant at this maximum peak number over a certain range. This range is also a measure for the method robustness. Based on the experimental design this study proposes the optimal separation conditions: 30 m*M* borate buffer at pH 10.0 containing 16% (v/v) methanol and 20°C. Spiking of the commercial sample with a synthetic derivative of

Table 1
Factorial analysis nominal values, corresponding to lower and higher levels of separation parameters under investigation

Variable	Experimental range investigated	
	Screening	Optimization
Borate (m*M*)	20–60	30
Methanol (%, v/v)	5–20	14–18
Temperature (°C)	15–30	15–25
pH	8–11	9.8–10.2
No. of experiments	$N=2^k+3=19$	$N=2^k+2k+3=17$

k=Number of variables.

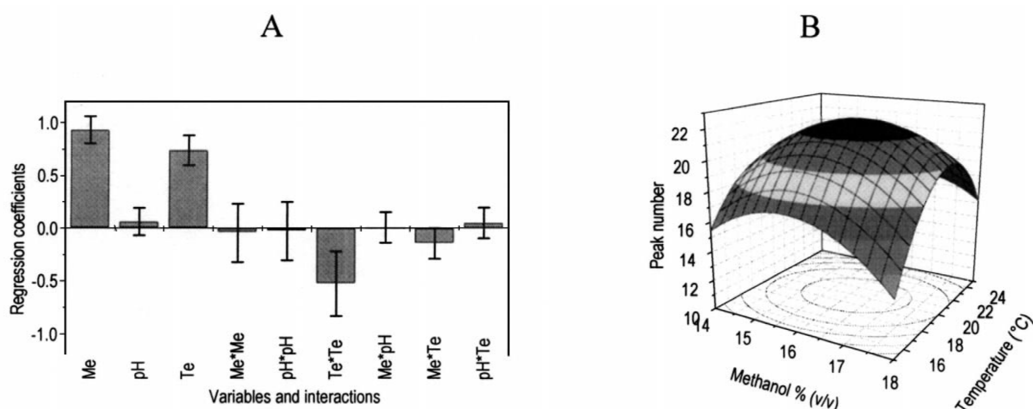

Fig. 2. (A) Regression coefficients plot and (B) response surface plot of the peak number as a function of significant influential factors. Me (methanol); Te (temperature); Te*Me (interactions) and Me*Me (non-linear effect).

kanamycin B showed that peak 7 in Fig. 3 co-eluted with 1-*N*-(1-hydroxymethyl-2-hydroxyethyl)kanamycin B. Since this product is a semi-synthetic compound and never described before to be present in fermentation products, this study cannot draw a definitive conclusion on the identity of peak 7.

Successful separation of kanamycin-related substances: kanamycins B, C and D, deoxystreptamine, paromamine, 6-*O*-(3-AG)DS and 4-*O*-(6-AG)DS from the main component kanamycin A was achieved in less than 15 min analysis time. Fig. 3 is a typical electropherogram of a commercial sample of kanamycin sulfate. Peak 1 is a reagent peak and it is well separated from kanamycin and its related substances. The selectivity of the previous described LC and this CE appears to vary for different substances. LC–PED performed well in separating paromamine and 4-*O*-(6-AG)DS while CZE offered better selectivity between 6-*O*-(3-AG)DS and paromamine [5]. This is due to the fact that paromamine and 4-*O*-(6-AG)DS are positional isomers making them to behave similarly in this electrophoretic environment. The short analysis time and the simplicity of the BGE make CZE more convenient compared to LC whose mobile phase contains more than two substances and which shows a longer analysis time (45 min). The UV detection used in CZE is also more convenient than the PED system known to be unstable.

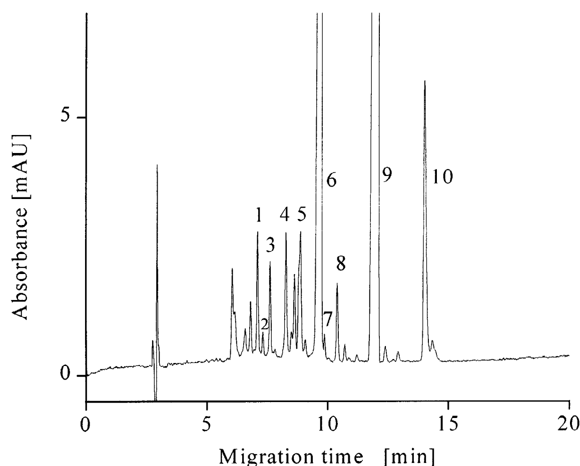

Fig. 3. Typical electropherogram of a commercial sample of 0.7 mg/ml kanamycin sulfate. 1: Reagent, 2: 2-deoxystreptamine, 3: kanamycin D, 4: 6-*O*-(3-AG)DS, 5: 4-*O*-(6-AG)DS+paromamine, 6: kanamycin A, 7: a peak co-eluting with 1-*N*-(1-hydroxymethyl-2-hydroxethyl)kamanycin B, 8 kanamycin C, 9: internal standard (picric acid) and 10: danamycin B.

