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Wai Siang Law[1, 2, 3]
Pavel Kubáň[3]
Ling Ling Yuan[1]
Jian Hong Zhao[2]
Sam Fong Yau Li[1]
Peter C. Hauser[3]

[1]Department of Chemistry, National University of Singapore, Republic of Singapore
[2]Singapore Institute of Manufacturing Technology, Republic of Singapore
[3]Department of Chemistry, University of Basel, Basel, Switzerland

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Research Article

Determination of tobramycin in human serum by capillary electrophoresis with contactless conductivity detection

A study on the determination of the antibiotic tobramycin by CE with capacitively coupled contactless conductivity detection is presented. This method enabled the direct quantification of the non-UV-absorbing species without incurring the disadvantages of the indirect approaches which would be needed for optical detection. The separation of tobramycin from inorganic cations present in serum samples was achieved by optimizing the composition of the acetic acid buffer. Field-amplified sample stacking was employed to enhance the sensitivity of the method and a detection limit of 50 μg/L (S/N = 3) was reached. The RSDs obtained for migration time and peak area using kanamycin B as internal standard were typically 0.12 and 4%, respectively. The newly developed method was validated by measuring the concentration of tobramycin in serum standards containing typical therapeutic concentrations of 2 and 10 mg/L. The recoveries were 96 and 97% for the two concentrations, respectively.

Keywords: Capillary electrophoresis / Capacitively coupled contactless conductivity detection / Field-amplified sample stacking / Tobramycin

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1 Introduction

Aminoglycoside antibiotics are used for the treatment of infections with susceptible Gram-negative microorganisms and, in combination with a β-lactam antibiotic, aminoglycosides can also be employed against infections of Gram-positive microorganisms [1]. Tobramycin (the structure is given in Fig. 1), a member of this group of antibiotics, was discovered in 1967 and is a product of *Streptomyces tenebrarius*. Tobramycin is also useful for the treatment of *Pseudomonas aeruginosa* in patients with cystic fibrosis. However, elevated levels of tobramycin can cause adverse or toxic reactions, such as nephrotoxicity and ototoxicity, and it is thus important to closely monitor the concentration of tobramycin in patients. The therapeutic concentrations in serum are in the range from 2 to 12 mg/L [1].

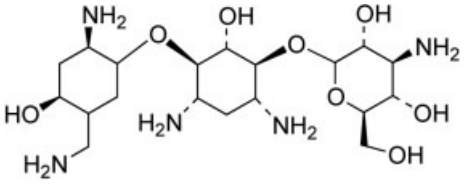

Figure 1. Chemical structure of tobramycin.

Analytical methods for the clinical analysis of tobramycin include enzymatic assays [2], immuno assays [3–7], and chromatography [8–15]. These methods either require a long incubation time, show poor sensitivity, or need relatively large amounts of sample. Commonly derivatization is required as tobramycin cannot be monitored by UV-absorption and the aminoglycosides are generally not well suited for HPLC because of their high hydrophilicity.

CE is an attractive alternative method for the analysis of drug components and for therapeutic drug monitoring due to its high resolution, the capability to analyze very small sample volumes, and its low cost of operation [16–18]. Several reports on the determination of tobramycin by CE have indeed been published [19–25]. As tobramy-

Correspondence: Professor Peter C. Hauser, Department of Chemistry, University of Basel, Spitalstrasse 51, CH-4004 Basel, Switzerland
E-mail: peter.hauser@unibas.ch
Fax: +41-61-2671013

cin has neither chromophore nor fluorophore it cannot be detected directly by UV-absorption or fluorescence measurements, the most common methods for CE. For this reason, precapillary derivatization to enable absorption detection, or indirect UV-detection, which works *via* the displacement of a charged dye, had to be employed [19–25]. Both of these approaches are not desirable. Chemical derivatization is a significant complication whereas indirect detection is suffering from unsatisfactory sensitivity. Two reports have also been presented on the analysis of tobramycin using amperometric detection with copper electrodes [21, 23].

