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Analysis of underivatized gentamicin by capillary electrophoresis with UV detection

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Abstract

A selective and rapid capillary zone electrophoresis method for determination of the multicomponent aminoglycoside antibiotic gentamicin is described. Baseline separation of gentamicin C1, C1a, C2, C2a and C2b components was achieved with a background electrolyte containing 0.35 mM cetyl trimethylammonium bromide, 3% methanol and 90 mM sodium pyrophosphate (pH 7.4) and detected directly with UV detection without derivatization. Quantitative analysis was performed and illustrated the potential use of capillary electrophoresis for the identification and quantitation of gentamicin components, but the application of this method is limited to a gentamicin concentration range of 2–6 mg/ml.

Keywords: Gentamicin; Direct UV detection; CTAB; CZE; Electrosmotic flow reversal

1. Introduction

Gentamicin is an aminoglycoside antibiotic complex mainly consisting of gentamicin C1, C1a, C2, C2a and the minor component C2b (Fig. 1) [1]. It is produced by the fermentation of *Micromonospora purpurea*, has a broad-spectrum antibacterial activity and is used for the treatment of serious bacterial infections [2]. Due to the multicomponent nature of gentamicin, it is a routine analysis requirement in pharmaceutical industry to measure the relative percentage of its major constituents. A reversed-phase LC method with electrochemical detection is prescribed by the Ph. Eur. for the determination of gentamicin sulphate composition, the amounts of C1, C1a and the sum of C2, C2a and C2b being limited to 20–40%, 10–30% and 40–60%, respectively [3]. The USP prescribes an LC method with UV detection after precolumn derivatization of gentamicin with 1,2-phthalic dicarboxyaldehyde (OPA). Gentamicin C1 is limited to 25–50%, C1a to 10–35% and the sum of C2 and C2a to 25–55% [4].

The analysis of gentamicin sulphate is difficult and challenging because it is a multicomponent mixture with lack of UV chromophore. Numerous analytical methods have been used to analyze gentamicin, such as TLC [5], ion-exchange chromatography [6,7], LC with spectroscopic detection [8–11], with electrochemical detection [12], with evaporative light scattering detection [13,14] and also CE [15–23]. Earlier LC methods and CE methods use precolumn or postcolumn derivatization by for example OPA/mercaptoacetic acid (MAA) or dansylchloride with either UV or fluorescence detection. Although these detection methods are quite sensitive, the derivatization step is time-consuming and needs well-controlled experimental conditions to obtain repeatable results. Ackermans et al. [20] reported the first CE analysis of aminoglycosides with indirect UV detection at low pH under reversed polarity. The formation of complexes between the hydroxyl groups and borate for direct UV detection at 195 nm was investigated by Flurer [21,22]. The gentamicin-borate complexes were used to enhance UV absorption at low wavelength and a conventional UV detector was used. But these methods did not reach a complete separation of major gentamicin components. Later, Phillip and Richard [23] developed a CE method with electrochemical detection using copper-based electrodes for the analysis of aminoglycoside

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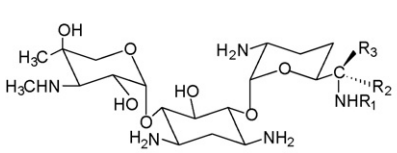

	R_1	R_2	R_3
Gentamicin C_1	CH_3	H	CH_3
Gentamicin C_2	H	H	CH_3
Gentamicin C_{1a}	H	H	H
Gentamicin C_{2a}	H	CH_3	H
Gentamicin C_{2b}	CH_3	H	H

Fig. 1. The chemical structure of five major gentamicin components.

antibiotics. This method suffered from poor selectivity as none of the individual components of the gentamicin complex could be resolved.

More recently, Yuan et al. [24] used CE with a newly developed detector, potential gradient detector (PGD), for analyzing gentamicin. This method was developed at low pH to render the gentamicin amine groups positively charged and used cetyl trimethylammonium bromide (CTAB) to cover the silanol groups and make the capillary wall positively charged in order to prevent positively charged gentamicin components from adsorbing on the wall. The use of CTAB in this method also reversed the direction of the EOF which helped in the resolution, since the gentamicin molecules moved against the EOF having more time to be separated. Although this method showed better sensitivity than previous CE methods for direct determination of gentamicin components, without derivatization, it only achieved the separation of C1, C1a and C2 plus C2a, failing in the separation of the latter two gentamicin components.

