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Optimization of capillary electrophoresis method with contactless conductivity detection for the analysis of tobramycin and its related substances

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ABSTRACT

A method was validated and optimized to determine tobramycin (TOB) and its related substances. TOB is an aminoglycoside antibiotic which lacks a strong UV absorbing chromophore or fluorophore. Due to the physicochemical properties of TOB, capillary electrophoresis (CE) in combination with Capacitively Coupled Contactless Conductivity Detection (C^{4}D) was chosen.

The optimized separation method uses a background electrolyte (BGE) composed of 25 mM morpholinoethane–sulphonic acid (MES) adjusted to pH 6.4 by L-histidine (L-His). 0.3 mM cetyltrimethyl ammonium bromide (CTAB) was added as electroosmotic flow modifier in a concentration below the critical micellar concentration (CMC). Ammonium acetate 50 mg L^{-1} was used as internal standard (IS). 30 kV was applied in reverse polarity (cathode at the injection capillary end) on a fused silica capillary (65/43 cm; 75 μm id).

The optimized separation was obtained in less than 7 min with good linearity (R^2 = 0.9995) for tobramycin. It shows a good precision expressed as RSD on relative peak areas equal to 0.2% and 0.7% for intraday and interday respectively. The LOD and LOQ are 0.4 and 1.3 mg L^{-1} corresponding to 9 pg and 31 pg respectively.

1. Introduction

The United States Pharmacopeia (USP) [1] defines validation of analytical methods as the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications. The USP has recommended a procedure for the validation of analytical methods.

Tobramycin (TOB) is an aminoglycoside antibiotic derived from nebramycin, an antibiotic complex produced by fermentation of the actinomycete *Streptomyces tenebrarius* [2]. It is a polycationic pseudo-oligosaccharide, which consists of two aminosugars joined by a glycosidic linkage in a central position to 2-deoxystreptamine. TOB is active against a broad spectrum of Gram-negative bacteria. It exerts a bactericidal activity against many bacterial strains involved in clinical infections. It is particularly indicated for the treatment of septicemia, complicated and recurrent urinary tract infections, lower respiratory infections, serious skin and soft tissue infections including burns and peritonitis, ophthalmic and central nervous system infections caused by organisms resistant to other antibiotics, including other aminoglycosides.

TOB is used in a variety of pharmaceutical applications, including ophthalmic suspensions, solutions and ointments, inhalation solutions and intravenous administrations. Like for other aminoglycosides, a narrow therapeutic index is implicated because of its potential oto- and nephrotoxicity encountered during its clinical use [3]. It has neither a chromophore nor fluorophore which limits optical detection (UV/vis and fluorescence). TOB can also be synthesized from kanamycin B [4]. The chemical structures of tobramycin and its major impurities are shown in Fig. 1.

TOB and kanamycin B are produced after base catalyzed hydrolysis of nebramine factor 5′ (6″-O carbamoyltobramycin) and 4 (6″-O carbamoylkanamycin B) respectively, produced by fermentation. Tobramycin and kanamycin B are hydrolyzed by acid to nebramine and neamine respectively. As TOB is produced by fermentation, beside the main component it also contains some related substances that result either from the incomplete purification or from degradation of the drug. Kanamycin B, nebramine and neamine (also known as neomycin A) are three known impurities of tobramycin reported in the Ph. Eur. [5].

The separation and detection of tobramycin and its impurities has been a great challenge, due to the polar basic nature and the lack of UV absorption properties. Several analytical methods have been used to determine it, such as paper chromatography [6], gas liquid chromatography after silylation [7] and spectrophotometry [8].

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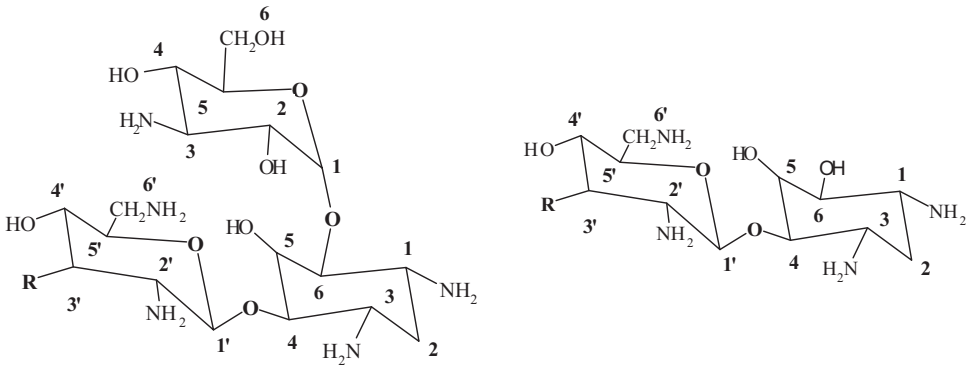

	R
Tobramycin	H
Kanamycin B	OH

	R
Nebramine	H
Neamine	OH

Fig. 1. Chemical structures of tobramycin and its major impurities.