Contactless conductivity detection has emerged over the last years as versatile new method for CE [26, 27]. In contrast to the optical methods of absorption and fluorescence, conductivity measurements can be considered universal in CE as all ionic species can be directly detected, and this eliminates the need for derivatization or indirect approaches. In our experience, contactless conductivity detection is also more reliable than amperometric detection [28]. For the determination of tobramycin, contactless conductivity detection therefore overcomes the disadvantages of the previously employed indirect optical and the amperometric detection methods [19–25].

The new detector is based on two external tubular electrodes placed side by side outside the capillary. The two electrodes serve to capacitively couple an excitation voltage into the solution inside the capillary and a resulting current, which is dependent on the conductivity of the solution, out to the detection circuitry. In conductivity measurements the signal is generated from the entire cell volume and not just the solution adjacent to the surface of an electrode and in the contactless approach the electrodes cannot deteriorate because they are not exposed to the solution. The method is therefore very robust. The design of the axial capacitively coupled contactless conductivity detection (C^4D) is simple because the electrodes are external and this imparts a high mechanical stability. Furthermore, the method is inexpensive. Although a relatively recent introduction, detailed studies of the influence of the cell design and its operating parameters on the performance of the C^4D have been carried out and its functioning is well understood [29–33]. Recent reviews, which give an overview of the reported applications of the C^4D, are available [34, 35]. The detection limits achieved are generally comparable to UV-detection, but the main advantage of contactless conductivity method is the determination of species which are not accessible by direct optical means. Herein, we propose a method which is suited for the routine analysis of the non-UV active tobramycin in clinical samples.

2 Materials and methods

2.1 Instrumentation

CE was conducted on a purpose-built instrument. Fused-silica capillaries (50 μm id and 360 μm od) were products of Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 60 cm and the effective length was 52 cm. A function generator (Model GFG 8019G, Goodwill Instrument, Taiwan) was employed to provide a sinusoidal excitation signal. The excitation frequency was set to 150 kHz and the amplitude was boosted to 350 V_{pp} (peak to peak) using a purpose-made amplifier. This signal was fed to the excitation electrode of 4 mm length and an internal diameter of approximately 400 μm. This electrode was separated by a gap of 1 mm from a second identical electrode which served to pick up the cell current. An operational amplifier (OPA655, Texas Instrument, Dallas, TX, USA) fitted with a feedback resistor of 2.2 MΩ was utilized to convert the cell current to an AC voltage. This raw output voltage was then rectified, offset, amplified, and low pass filtered before passing to the data acquisition system. More details on the C^4D used have been presented previously [36, 37]. Data were acquired and analyzed with a Maclab/4e system (AD Instrument, Hastings, UK).

2.2 Reagents and samples

All chemicals were either of analytical grade or reagent grade. Tobramycin sulfate salt and kanamycin A and B sulfate salt, were purchased from Sigma (St. Louis, MO, USA). Methanol, acetic acid, CTAB, sodium chloride, potassium chloride and ammonium chloride were obtained from Fluka (Buchs, Switzerland). Lithium chloride and magnesium sulfate heptahydrate were products of Merck (Darmstadt, Germany). COBAS-FP Tobramycin Calibrators were bought from Roche (Indianapolis, IN, USA). Water used throughout this experiment had a resistivity $\geq$18 MΩ and was obtained from a NANOpure ultrapure water purification system (Barnstead, IA, USA). Blank serum samples were obtained from the Clinical Chemistry Laboratory of the University Hospital Basel.

The running buffer consisted of 300 mM acetic acid and 75 μM CTAB unless stated otherwise. Tobramycin and kanamycin B (internal standard) stock solutions were prepared at a concentration of 1 mg in 1 mL of water, and are stable for at least 1 year at 4°C. Working standard solutions for the calibration curves were made with concentrations ranging from 5 to 0.06 mg/L. Individual cation stock solutions (Na^+, Li^+, Ca^{2+}, Mg^{2+}, and K^+) were prepared with a concentration each of 1 mg/mL. The tobramycin and kanamycin B stock solutions were mixed with

cation stock solutions and further diluted with H_2O to obtain the desired concentrations for the test solutions. All solutions were prepared daily and were filtered with 0.20 μm Minisart filters (Göttingen, Germany). Serum samples (with internal standard added) were deproteinized by adding 500 μL of methanol and then 400 μL of water to 100 μL of serum (or as stated otherwise). After vortexing for 30 s, the mixture was then centrifuged at 4000 rpm for 5 min. The supernatant was ready for injection [38].