In this work we report the results of CE analysis of gentamicin using CTAB and sodium pyrophosphate as background electrolyte (BGE) under reversed polarity to achieve a complete baseline separation between the major components (C1, C1a, C2 and C2a) and the minor component C2b with direct UV detection without derivatization.

2. Materials and methods

2.1. Chemicals and solutions

SDS, sodium pyrophosphate, α-cyclodextrin (α-CD), β-CD, γ-CD and hydroxypropyl-beta-CD (OH-propyl-β-CD) were obtained from Acros Organics (Geel, Belgium), heptakis(2,6-di-*O*-methyl)-β-CD (hept-di-methyl-β-CD), hexakis(2,3,6-tri-*O*-acetyl)-α-CD (hexa-tri-acetyl-α-CD), heptakis(2,3,6-tri-*O*-acetyl)-β-CD (hept-tri-acetyl-β-CD) and methyl-β-CD from Sigma–Aldrich (Steinheim, Germany), CTAB and dodecyl trimethylammonium bromide (DTAB) from Merck (Darmstadt, Germany), sodium hydroxide pellets from BDH (Poole, England), methanol and acetonitrile from Fisher Scientific (Loughborough, Leicestershire, UK). The gentamicin components C1a, C1 and a mixture of C2 and C2a were obtained from Pierrel (Capua, Italy), gentamicin C2b was provided by Kyowa Hakko Kogyo (Tokyo, Japan). Gentamicin sulphate standard was obtained as CRS from the Ph. Eur. Laboratory. Three recent bulk samples were from Schering-Plough (Heist op den Berg, Belgium). All solutions were made with ultrapure Milli-Q water (Millipore, Milford, MA, USA) and filtered with a 0.2 μm filter (Euroscientific, Lint, Belgium).

2.2. Instrumentation

Experiments were performed on a Beckman P/ACE MDQ instrument equipped with a photo diode array detector from Beckman Coulter (Fullerton, CA, USA). Data acquisition was done by means of 32 Karat^{TM} version 5.0 software (Fullerton, CA, USA). Uncoated fused-silica capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA). The pH measurements were performed on a Metrohm 691 pH meter (Herisau, Switzerland).

2.3. Electrophoresis

Before use, a new capillary was conditioned at 30 °C by washing with 1 M NaOH for 5 min and keeping it in 1 M NaOH for 2 h. Then, it was washed with 0.1 M NaOH and water for 5 min each. It was further equilibrated by flushing with the separation buffer for 20 min. At the beginning of each day, prior to the analyses, the capillary was activated by washing in the following sequence: 1 M NaOH, 0.1 M NaOH and water for 5 min each, and finally equilibrated with running buffer for 10 min followed by an applied voltage of −6 kV for 10 min. To ensure repeatability of the migration times, the first few runs were disregarded and the capillary was rinsed with 0.1 M NaOH and water for 2 min each and BGE for 3 min in between runs. The inlet/outlet vials were renewed every three runs. All the washings were performed by applying a pressure of 137.9 kPa.

The finally optimized electrophoretic conditions include the use of an uncoated fused-silica capillary with a total length of 40.2 cm, effective length of 30 cm, and an ID of 75 μm, a BGE containing 0.35 mM CTAB, 3% methanol and 90 mM sodium pyrophosphate adjusted to pH 7.4 using *ortho*-phosphoric acid, an applied voltage of −6.0 kV (reverse polarity) and the capillary temperature maintained at 30 °C using liquid coolant. The current corresponding to these conditions was about 110 μA. The samples were hydrodynamically injected for 10 s × 5.5 kPa. On-line detection was performed by UV at 195 nm.

2.4. Spiked standard and samples

Solutions of gentamicin sulphate standard and gentamicin sample were prepared at a concentration of 4 mg/ml in water, unless mentioned otherwise. Electrophoretic parameters were determined using the standard solution and both solutions were used for quantitation purposes. For identification purposes solutions of gentamicin sulphate standard 4 mg/ml were spiked separately with C1, C1a, C2, C2b and a mixture of C2 and C2a.