LC and CE using pre and post-column derivatization of tobramycin [9–14] have been performed using o-phthalaldehyde (OPA) [9–11], 2,4,6-trinitrobenzenesulphonic acid [12] and 2,4-dinitrofluorobenzene [13]. However, these techniques can be tedious, time consuming, not safe and give problems with quantitation because of additional sample processing, variability of reaction completeness, possible instability of derivatized products and toxicity of some derivatization agents. Therefore it presents a limitation to its routine use. Direct detection using evaporative light scattering detection (ELSD) [15], mass spectrometry (MS) [16,17] and pulsed electrochemical detection (PED) [18,19] have also been described.

An ion-pair LC method using a poly(styrenedivinylbenzene) column as stationary phase combined with PED has been developed [18]. This method is currently prescribed in the Ph. Eur. [5] for the analysis of tobramycin. Beside with reversed phase chromatography, PED has also been used in combination with anion exchange chromatography for the detection of tobramycin and its impurities [19]. LC methods with electrochemical detection have shown good separation performance, but stability and operator experience are the major limitations. Because of that CE is increasingly being viewed as an alternative and a complement to LC for determination of drug related impurities [20]. Many investigators have used CE in the analysis of aminoglycosides combined with borate complexation [21], indirect detection methods [22] and amperometric detection [23]. Micellar electrokinetic capillary chromatography (MEKC) with UV detection for simultaneous determination of amikacin, tobramycin and kanamycin A, was performed in Tris buffer at pH 9.1 with a high concentration of sodium pentanesulphonate as an anionic surfactant [24]. However, these methods are less sensitive and selective for the related substances. CZE with amperometric detection has been reported for analysis of kanamycin and amikacin, but could only show selectivity for three components [23]. The determination of tobramycin in human serum has been reported using CE with Capacitively Coupled Contactless Conductivity Detection (CE-C^4D) [25].

The official method for the analysis of tobramycin in the European Pharmacopoeia (Ph. Eur.) [5] prescribes the use of thin layer chromatography (TLC) for identification and the test on kanamycin B and the assay are performed by LC-PED.

This work investigates the sensitivity and selectivity of CE with C^4D as a direct detection method for the analysis of tobramycin and its related substances. This mode of detection can be useful in capillary electrophoretic analyses of a broad scale of compounds, from low-molecular-mass highly mobile small inorganic and organic ions (e.g. halogenides, alkali metal ions, trifluoroacetic acid (TFA)) to alcohols, carbohydrates, proteins, aminoglycosides, etc. [26–28]. It can also be a good alternative to derivatization in case of non UV-absorbing substances.

2. Material and methods

2.1. Reagents, samples and solutions

All chemicals used were of analytical grade. 2-(N-morpholino)ethanesulphonic acid monohydrate (MES) and L-histidine (L-His) were purchased from Fluka (Sigma-Aldrich, Schnelldorf, Germany). N-cetyltrimethyl ammonium bromide (CTAB) and ammonium acetate were from Merck (Darmstadt, Germany). Sodium hydroxide was from Riedel-deHaën (Seelze, Germany), sodium chloride was from Fisher chemicals (Leicestershire, UK). Ammonium formate was from Fluka AG (Buchs Switzerland), formic acid from Acros Organics (Geel, Belgium) and sodium acetate from Applichem GmbH (Darmstadt, Germany). Tobramycin reference CRS was obtained from the European Pharmacopoeia EDQM (Strasbourg, France), tobramycin samples were obtained from different sources as Alcon Cusí (Barcelona, Spain), Biogal (Debrecen, Hungary) and Chongqing Daxin Pharmaceutical Co. Ltd. (Chongqing, China). Kanamycin B was acquired from WHO (Geneva, Switzerland). Neamine was prepared in the laboratory [29] and nebramine was obtained from Dr. Istvan Fabian (Lajos Kossuth University, Debrecen).

All solutions were prepared by using ultrapure MilliQ-water (Millipore, Milford, MA, USA) and were filtered with a 0.2 μm membrane filter syringe (Dassel, Germany).

The pH value of the buffers was measured and adjusted with the aid of a pH-meter Metrohm 691 (Herisau, Switzerland). MES buffers with pH values equal to or higher than 6.0 were prepared with MES ($pK_a = 6$) and the pH was adjusted with solutions of L-His. Stock solutions of 100 mM of MES, L-His and 10 mM of CTAB were prepared.

During method development, sample stock solutions of tobramycin and related impurities were individually prepared at a concentration of 1.0 g L^{-1} in water and stored at 7 °C, for at most one week.

2.2. Instrumentation and operating conditions

The experiments were performed on a P/ACE MDQ instrument (Beckman Coulter, Inc., Fullerton, CA, USA), coupled with an eDAQ

C^4D system (eDAQ, Denistone East, Australia). Data acquisition was done by 32 Karat™ 4.0 software (Beckman Coulter, Inc., Fullerton, CA, USA).

Uncoated fused silica capillaries of 75 μm I.D. and 375 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). The total length was 65 cm and effective length 43 cm. New capillaries were conditioned at 45 °C by rinsing with 1 M NaOH (10 min), 0.1 M NaOH (30 min), wait for 30 min and water (5 min). Daily at the beginning of analysis, the capillary was rinsed with 1 M NaOH (5 min), 0.1 M NaOH (3 min), water (1 min) and BGE (2 min); all the steps were performed at 25 °C and 138 kPa pressure. The inlet/outlet vials were replaced every 3 runs.