2.3 Separation procedure

Capillaries were rinsed with 0.1 M NaOH (3 min), followed by water (5 min), and running buffer (15 min), respectively, before commencing experiments every morning or when peak distortion and poor peak shapes were observed. The capillary was flushed with running buffer for 2 min between runs. Normal injection was performed by siphoning at 10 cm height difference for 10 s. When sample stacking was employed, the following procedure was used. A plug of water/methanol mixture was introduced into the capillary by siphoning at 10 cm for a duration of 150 s or as stated otherwise. This was followed by electrokinetic sample injection at 10 kV positive polarity at the injection end for 120 s or as stated otherwise. After sample introduction, a positive separation voltage of 20 kV was applied.

3 Results and discussion

3.1 Composition of the BGE solution

The pK_as of the amine groups of the aminoglycoside antibiotics range from about 7 to about 11; hence, it is best to use an acidic buffer to achieve full protonation and highest sensitivity in the detection of the species. The use of a buffer with low pH-value also leads to a relatively low EOF, thereby increasing the residence time and resolution, prevents adsorption of the analyte (as cation) on the inner surface of the capillary, and facilitates the stacking process (see below for details). Good results had previously been obtained for CE-C^4D with a BGE solution consisting solely of acetic acid [39, 40]. For conductivity detection, the analytical signal largely arises from the difference in limiting equivalent conductivity between the analyte ion and the buffer ions of the same charge as the latter are displaced by the analyte zone. For solutions of acetic acid the only type of cation present is the proton which leads to a high sensitivity because of the high conductivity of this ion. Low pH-values can be achieved by employing relatively high concentrations of acetic acid.

The effect of the concentration of acetic acid between 100 and 500 mM (pH 3 and 2.5, respectively) on the determination of tobramycin was thus studied. The results are shown in Fig. 2. In order to simulate the contents of serum, the five alkali and alkaline earth metal cations which are present at significant concentrations in serum, namely Na^+, Li^+, Ca^{2+}, Mg^{2+}, and K^+, were added to the standard solutions. Besides tobramycin also kanamycin A and B were added to the standard solution as these other aminoglycoside antibiotics with similar structure and properties were considered for use as internal standards for quantification. As depicted in Fig. 2, the relatively large organic species show fast migration times comparable to the small inorganic cations of high electrophoretic mobility. This must be due to the multiple charges giving the organic species a high mobility. At the relatively low concentration of 100 mM of the acetic acid, there is no separation between kanamycin A and Ca^{2+} and the resolution R between tobramycin and kanamycin B was approximately 1, which is not quite adequate for quantification. Overall, better resolution could be achieved with an increase in the concentration of acetic acid (decrease of the pH-value). It appears that the acid

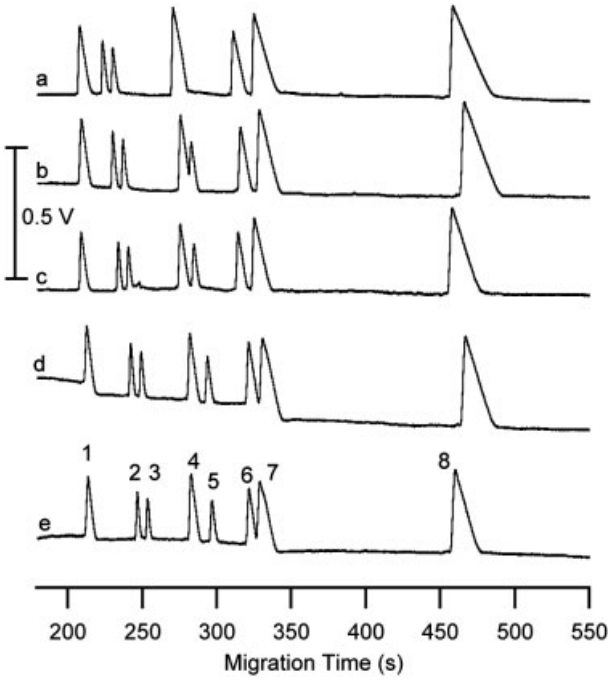

Figure 2. Electropherograms of the separation of tobramycin, kanamycin A and B and five cations all at 25 mg/L by CE-C^4D at different concentrations of acetic acid: (a) 100 mM, (b) 200 mM, (c) 300 mM, (d) 400 mM, and (e) 500 mM. Hydrodynamic injection at 10 cm height difference for 10 s. Separation voltage: 20 kV. L_{tot} = 60 cm, L_{effec} = 52 cm. Peak identifications: (1) K^+, (2) tobramycin, (3) kanamycin B, (4) Ca^{2+}, (5) kanamycin A, (6) Mg^{2+}, (7) Na^+, (8) Li^+.