3.2. Optimization of derivatization conditions

A central composite design experiment and a multilinear regression analysis using Modde 4.0 statistical software enabled optimization of the derivatization reaction. As part of initial experiments screening was performed to establish what are the most influential factors to be included in the response surface modeling. See Table 2. Four factors investigated by two-level full factorial design were reaction temperature, time and concentration of OPA and

Table 2
Factorial analysis nominal values, corresponding to lower and higher levels of derivatization reaction

Variable	Experimental range investigated	
	Screening	Optimization
OPA (mg/ml)	2.0–50.0	2.0–38.0
MAA (μl/ml)	1.0–45.0	1.75–33.25
Temperature (°C)	40.0–80.0	40.0
Time (min)	2.0–20.0	5.0
No. of experiments	$N=2^k+3=19$	$N=2^k+2k+3=11$

k=Number of variables.

MAA. As a response the ratio of corrected peak area of kanamycin A to picric acid (an internal standard, I.S.) was calculated. This preliminary study established that only the concentration of OPA and MAA have significant influence on the derivatization yield. Throughout this study sample solution contained 0.7 mg/ml kanamycin sulfate and 1.0 mg/ml picric acid in water.

For response surface modeling a total of 11 experiments were performed with the concentration of OPA and MAA as variables. The model was fitted with multiple linear regression. The regression coefficient plot generated confirms the importance of OPA and MAA (Fig. 4A). Similar results were obtained in the previous study on gentamicin [17]. In the presence of large excess of reagent the reaction kinetics are quite fast. Increasing the concentration of the OPA and MAA significantly enhances the yield, to a certain point however; further increase results into a decrease of the yield (Fig. 4B). Excess reagent is known to render instability to the reaction products [19,20]. This design produced a derivatization method featuring better-optimized yield in terms of short reaction time and low reaction temperature. The design proposes an optimum derivatization conditions to be: reagent with 27 mg/ml OPA and 25 μl/ml MAA, 40°C reaction temperature and 5 min reaction time.

3.3. Quantitative aspects

A solution of 0.7 mg/ml kanamycin sulfate was used. The limit of quantitation (LOQ) at signal-to-noise ratio $(S/N)=10$ is 0.001 mg/ml (0.14%) with RSDs of kanamycins A, B, C and D being 13, 10, 6, and 8%, respectively. The limit of detection (LOD) at $S/N=3$ is 0.0003 mg/ml (0.04%) relative to 0.7 mg/ml kanamycin sulfate. This shows that sensitivity of the CZE is comparable to LC–PED [5]. The intra-day and inter-day RSD values were 2% ($n=6$) and 2.9% ($n=18$, 6 days), respectively. The calibration curves show a linear relationship in the range investigated. Kanamycin A, range 0.007–1.01 mg/ml, $y=555\,389x-169$, $r=0.9968$ and $S_{y,x}=656$. Related substances were investigated in the range 0.003–0.1 mg/ml, thus kanamycin B: $y=116\,915x-471$, $r=0.9981$ and $S_{y,x}=215$, kanamycin C: $y=113\,843x+26$, $r=0.9998$ and $S_{y,x}=67$ and kanamycin D: $y=84\,382x-494$, $r=0.9981$ and $S_{y,x}=170$. Results were calculated from corrected peak area of