During method development and later on, for method application to tobramycin analysis, the capillary was rinsed between runs for 1 min with 0.1 M NaOH, 1 min with water and 3 min with buffer at 138 kPa. Samples were hydrodynamically introduced at a pressure of 3.45 kPa for 5 s injection time and a separation voltage of −30 kV (cathode at the injection capillary end) at 25 °C was applied.

The eDAQ C^4D detector was employed at a peak-to-peak amplitude of 80 V and the frequency was 600 kHz. The data were processed using licensed PowerChrom v2 software (EDAQ, Denistone East, Australia). Further data acquisition was done by both PowerChrom v2 and 32 Karat™ 4.0 softwares.

Table 1
Electrophoretic parameter settings applied in the method optimization, corresponding to low (−), central (0) and high (+) levels.

Parameter	Low value (−)	Central value (0)	High value (+)
pH	6.2	6.4	6.6
BGE (mM)	20	30	40
CTAB (mM)	0.2	0.4	0.6
Temp (°C)	22.5	25	27.5

2.3. Experimental design

Four experimental parameters (factors) were varied at levels under and above the central value: concentration of BGE (mM), pH, concentration of CTAB (mM) and capillary temperature (°C) (as shown in Table 1). The levels were chosen based on some pre-experiments and knowledge about the system. Factors were varied at the same time, making it possible to distinguish between the effects of a single variable and of interacting variables. Replicating center points are added to check for curvature (quadratic effects), and to obtain an independent estimate of the error to illustrate the repeatability of the method. A two level full factorial design was applied. For this design, the number of runs is equal to $2^k + n$, where k is the number of parameters and n is the number of

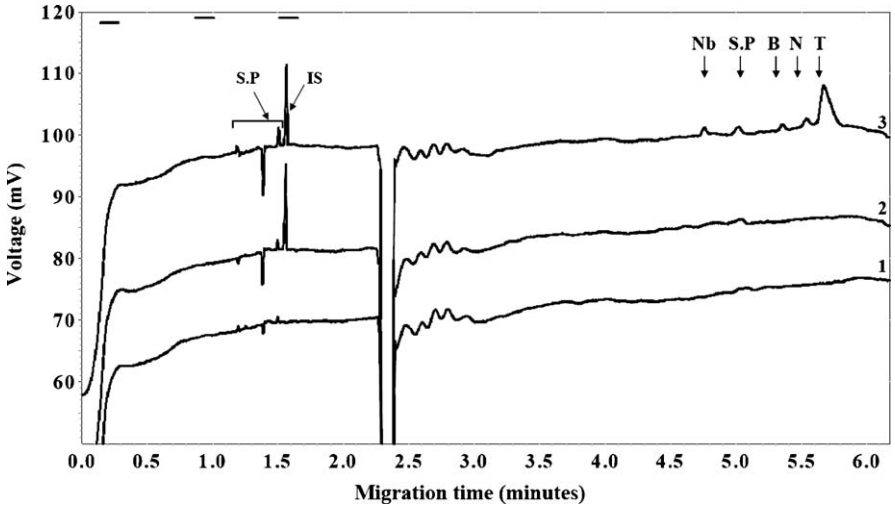

Fig. 2. Typical CE-C^4D electropherograms showing the separation of tobramycin from its impurities using CE; (1) blank (H_2O), (2) internal standard (ammonium acetate 50 mg L^{-1}) only and (3) samples dissolved in water: kanamycin B, nebramine and neamine 2.5 mg L^{-1} each and tobramycin 50 mg L^{-1}. S.P, system peak; IS, internal standard; B, kanamycin B; Nb, nebramine; N, neamine; T, tobramycin. Capillary, uncoated fused silica 75 μm I.D., 375 μm O.D. (65 cm total length, effective 43 cm to C^4D detector); voltage, 30 kV in reverse polarity; temperature, 25 °C; injection, inlet pressure 3.45 kPa for 5 s; BGE, 20 mM MES and 0.3 mM CTAB, adjusted by L-His to pH 6.4.

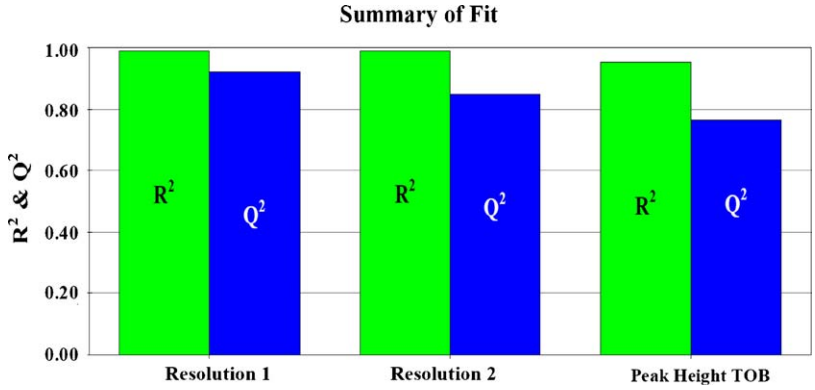

Fig. 3. Variations of the responses explained by the experimental design. The light shaded bars, R^2, denote the fraction of variation of the responses explained by the model and the dark shaded bars, Q^2, denote the fraction of variation of the responses that can be predicted by the model. Responses: resolution 1, resolution between kanamycin B and neamine; resolution 2, resolution between neamine and tobramycin; peak height TOB, peak height of tobramycin.