has an effect on the apparent mobility of the antibiotics (possibly through ion-pairing) but not of the metal cations and thus an improvement of the separation is achieved. At a concentration of 300 mM acetic acid separation is possible, and the *R*-value between tobramycin and kanamycin B was determined as 1.4. Kanamycin B was chosen as internal standard for the further experiments.

The detection limit for tobramycin was found to be approximately 5 mg/L which does not quite provide adequate sensitivity to quantitatively determine tobramycin in real serum samples. Thus, an on-line electrophoretic preconcentration method, namely field-amplified sample stacking (FASS), was adopted in order to achieve the low limits of detection required for clinical analysis (details on the procedure see below). As the approach chosen requires a slow EOF toward the anode, a small amount of CTAB was added to the buffer. Three levels of CTAB, 50, 75, and 100 μM, were investigated and the best separation was achieved with a BGE solution consisting of 300 mM acetic acid and 75 μM CTAB. The resulting electropherogram is shown in Fig. 3a. As evident from a comparison of Figs. 2, 3a, the reversal of the EOF by addition of the modifier leads to a delay of the peaks for all cations and the determination of tobramycin and kanamycin B is possible without overlaps with an *R*-value of 2.5. Note that when using conductivity detection it is not possible to measure the EOF directly by addition of a neutral marker compound, as commonly done for optical detection methods.

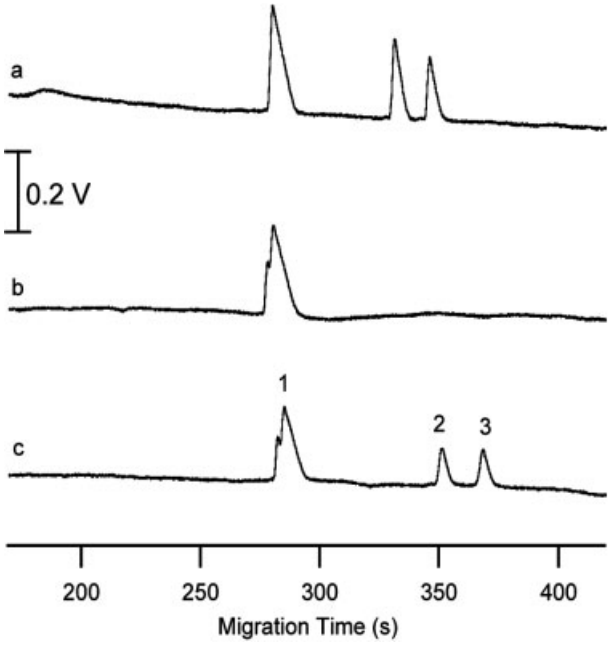

Figure 3. Electropherograms of the separation of tobramycin and kanamycin B in (a) standard solution, (b) blank serum, (c) serum spiked with 15 mg/L both substances. Buffer: 300 mM acetic acid, 75 μM of CTAB. Other conditions as for Fig. 2. Peak identifications: (1) K^+, (2) tobramycin, and (3) kanamycin B.