2.5. Software

The experimental design and multivariate analysis for optimization and method robustness were performed with the support of Modde 5.0 software (Umetrics AB, Umeå, Sweden).

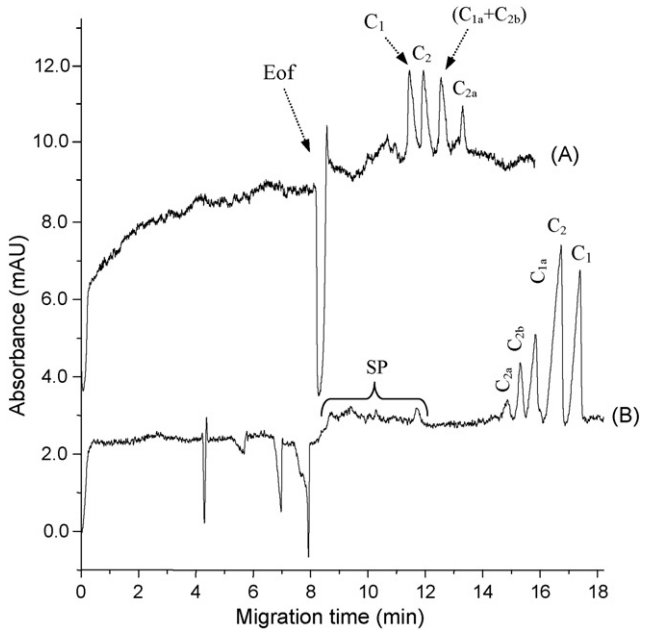

Fig. 2. Typical electropherograms of a standard sample of gentamicin sulphate. (A) BGE, pyrophosphate 60 mM, pH 7.5; capillary, 50 μm i.d., 40.2 cm total and 30 cm effective length; injection pressure, 10 s × 5.5 kPa; temperature, 30 °C; voltage, 10 kV; current generated, 95 μA; UV detection, 195 nm. (B) Under optimum conditions: BGE, pyrophosphate 90 mM, CTAB 0.35 mM, pH 7.4, 3% (v/v) methanol; capillary, 75 μm i.d., 40.2 cm total and 30 cm effective length; injection pressure, 10 s × 5.5 kPa; temperature, 30 °C; voltage, −6 kV; current generated, −110 μA; UV detection, 195 nm (SP: system peaks).

3. Results and discussion

3.1. Method development

Previous studies in our laboratory showed that sodium pyrophosphate allows a separation, though incomplete, of the gentamicin components (data not shown) with direct UV detection without derivatization [25]. In the initial investigation on the influence of pH (pH 7.0–8.0 with steps of 0.1 units) and concentration (35–70 mM) of the same BGE, a capillary zone electrophoresis method with normal polarity (using a capillary of 50 μm i.d.) was developed. Although a better resolution (Fig. 2A) was achieved, this method gave poor sensitivity and the current generated was between 75 and 95 μA.

A lot of different additives, such as chiral selectors (α-CD, β-CD, γ-CD, OH-propyl-β-CD, methyl-β-CD, hept-di-methyl-β-CD, hept-tri-acetyl-β-CD, hexa-tri-acetyl-α-CD at 3, 5 and 10 mM), organic modifiers (methanol and acetonitrile from 3 to 5%) and surfactants (SDS from 10 to 100 mM, CTAB and DTAB at 10 and 20 mM), were investigated as buffer modifiers, but they did not improve the resolution. Moreover, it was not possible to increase the internal diameter of the capillary in order to obtain better sensitivity since this not only increases the sensitivity but also the current.

In a second stage of method development, it was decided to reverse the electrosmotic flow using CTAB as buffer additive, since the use of CTAB in low concentration (0.2–0.6 mM) has been described for the analysis of gentamicin using potential gradient detection [24] and its use in this range of concentrations can lead to electrosmotic flow reversal [26]. At this stage the capillary internal diameter was increased to 75 μm to obtain better sensitivity.