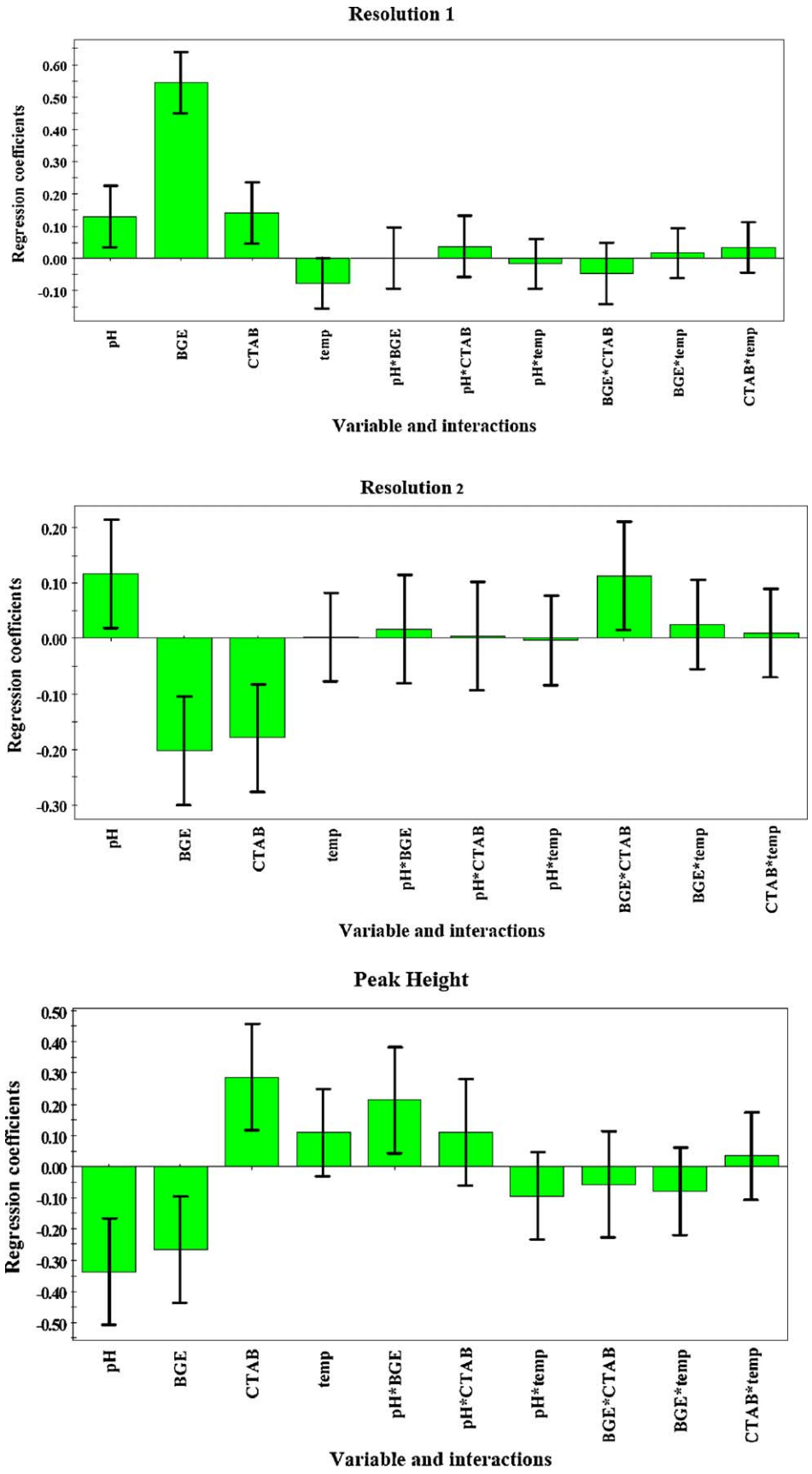

Fig. 4. Regression coefficient plots obtained from the optimization study. Rs1, resolution between kanamycin B and neamine and Rs2, resolution between neamine and tobramycin. pH, pH of the BGE; BGE, BGE concentration (mM); CTAB, CTAB concentration (mM) and temp, capillary temperature (°C).

center points, giving 19 experiments (i.e. 16 + the center point was replicated three times).

The responses investigated included the resolution between kanamycin B and neamine (Rs1), the resolution between neamine and tobramycin (Rs2) and the peak height of tobramycin (HT) in mV. With this it was concluded that all factors except capillary temperature had a significant effect on one or more responses at 95% confidence.

The statistical relationship between a response Y and the experimental variables X_i, X_j, ... is of the following form:

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \cdots + E \quad (1)$$

where the β's are the regression coefficients and E is the overall experimental error.

The linear coefficients, β_i and β_j, describe the quantitative effect of the experimental variables in the model. The cross coefficient β_{ij} measures the interaction effect between the variables i and j.