3.2 Detection in serum

In first tests with spiked samples it was found that tobramycin is not detected when measured directly in serum. Presumably this is due to binding to serum proteins. The determination of tobramycin is indeed usually carried out after precipitation of serum proteins by addition of an organic solvent [38]. At first, a ratio of serum to methanol of 10:30 was investigated, but it was found that the recovery was low (≈60%). The recovery was calculated by comparing the relative peak area of tobramycin in serum with the relative peak area of tobramycin measured in an aqueous standard solution. Best results in terms of recovery were obtained for a ratio of serum to methanol of 10:50 and subsequent addition of water to obtain a total dilution factor for serum of 10. The recovery of tobramycin at 20, 15, and 10 mg/L was 85, 80, and 70%, respectively. For kanamycin B the corresponding values were determined as 90, 85, and 74%, respectively. This indicates that kanamycin B can indeed be used as internal standard as the recoveries for the two species are very similar. Typical electropherograms of a blank serum and serum spiked with 15 mg/L of tobramycin and kanamycin B are shown in Fig. 3b and c, respectively. As evident, for serum no additional peaks are observed across the window for tobramycin and kanamycin B, but note the small shoulder caused by an unknown constituent of serum on the peak for potassium. Serum samples from four different healthy volunteers were investigated and the peak patterns were all similar to the ones shown in Fig. 3b.

3.3 FASS

FASS was first used in CE by Mikkers *et al.* [41] and intensively studied by Chien and Burgi [42]. Briefly, this method is based on the injection of a relatively large volume of sample to boost the sensitivity. This is possible because the ionic analytes are electrokinetically concentrated at the boundary between two zones of high and low conductivity created by the prior injection of a plug of solvent without electrolyte. The zone of low conductivity is subsequently removed from the capillary at the injection end by the reversed EOF and thus does not interfere in the further separation and detection of the analytes. The protocol followed for this work is detailed in Section 2.

Initially stacking was attempted using pure water as preinjection plug. However, it was found that the resolution between kanamycin B and Ca^{2+} was not adequate under

these conditions. This could be improved by employing a water–methanol mixture rather than pure water. As is illustrated in Fig. 4, the separation is dependent on the ratio of water to methanol. Note also the presence of a small unidentified peak for the serum sample, evident in traces (c) to (e) of Fig. 4, on the shoulder of the Ca^{2+}-peak, which could also interfere if the resolution is not adequate. The quantitative data is given in Table 1. For a 70:30 ratio of water to methanol best compromise conditions in terms of peak height (highest stacking efficiency at 20-fold) and resolution were obtained. At a mixing ratio higher than this, the enhancement factor decreased while a still slightly increased resolution could be observed. The improved peak separation is presumably due to an effect of the methanol content on the EOF, which is generally suppressed in the presence of organic solvents.

The size of the preinjection plug also has an effect on the peak shape and hence on the detection limits and the resolution [43–45]. Electropherograms obtained when varying the time for the hydrodynamic injection of the solvent plug between 120 and 240 s are shown in Fig. 5 and the quantitative data is given in Table 2. Considering both, the resolution and enhancement factor, an injection time of 150 s for the solvent plug was adopted for the remaining part of this study.

With FASS the amount of sample injected into the capillary, as controlled by the duration of the electrokinetic injection, principally determines the enhancement factor.

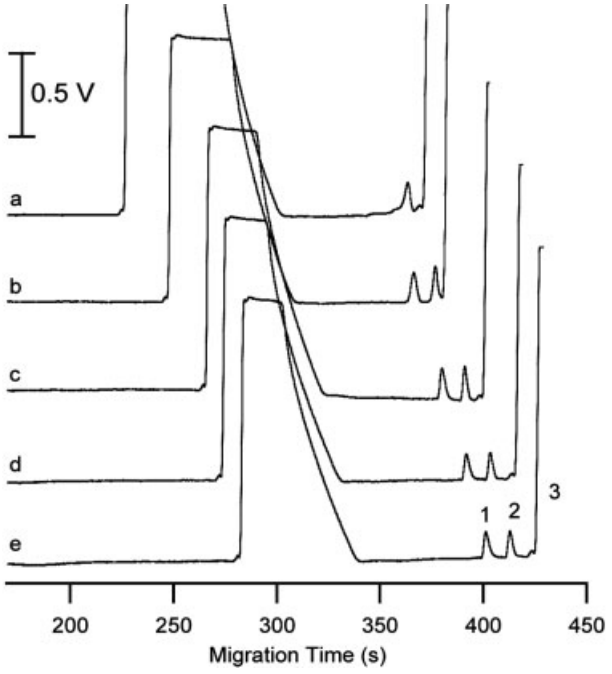

Figure 4. Effect of the composition of the solvent plug used in FASS on the resolution and sensitivity enhancement for serum spiked with tobramycin and kanamycin B. H_2O:MeOH ratios: (a) pure water, (b) 80:20, (c) 70:30, (d) 60:40, and (e) 50:50. Buffer: 300 mM acetic acid, 75 μM CTAB. Solvent plug was hydrodynamically injected from a height of 10 cm for 120 s. Sample was injected electrokinetically at 10 kV for 120 s. Concentration of tobramycin and kanamycin B: 0.5 mg/L; concentration of inorganic cations: 25 mg/L. Other conditions as for Fig. 2. Peak identifications: (1) tobramycin, (2) kanamycin B, and (3) Ca^{2+}.