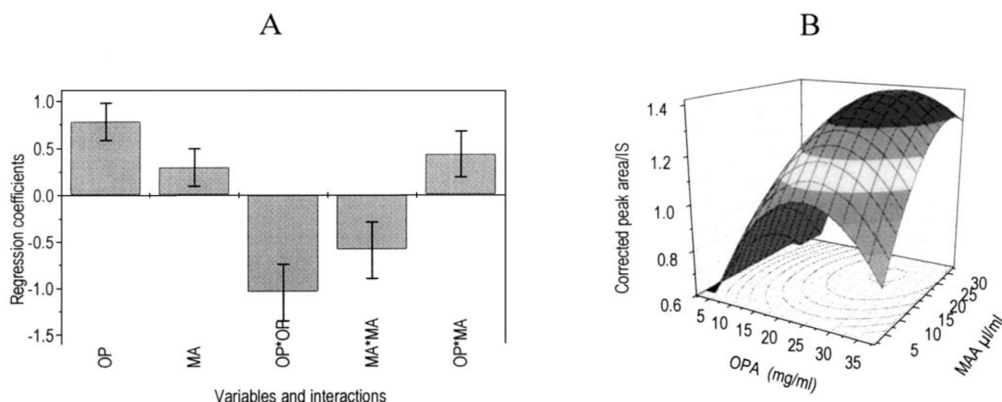

Fig. 4. (A) Regression coefficients plot and (B) response surface plot of the derivatization yield as a function of significant influential factors. OP (1,2-phthalic dicarboxaldehyde); MA (mercaptoacetic acid); OP*MA (interaction) and OP*OP (non-linear effect).

kanamycin component/I.S. y=corrected peak area/I.S., x is the concentration of kanamycin sulfate, r is the coefficient of correlation, $S_{y,x}$=standard error of y estimate, three injections per concentration for six different concentrations were performed.

3.4. Analysis of bulk samples

Six samples of kanamycin were analyzed using the described method. Table 3 gives a summarized comparison of the composition of these samples by CZE and LC–PED. The percentage compositions are expressed as kanamycin A base, calculated with reference to the kanamycin house standard (94.86%, m/m, kanamycin A, as is). For CZE, the content in the six samples varied as follows: 2-deoxystreptamine (<LOQ–0.4%), kanamycin D (0.5–1.8%), 6-*O*-(3-AG)DS (1.8–3.6%), 4-*O*-(6-AG)DS+paromamine (1.6–3.2%), kanamycin A (56.1–84.2%), kanamycin C (0.1–1%) and kanamycin B (0.2–3.9%). There is a difference compared to those obtained by Adams et al. using LC–PED [5], without showing however a definitive and regular trend for kanamycin A. The difference in the detection modes (CZE is based on the UV chromophore detection and LC–PED is based on the oxidation of the hydroxyl groups) could only partly be implicated in this difference. The slopes of the calibration curves were different for the kanamycin components implying different detector response towards these agents. An explanation for the irregular trend when comparing CZE and LC–PED data obtained for kanamycin A is not obvious.

4. Conclusion

A simple, selective and fast CZE method for kanamycin analysis has been developed and validated. The composition of the BGE is simple and easy to prepare. The overall optimal conditions were determined by experimental design. Successful separation of eight related substances of kanamycin and several minor unknowns from the main component was achieved in short analysis time (15 min) compared to LC, which needs 45 min after a two-step gradient. The assay method was used to determine the composition of six commercial samples. The quantitative feature of this assay makes it a suitable alternative to the labor-intensive microbiological assays used in official compendia [15,21].

Table 3
Composition of six different commercial samples expressed as kanamycin A base (%, m/m) as is

Sample	Method	DS	Kanamycin D	6-*O*-(3-AG)DS	4-*O*-(6-AG)DS + paromamine	Kanamycin A	Kanamycin C	Kanamycin B
A	CZE	0.4	1.4	3.6	3.2	56.1 (2.9)	1.0	3.9
	LC–PED	0.6	2.1	1.1	3.6	57.8 (0.3)	0.4	3.3
B	CZE	NQ	1.8	1.8	1.6	64.8 (0.5)	NQ	0.2
	LC–PED	NQ	2.9	0.2	0.9	61.4 (1.5)	NQ	0.2
C1	CZE	NQ	0.9	2.1	2.2	84.2 (0.4)	0.1	0.7
	LC–PED	0.2	1.5	0.4	1.0	75.6 (0.8)	0.02	0.6
C2	CZE	NQ	1.0	2.4	2.5	78.9 (1.5)	0.2	0.4
	LC–PED	0.1	1.8	0.3	0.6	75.9 (0.6)	0.1	0.4
C3	CZE	NQ	0.7	2.1	2.1	76.6 (1.6)	NQ	0.5
	LC–PED	NQ	1.2	0.1	0.4	78.2 (0.4)	0.1	0.4
C4	CZE	NQ	0.5	3.6	2.0	62.8 (2.2)	NQ	0.4
	LC–PED	NQ	0.9	0.4	0.6	70.8 (1.0)	0.02	0.9

Comparison of CE and LC–PED. NQ=Not quantified, i.e., below LOQ, and LC–PED=liquid chromatography with pulsed electrochemical detection [5]. A, B, C represent different sources. The values in parentheses represent RSD values. DS, 2-deoxystreptamine.

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