All the experiments were carried out in a random order. Multiple linear regression (MLR) of the program Modde 5.0 software (Umetri, Umeå, Sweden) was used to calculate quantitative relations between the responses and the factors.

The statistical significance of the variables and interaction terms was tested at a significance level of $\alpha = 0.05$.

3. Results and discussion

3.1. Method development—separation BGE

The buffer pH as well as the ionic strength influence the electrophoretic mobility of the analytes through the capillary. Hence the choice of the BGE constituents is crucial. Accordingly in the present work, the buffer constituents MES-monohydrate and L-histidine were used and CTAB was added to the mixture as an electroosmotic flow modifier in a concentration less than 1.3 mM, the critical micellar concentration (CMC).

The mixture of MES and L-His is used for keeping the background conductivity as low as possible. MES and L-His both produce the necessary pH buffering.

According to the aim of the study different combinations were prepared from the stock solutions and tested in order to define the best combination for getting good selectivity and sensitivity for tobramycin and its related substances. The relevant responses evaluated were: the sensitivity by peak height and resolutions between kanamycin B and neamine (Rs1) and between neamine and tobramycin (Rs2).

A series of experiments was carried out in order to find the optimal pH and concentration of MES, L-His and CTAB for determining tobramycin. A BGE containing the following combination: 25 mM MES and 0.3 mM CTAB, at pH 6.4 adjusted by adding L-His, was chosen because it gave the highest peak height and good resolution between kanamycin B and neamine and good resolution between neamine and tobramycin.

The pH influence on the resolutions between tobramycin and its related substances was such that at higher pH best resolutions for Rs1 and Rs2 were obtained, but at the same time lowest peak height and so less sensitivity.

Under the optimized conditions of reverse polarity (cathode at the injection capillary end) and pH 6.4, the migration order was as follows: nebramine, kanamycin B, neamine and tobramycin. These compounds differ in the degree of substitution (number of amino

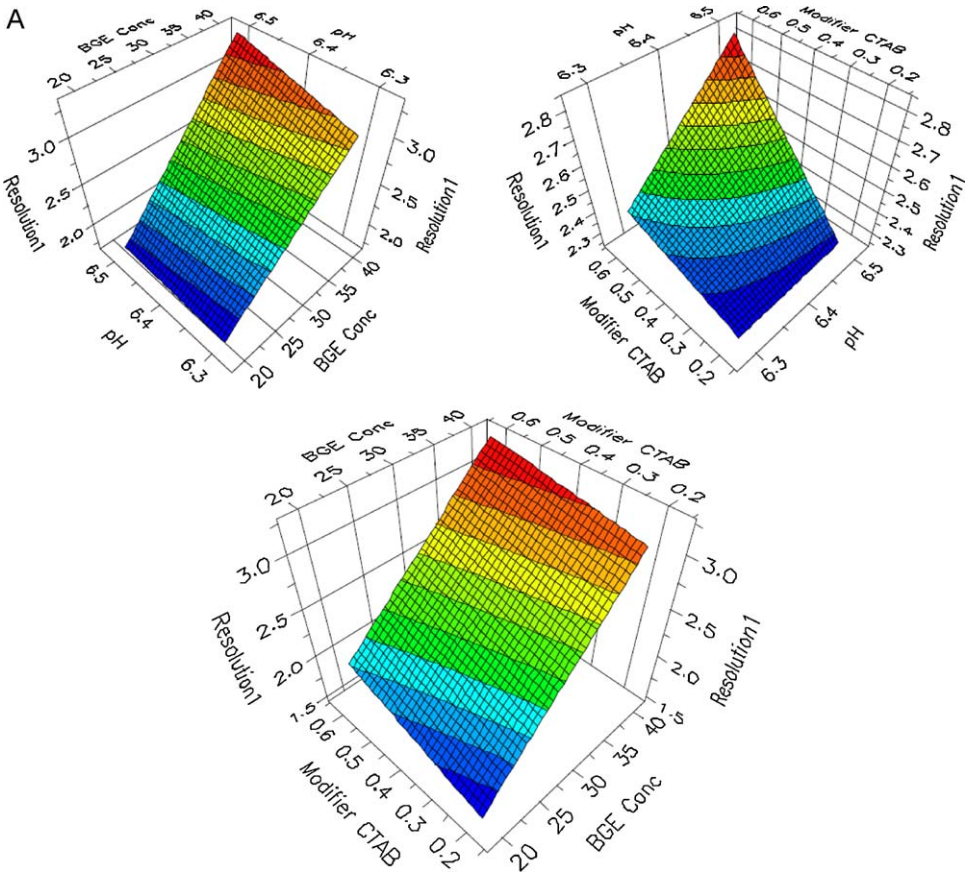

Fig. 5. (A) Response surface plot as a function of pH and BGE concentration (mM), pH and CTAB concentration (mM) and BGE concentration (mM) and CTAB concentration (mM) for Rs1. The other parameters are kept at their central values. (B) Response surface plot as a function of pH and BGE concentration (mM), pH and CTAB concentration (mM) and BGE concentration (mM) and CTAB concentration (mM) for Rs2. The other parameters are kept at their central value. (C) Response surface plot as a function of BGE concentration (mM) and CTAB concentration (mM), pH and CTAB concentration (mM) for peak height HT (mV). The other parameters are kept at their central values.