Then, the influence of CTAB concentration (0.2–0.6 mM, steps of 0.05 mM), the influence of sodium pyrophosphate concentration (20–120 mM, steps of 10 mM) and the influence of pH (7.20–7.60, steps of 0.05 units) were tested. The influence of methanol and acetonitrile (both at 1, 3, 5, 7 and 10%) was also investigated. The best results were achieved with pyrophosphate concentrations higher than 60 mM, especially between 80 and 100 mM, CTAB concentration between 0.25 and 0.45 mM, 1–5% (v/v) of methanol and a pH range between 7.35 and 7.45. Due to the complex composition of the BGE, further experimental design studies were carried out to achieve the optimum conditions for the analysis of gentamicin using UV detection without derivatization.

Fig. 2B shows a typical electropherogram of gentamicin sulphate standard solution under the final optimum conditions. Some negative and positive system peaks appear in the first 12 min. This was demonstrated by analyzing sulphuric acid and blank solutions, where these peaks appeared.

3.2. Optimization of selectivity

The initial method development was followed by a more systematic step in the optimization process, namely the execution of screening experiments where a relatively large number of variables are examined concerning their significance [27]. Screening experiments, which involved a full factorial design, were performed to study the influence of several electrophoretic parameters (concentration of sodium pyrophosphate from 80 to 100 mM, methanol from 3 to 5%, and CTAB from 0.25 to 0.45 mM; pH from 7.35 to 7.45) on the selectivity between critical peak pairs. It was established from these screening experiments that only the concentration of sodium pyrophosphate and methanol and the pH have a significant effect while the CTAB concentration was non-significant.

The screening experiment results were used for further optimization by a central composite response surface modelling (RSM) experiment. The RSM experiment included the parameters with significant effect, and as response variables, selectivities $S_{C2a–C2b}$, $S_{C2b–C1a}$ and $S_{C1a–C2}$ corresponding to critical peak pairs C2a–C2b, C2b–C1a and C1a–C2 were chosen. The factor levels that showed to be optimal were set as center point values in the RSM experiment. Factorial analysis nominal values, applied for optimization of selectivity are shown in Table 1 and a summarized work sheet is shown in Table 2. The CTAB concentration was kept constant at 0.35 mM, the temperature at 30 °C and the voltage at −6 kV.

The central composite face-centered (CCF) design permits the response surface to be modeled by fitting a second order polynomial model. In particular, the CCF consists of points of a full factorial design ($2^k + n$), which have been augmented, with ($2k$) star points to enable this model estimate the response curvature plot. The star points are located at the edges of the experimental domain in the sense that each point is a combination of variables,

Table 1
Factorial analysis nominal values, corresponding to (−), (0) and (+) levels of separation conditions

Electrophoretic variables	Low value (−)	Central value (0)	High value (+)
Sodium pyrophosphate (mM)	80	90	100
Methanol (%, v/v)	1	3	5
Buffer pH	7.35	7.40	7.45

such that two out of three variables are at the center level and one is at the extreme level (see experiments nos. 9–14 in Table 2). In total $2^k + 2k + n = 17$ experiments were carried out where k is the number of factors ($k = 3$) and n, the number of central points ($n = 3$).

Since this model has three responses, which have to be determined simultaneously, it is important to develop a model representing the relationship of all responses to the variables. To determine if the data fit well with the model, the response of the model has to be checked. In this case, the model was fitted using PLS.

The R^2 values for the three responses $S_{C2a–C2b}$, $S_{C2b–C1a}$ and $S_{C1a–C2}$ were 0.869, 0.909 and 0.826. The Q^2 values were 0.176, 0.489 and 0.361, respectively. R^2 is the percent of the variation of the response explained by the model and Q^2 is the percent of the variation of the response that can be predicted by the model. This predictive performance is evaluated by leave-one-out cross validation. The Q^2 values were found to be less than the R^2, which illustrates the low predictive power of the method. Fig. 3 shows the regression coefficient plots with confidence intervals for all the responses. The coefficients of the model represent the relationship between the response variables measured and the factors studied. In these plots, the effect of a factor is denoted by a coefficient bar and the 95% confidence limits by an error bar. A regression coefficient smaller than the error bar interval shows that the variation in the response caused by change of that variable is smaller than the experimental error. Therefore, the effect of that variable is considered to be negligible. The positive or negative coefficient in the chart indicates that the corresponding variable shows a positive or negative effect on the response, respectively.