Table 1. Effect of the composition of the solvent plug on resolution and enhancement factor

Composition (H_2O:MeOH)	R_1	R_2	Enhancement factor
Pure water	–	–	15
80:20	1.9	1.1	18
70:30	2	2	20
60:40	2.1	2.3	17
50:50	2.1	2.5	16

R_1 = resolution between tobramycin and kanamycin B.
R_2 = resolution between kanamycin B and Ca^{2+}.

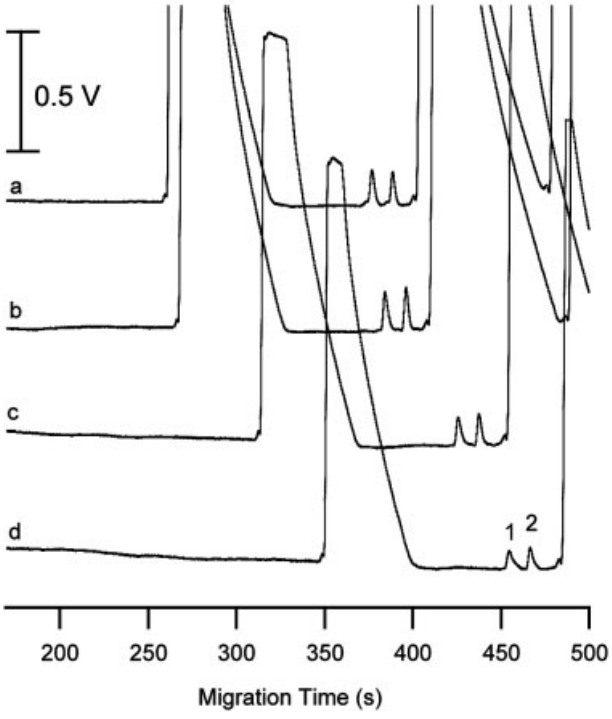

Figure 5. Effect of the size of the solvent plug on resolution and sensitivity enhancement for serum spiked with tobramycin and kanamycin B. Solvent plug (H_2O:MeOH, 70:30) was hydrodynamically injected from a height of 10 cm for different durations: (a) 120 (b) 150, (c) 180, and (d) 240 s. Sample was injected electrokinetically at 10 kV for 120 s. Other conditions as for Figs. 2, 4. Peak identifications: (1) tobramycin, (2) kanamycin B.

Table 2. Effect of the duration of the injection of the solvent plug on resolution and enhancement factor

Duration of injection (s)	R_1	R_2	Enhancement factor
120	1.8	2	20
150	2	2	25
180	2.1	2.1	18
240	1.9	2.3	13

R_1 = resolution between tobramycin and kanamycin B.
R_2 = resolution between kanamycin B and Ca^{2+}.

A plot of this parameter against different injection times between 90 and 150 s is given in Fig. 6. As can be seen in the figure, for an injection time of 150 s for the sample the sensitivity was boosted by factor of close to 40. The enhancement factors could be reproduced to 2.0, 2.4, 3.1, and 2.1% for repeated injections of the same solution for injection times of 90, 120, 135, and 150 s, respectively (RSDs, n = 3). However, the injection of an excessive amount of sample can lead to peak broadening and thus to inadequate resolution as is also illustrated in Fig. 6. For the highest enhancement factor of 40 the resolution between the internal standard kanamycin B and Ca^{2+} was not adequate for quantitation. Thus, an injection time of 120 s for the sample was adopted as best compromise condition.