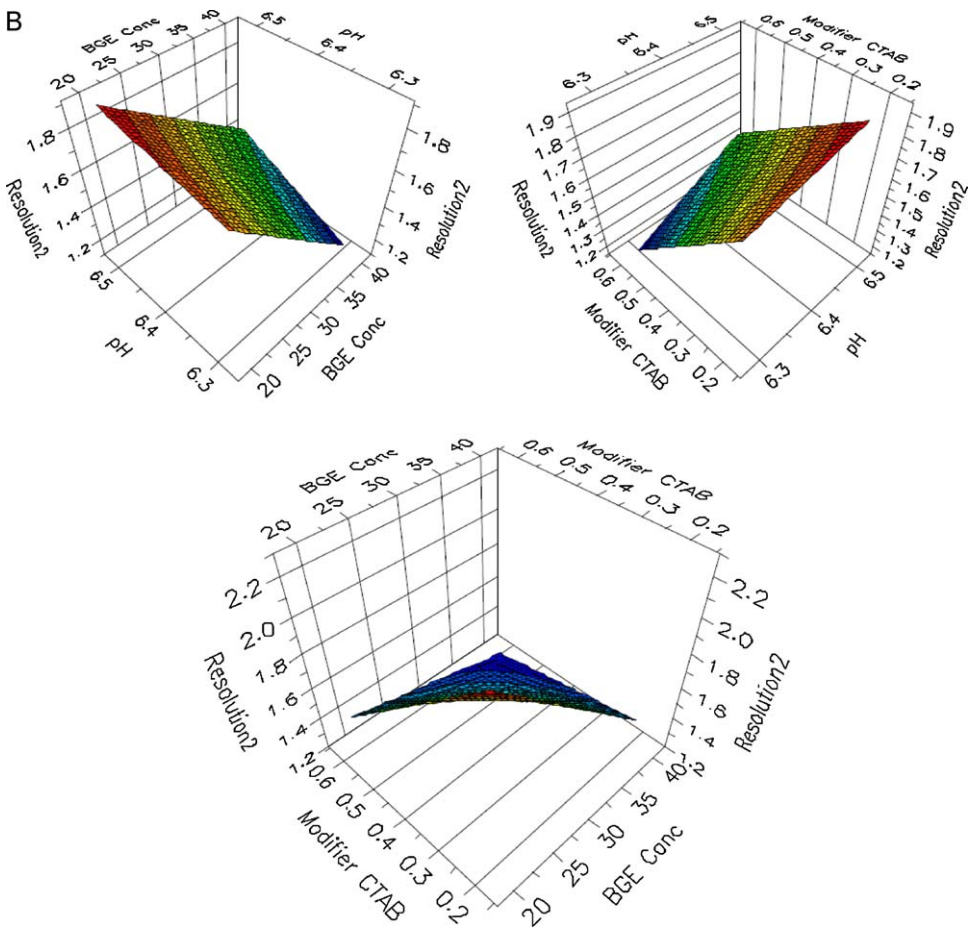

Fig. 5. (*continued*)

groups) which greatly affects the degree of compound ionization and thus the migration order. Tobramycin has five NH_2– groups in the molecular structure with pK_{a1} to pK_{a5} as 6.2, 7.4, 7.8, 8.3 and 8.9 [30]. Kanamycin B has five NH_2– groups with pK_{a1} to pK_{a5} as 5.79, 6.61, 7.66, 8.11 and 9.26. Neamine has four NH_2– groups in the molecular structure with pK_{a1} to pK_{a4} as 5.7, 7.6, 8.1 and 8.6.

Tobramycin migrates last because it has five protonated amino groups and one hydroxyl group less, nebramine migrates faster because it has four amino groups, one less than tobramycin and kanamycin B. The rank order of charge to mass ratio is TOB > neamine > kanamycin B > nebramine.

The separation voltage was optimized by plotting a curve of the generated current in function of the applied voltage. The linear range (where Ohm's law is valid) is the working range and the maximum voltage in this range (−30 kV) was adopted as the optimal separation voltage. To correct injection volume imprecision common in CE, an internal standard was used. Different compounds were tested during method development and finally ammonium acetate was chosen as internal standard at a concentration of 50 mg L^{-1}. Fig. 2 shows a separation obtained with the optimized parameters.

3.2. Method optimization

Performing the experiments according to a $2^k + n$ design as mentioned in Section 2.3, Table 1 produced the conclusion that most factors had a significant effect on the separation of one or more responses at 95% confidence.

A mathematical model was created, based on the total of 19 experiments. The variances of the responses were stabilized by a logarithmic transformation, which improved the model. Fig. 3 shows the fraction of variation of the responses explained by the model, R^2 and the fraction of variation of the responses that can be predicted by the model, Q^2. The possible values are in the range 0–1.0, with 1.0 revealing the existence of a model with an excellent predictive power. R^2 in our model was found to be between 0.99 and 0.96, and the values for Q^2 between 0.76 and 0.94.