3.3. Effect of factors on responses

In the range investigated, it has been observed (see Fig. 3) that the concentration of pyrophosphate buffer is positively correlated to the selectivity $S_{C2a–C2b}$ (increased selectivity), however, negatively correlated to the selectivities $S_{C2b–C1a}$ and $S_{C1a–C2}$ (decreased selectivity). The buffer pH is positively correlated to the selectivities $S_{C2a–C2b}$ and $S_{C1a–C2}$ but negatively correlated to the selectivity $S_{C2b–C1a}$. The pH had much influence on the selectivity and this could be correlated on the one hand to the characteristics of the amino groups, and on the other hand to a possible complexation with pyrophosphate. Indeed, the experiments in pyrophosphate without CTAB show that gentamicin is negatively charged (Fig. 2A) which points to an interaction between gentamicin and pyrophosphate. When CTAB is added to the pyrophosphate BGE, gentamicin remains negatively charged. This could be deduced from the observation that the electrosmotic flow migrates after the gentamicin components under the conditions of Fig. 2B ($\mu_{eo} = 0.152\,cm^2/kV\,s$). Increasing methanol has a negative effect only on the selectivity $S_{C2b–C1a}$.

For the selection of the overall optimum point, balancing of all these effects was necessary. This can be concluded from Fig. 4 showing the influence of the significant parameters on the selectivity.

3.4. Robustness

Robustness is an important feature of analytical method development. It evaluates the influence of small changes in the

Table 2
CCF design used in the method optimization and robustness

Exp. no.	Variables			Responses		
	Pyrophosphate (mM)	pH	Methanol (%, v/v)	$S_{C2a–C2b}$	$S_{C2b–C1a}$	$S_{C1a–C2}$
1	80	7.35	1	1.000	1.059	1.077
2	100	7.35	1	1.043	1.053	1.074
3	80	7.45	1	1.025	1.057	1.080
4	100	7.45	1	1.048	1.050	1.075
5	80	7.35	5	1.019	1.057	1.076
6	100	7.35	5	1.032	1.047	1.067
7	80	7.45	5	1.022	1.052	1.079
8	100	7.45	5	1.044	1.043	1.071
9	80	7.40	3	1.027	1.056	1.076
10	100	7.40	3	1.039	1.047	1.073
11	90	7.35	3	1.031	1.052	1.073
12	90	7.45	3	1.042	1.042	1.080
13	90	7.40	1	1.034	1.049	1.073
14	90	7.40	5	1.030	1.048	1.074
15	90	7.40	3	1.038	1.048	1.075
16	90	7.40	3	1.036	1.049	1.077
17	90	7.40	3	1.026	1.050	1.078