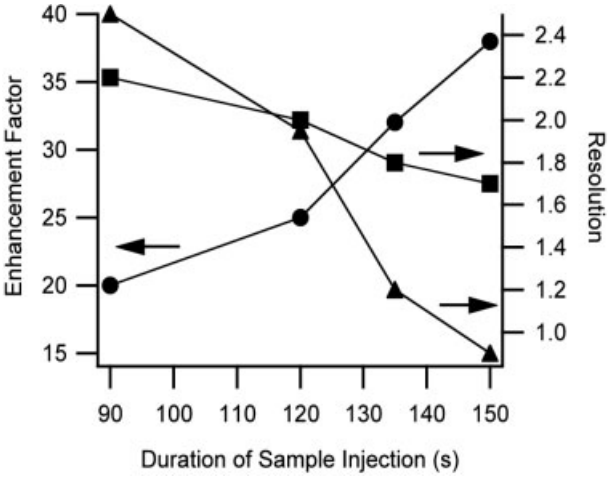

Figure 6. Effect of the duration of the sample injection on resolution and sensitivity enhancement for serum spiked with tobramycin and kanamycin B. Solvent plug (H_2O:MeOH, 70:30) was hydrodynamically injected from a height of 10 cm for 150 s. Other conditions as for Figs. 2, 5. (●): Enhancement factor for tobramycin; (■) resolution between tobramycin and kanamycin B; (▲) resolution between kanamycin B and Ca^{2+}.

3.4 Method validation

Under optimal separation and stacking conditions, the sensitivity was enhanced by a factor of 25 in comparison with normal hydrodynamic injection. The LOQ using an S/N criterion of 10, was determined as 0.1 mg/L whereas the LOD based on S/N of 3, was 50 μg/L (referring to the concentrations in the untreated sample). The linearity of the detector response was within the range from 3 to 0.1 mg/L when employing kanamycin B as internal standard. The correlation coefficient of the calibration plot was 0.9993. The RSDs of the corrected peak areas and migration times measured over the course of a working day (intraday precision) were 4 and 0.12%, respectively (n = 6). The measurements could be reproduced to a SD of 6.3 and 0.4% over the course of three consecutive days (n = 3) for peak area and migration time, respectively.

The present method was then verified by measuring the concentrations of tobramycin in certified standard solutions which are composed of a human serum matrix. Two calibration solutions with certified concentrations of 10.0 and 2.00 mg/L, which encompass the usual therapeutic range of tobramycin in serum, were tested. A concentration of 9.7 ± 0.2 mg/L was determined for the standard of 10 mg/L and a value of 1.92 ± 0.04 mg/L was obtained for the standard of 2 mg/L employing kanamycin B as internal standard (confidence limits for n = 3). The recoveries for the two certified standards were thus 97 and 96% for the two concentrations, respectively. The electropherogram for one of the solutions is given in Fig. 7.

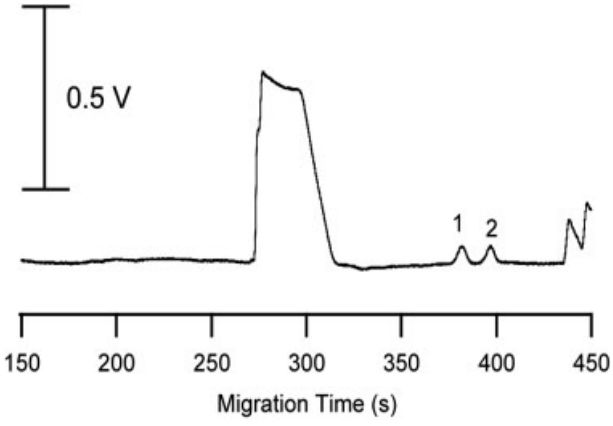

Figure 7. Electropherogram for a certified serum standard (2.0 mg/L of tobramycin) spiked with kanamycin B as internal standard. Sample was injected electrokinetically at 10 kV for 120 s. Other conditions as for Figs. 2, 6. Peak identifications: (1) tobramycin and (2) kanamycin B.

4 Concluding remarks

A member of the aminoglycoside antibiotics, tobramycin, was successfully determined in a serum matrix by using CE with capacitively coupled conductivity detection. This could be achieved by direct detection without the derivatization needed for optical methods. It is hoped that this approach will prove to be useful not only for the other antibiotics of the same class but also for the determination of further non-UV-absorbing species in clinical samples.

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