The responses investigated included the resolution between kanamycin B and neamine (Rs1), resolution between neamine and tobramycin (Rs2) and the peak height of tobramycin (HT). The results expressed as regression coefficient plots are summarized in Fig. 4. These plots consist of bars, which are proportional to the regression coefficients. The bars denoted by one variable reflect the regression coefficient for the linear effect of that particular variable and the bars denoted by variable1 × variable2 the interaction between the two variables concerned. The 95% confidence interval is expressed in terms of an error line over the coefficient. When the interval includes zero, the variation of the response caused by changing the variable is smaller than the experimental error and the effect is considered to be not significant.

A positive regression coefficient stands for a positive effect on the responses studied, while a negative regression coefficient indicates a negative effect, as shown in Fig. 4. It can be observed that the pH of the BGE has a positive significant effect on Rs1, Rs2 and a negative significant effect on the HT of tobramycin. It means that increasing the pH of the BGE will increase Rs1, Rs2 and decrease the peak height of tobramycin causing less sensitivity. The concentration of the BGE was found to have a positive significant effect on Rs1 and negative significant effect on Rs2 and HT. This means that increasing the BGE concentration will improve Rs1 and

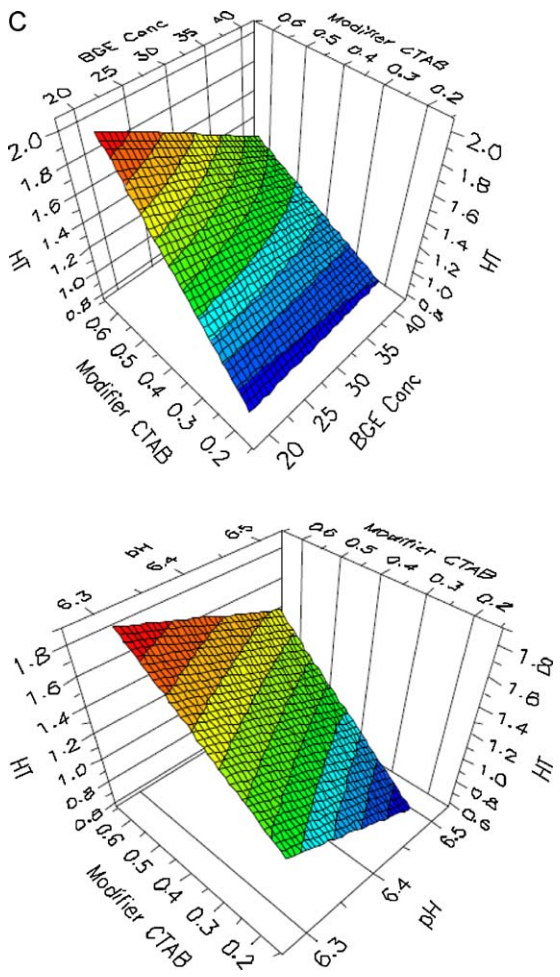

Fig. 5. (*continued*).

deteriorate Rs2 and HT. The concentration of CTAB was found to have a positive significant effect on Rs1, HT and a negative significant effect on Rs2. This means that increasing the CTAB concentration will improve Rs1 and peak height, but decrease Rs2. The capillary temperature was found to have no significant effect on Rs1, Rs2 and peak height. No significant interactions for Rs1, Rs2 and HT were found between the parameters studied, except a positive significant interaction for Rs2 between the concentration of BGE and CTAB and for peak height between pH and BGE concentration.

In order to better estimate the influence of the most important parameters on Rs1, Rs2 and HT, response surface plots were constructed. Fig. 5 shows the variation of Rs1, Rs2 and HT as a function of two significant parameters while the other parameters are kept constant at their central values. It is observed that in the range examined, the minimum resolution is 1.2 for Rs2. Therefore it can be concluded that selectivity is sufficient in the range examined. Hence the Rs1 will not be affected that much, and stay above 1.8. In order to get a good resolution for Rs2 that was considered as critical response, the factors were set to just below the center point value for the concentration of BGE and the concentration of CTAB. The pH was set to the center point level to compromise between resolutions 1, 2 and HT.

Based on those considerations, we define an optimal separation when the factors are 25 mM BGE at a pH of 6.4, an addition of 0.3 mM CTAB, 25 °C and −30 kV. This is adopted as optimized conditions for analyzing the commercial samples of tobramycin.

Table 2
Composition of commercial tobramycin and CRS expressed as is (% m/m).

Sample	Tobramycin	Kanamycin B	Neamine	Nebramine	Water content
CRS	91.6	ND	ND	ND	9.9
1	91.4	ND	ND	ND	9.5
2	91.6	ND	ND	ND	7.4
3	88.1	1.8	ND	ND	10.0

ND, not detected.

Electropherograms of the separations obtained by using these conditions are shown in Figs. 2 and 6.

3.2.1. Quantitative aspects

The precision, sensitivity and linearity of the method were evaluated as follows:

3.2.1.1. Sensitivity. For calculation, relative corrected peak areas were used. LOD and LOQ values correspond to 3 and 10 times the S/N. LOD and LOQ are expressed as percentage of a sample containing 1.0 g L^{-1} of tobramycin. Good sensitivity was observed, with LOD and LOQ estimated values of 0.4 mg L^{-1} (0.04% m/m) and 1.3 mg L^{-1} (0.1% m/m), these correspond to 9 pg and 31 pg, respectively.