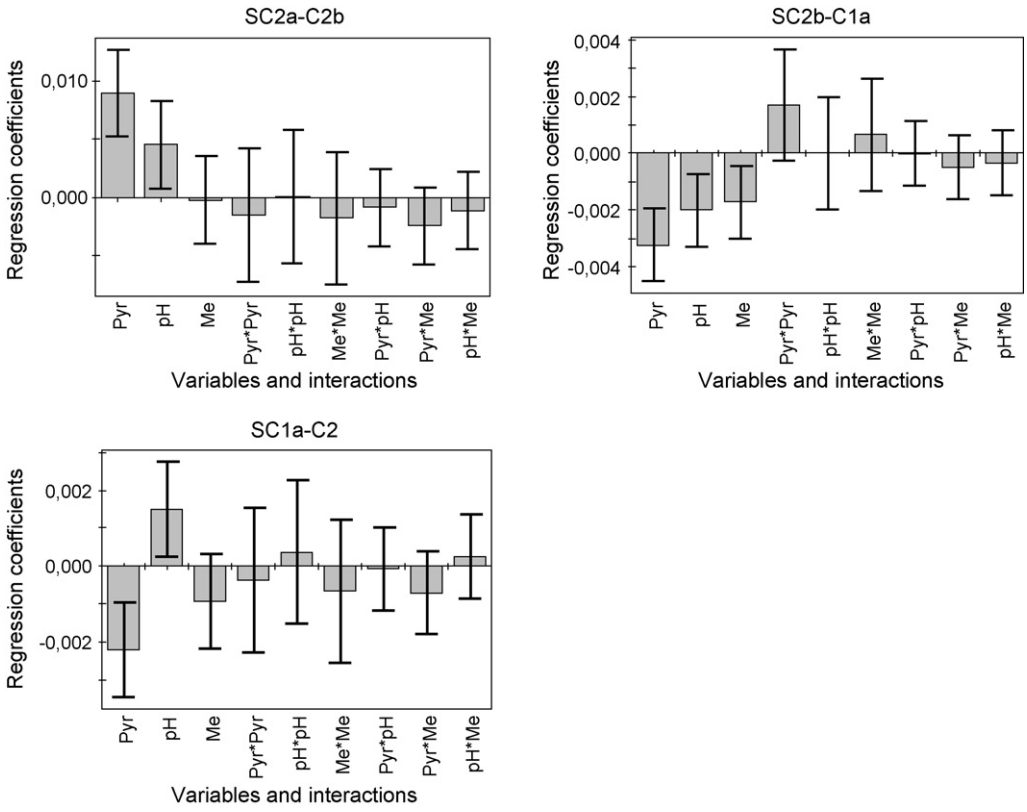

Fig. 3. Regression coefficient plots for the separation selectivity. $S_{C2a–C2b}$: selectivity between critical peak pair C2a and C2b; $S_{C2b–C1a}$: selectivity between critical peak pair C2b and C1a; $S_{C1a–C2}$: between critical peak pair C1a and C2 (Pyr: sodium pyrophosphate; Me: methanol; pH: buffer pH).

operating conditions of the analytical procedure on measured or calculated responses. The experimental design described in above sections was used to evaluate the response surface plot constructed by plotting the responses individually as a function of the most important variables. Fig. 4 shows that selectivity was good enough for all the responses in the buffer pH range from 7.35 to 7.45, methanol concentration from 1 to 5% (v/v) and sodium pyrophosphate concentration from 80 to 100 mM but for the response $S_{C2a–C2b}$ the range for pyrophosphate concentration is restricted from 87 to 100 mM in order to have a selectivity higher than 1.03. It means that the method is robust in this range.

3.5. Method validation

Under the optimum conditions, quantitative features of this analytical CE method were tested (using the corrected peak area of gentamicin). The corrected areas were obtained by dividing the area of the peak by its migration time. The precision was checked by injecting 36 nl (144 ng) of a 4 mg/ml solution of gentamicin sulphate standard. R.S.D. values of less than 3.5% intraday ($n = 6$) and ≤5.0% interday ($n = 12$) were obtained for the five components. The intraday R.S.D. value at 2 mg/ml was <4.9% ($n = 3$) and at 8 mg/ml it was <8.4%.

Gentamicin sulphate standard solutions were used for LOQ (at a signal-to-noise ratio S/N = 10) and LOD (S/N = 3) analysis because pure reference substances were not available in sufficient quantities. The total concentration of gentamicin solutions corresponding to LOQ and LOD was 2.09 and 0.67 mg/ml, respectively, taking into account S/N values of 10 and 3 for the smaller peak (C2a) in the electropherograms.

Calibration curves for each of the gentamicin components (C2a, C2b, C1a, C2 and C1) were obtained by triplicate analyses of solutions containing 2, 3, 4, 5 and 6 mg/ml of gentamicin sulphate standard (the lowest point of the calibration curve was the LOQ). Fig. 5 shows that the linearity range of this method

Table 3
Results for sample analysis

	Sample				
	C2a	C2b	C1a	C2	C1
Standard					
Corr. area (%) ($n = 3$)	2.73	9.08	16.31	43.08	28.79
R.S.D. (%) ($n = 3$)	4.5	1.1	0.9	0.3	0.8
Sample 1					
Corr. area (%) ($n = 3$)	1.83	1.52	28.85	36.53	31.27
R.S.D. (%) ($n = 3$)	11.3	7.6	0.8	0.8	0.5
Sample 2					
Corr. area (%) ($n = 3$)	1.80	1.68	26.09	38.19	32.24
R.S.D. (%) ($n = 3$)	13.5	13.1	1.1	0.8	1.3
Sample 3					
Corr. area (%) ($n = 3$)	1.59	1.37	27.99	36.24	32.82
R.S.D. (%) ($n = 3$)	7.9	8.3	0.4	0.1	0.3

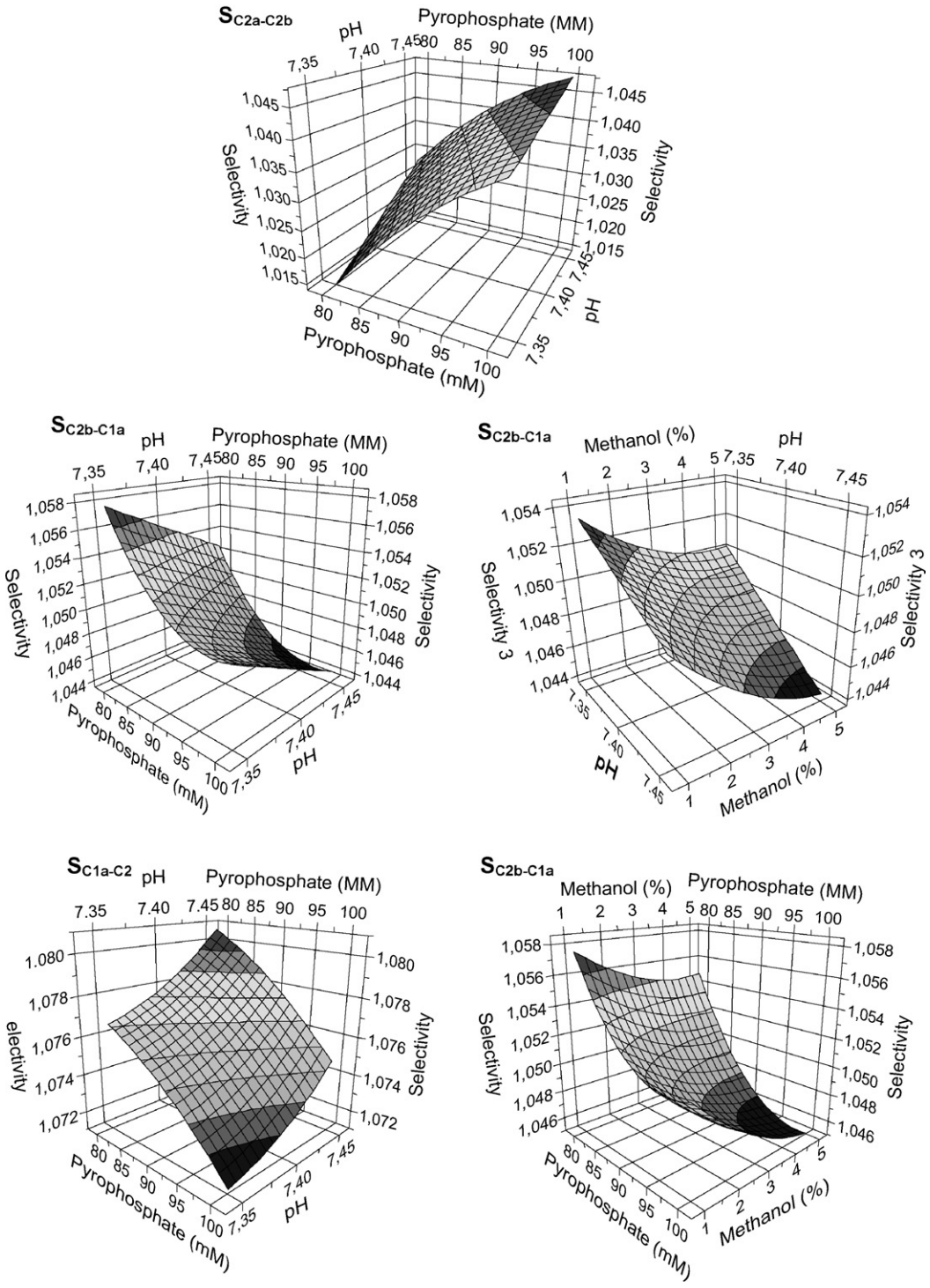

Fig. 4. Response surface plots of selectivity as a function of significant separation parameters.

for gentamicin was from 2 mg/ml (50%) to 6 mg/ml (150%) relative to 4 mg/ml total concentration of gentamicin. A good linear relationship was established in this range. For samples with a concentration higher than 6 mg/ml, overloading would occur.