3.2.1.2. Linearity for tobramycin. The optimized separation was obtained in less than 7 min with good linearity ($R^2 = 0.9995$) for tobramycin, with 9 concentration points injected in triplicate for the concentrations range from 1.5 to 1000 mg L^{-1}. Ammonium acetate was used as internal standard at 50 mg L^{-1}. For calculation, relative corrected peak areas were used.

The following regression equation was obtained: $y = 0.0131x - 0.0072$, $S_{yx} = 0.13$, where y: relative corrected peak area, x: concentration (mg L^{-1}), and S_{yx}: standard error of estimate. The intercept was found to be not statistically different from zero.

3.2.1.3. Repeatability for tobramycin. The system repeatability was performed by using the same sample, BGE and expressed as the relative standard deviation (% RSD) of the relative corrected peak areas, migration time and relative migration time. Intraday precisions were 0.2%, 0.6% and 0.5% ($n = 6$) respectively, and interday precisions were 0.7%, 0.8% and 0.8% ($n = 18$) respectively. The mean of migration time and relative migration time were 5.7 min, 3.5 ($n = 6$) and 5.7 min, 3.5 ($n = 18$) for intraday precision and interday precision respectively.

3.3. Application of the optimized CE-C^4D method for analysis of tobramycin in real samples

The water content in the tobramycin CRS and commercial samples was determined by Karl Fischer titration as shown in Table 2.

The optimized method was applied for the analysis of tobramycin and its related impurities in commercial bulk samples.

A solution of 0.2 g L^{-1} was investigated for each commercial sample. The content of each sample was determined by using Tobramycin CRS (91.6% as is) and expressed as percent content of the main compound. Data obtained were in the range of 88.1–91.6%. There are no related substances above the detection limit in two samples, but sample number 3 which was obtained from Chongqing Daxin Pharmaceutical Co. Ltd. (Chongqing, China) has around 1.8% of kanamycin B as shown in Fig. 6 (electropherogram 3).

Improvement in analysis time was obtained compared to the method of Kaale et al. [14] using UV detection after derivatization, which needs 30 min (10 min for derivatization plus 20 min for separation) and to the LC-PED method, which needs more than 20 min

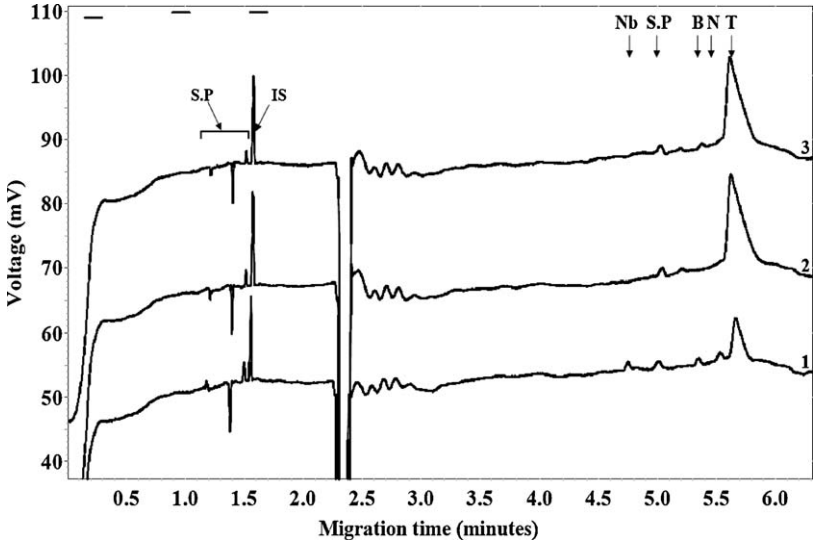

Fig. 6. Typical CE-C^4D electropherograms showing the separation of tobramycin from its impurities using CE; (1) samples dissolved in water: kanamycin B, nebramine and neamine 2.5 mg L^{-1} each and tobramycin 50 mg L^{-1}. (2) Tobramycin 0.2 g L^{-1} CRS and (3) commercial bulk sample, S.P, system peak; IS, internal standard (ammonium acetate 50 mg L^{-1}); B, kanamycin B; Nb, nebramine; N, neamine; T, tobramycin; CE conditions see Fig. 2.

for a two-step gradient for separation plus equilibration time of the PED instrument [7].

4. Conclusion

The goal of this work was the development, optimization and evaluation of a selective, simple and fast CZE method for the analysis of tobramycin and its related impurities in commercial samples.

A CZE method with C^4D detection was developed and validated for linearity, inter- and intra-day precision and sensitivity. Successful separation and good resolution between tobramycin and related substances were achieved. The described method yielded an effective improvement in simplicity and analysis time (7 min), which is important for routine application. The assay method was used to determine the content of commercial samples. The quantitative feature of this assay makes it a suitable assay for tobramycin.

This mode of detection can be useful for the analysis and assay of aminoglycosides such as tobramycin. It can also be a good alternative to derivatization in case of non UV-absorbing substances.

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