It can be concluded from these validation data that high levels of gentamicin concentrations need to be used, but it should be emphasized that direct UV detection is performed. The range of application of the method from 2 to 6 mg/ml is rather narrow.

3.6. Sample analysis

To calculate the relative percentages of the main components of gentamicin, corrected areas were used. The relative percentages were obtained by dividing the individual corrected area

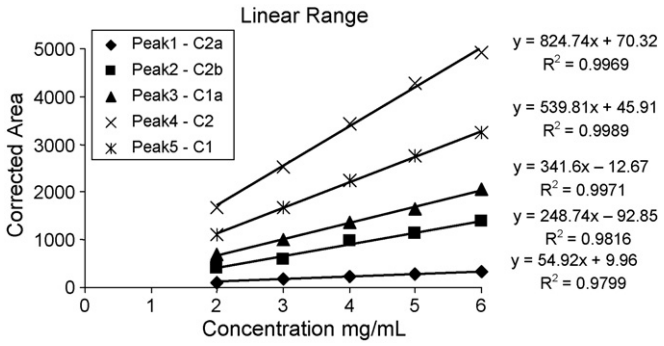

Fig. 5. Linearity for gentamicin sulphate standard.

by the total corrected area of the five peaks (normalization of corrected peak areas to 100%). It was not possible to determine response factors because pure reference substances were not available in sufficient quantities. Fig. 6 and Table 3 show the results of applying the method to 4 mg/ml gentamicin sulphate standard solution and three different gentamicin sample solutions. The same separation profile is obtained for standard and real samples. The R.S.D. values obtained for the smaller peaks (C2a and C2b) were much higher than those for the major components C1a, C2 and C1 where the maximum R.S.D. value obtained was 1.3% (Table 3).

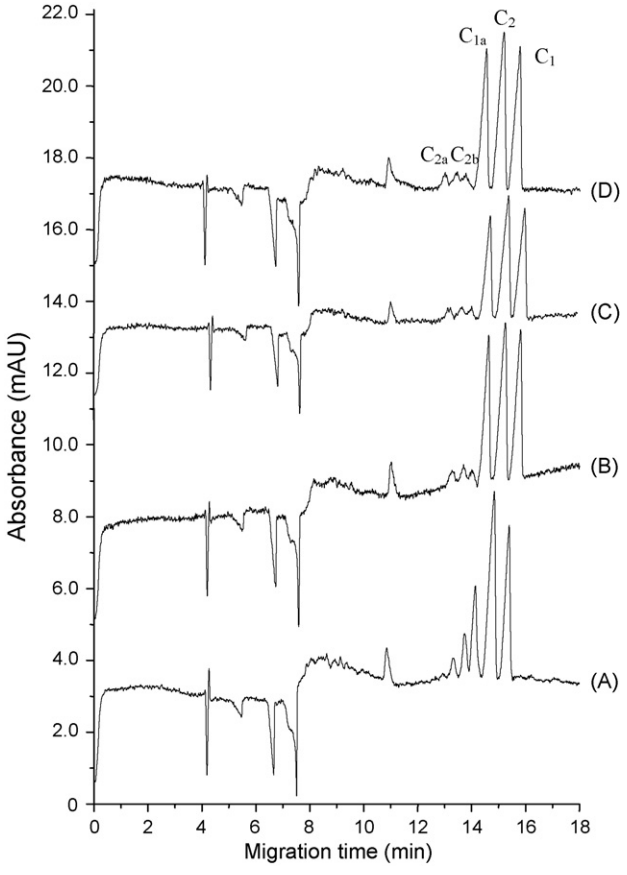

Fig. 6. Analysis of gentamicin sulphate. (A) Standard; (B)–(D) three different real samples.

4. Concluding remarks

A simple and fast method was developed to determine major components (C1, C1a, C2, C2a) and C2b in gentamicin using CZE with direct UV detection. The use of pyrophosphate buffer as BGE enabled the direct UV detection of gentamicin. The method has shown better selectivity than previous CE methods for direct determination of major components of gentamicin, without derivatization. Optimization and robustness were evaluated with an experimental design. The method showed good selectivity, repeatability and linearity. The quantitative analysis established that the method is suitable for the analysis of the composition of the active components of gentamicin in bulk drugs. The method is not sufficiently sensitive to analyze for impurities.

Acknowledgements

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