ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 809–816

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

The analysis of aminoglycoside antibiotics by capillary electrophoresis

Cheryl L. Flurer

National Forensic Chemistry Center, US Food and Drug Administration, 1141 Central Parkway, Cincinnati, OH 45202, USA

Received for review 19 October 1994; revised manuscript received 28 December 1994

Abstract

The analyses of aminoglycoside antibiotics by capillary electrophoresis utilizing borate complexation and direct UV detection are discussed. Twelve aminoglycosides were studied and separated to demonstrate identification capabilities, with migration time RSDs from 0.21 to 0.44% ($n = 6$) for individual components. This buffer system permitted the detection of minor impurities such as precursors or closely related fermentation products. Quantification of dihydrostreptomycin and streptomycin was accomplished in 160 mM sodium tetraborate decahydrate with linearity over the range 0.050–1.0 mg ml^{-1}. Determination of the purity of bulk dihydrostreptomycin was possible by the addition of the cationic surfactant myristyltrimethylammonium bromide. This reversed the electroosmotic flow, thereby reversing the migration order, and causing the streptomycin impurity to migrate before the dihydrostreptomycin main peak. Quantification was also demonstrated with the closely related compounds amikacin, bekanamycin, kanamycin A, and tobramycin, using sisomicin as an internal standard. The reproducibility of the method was typically 2–3% over 1 day, and 2% day-to-day. These studies illustrate the use of capillary electrophoresis for the identification and quantification of selected aminoglycosides as potential alternative methods to the assays given by the US Pharmacopeia.

Keywords: Aminoglycoside antibiotics; Borate complexation; Capillary electrophoresis; Potency determination

1. Introduction

The class of antibiotics known as aminoglycosides encompasses compounds that exhibit activity against both Gram-negative and Gram-positive organisms. Some of these antibiotics, such as gentamicin, kanamycin A and bekanamycin, and butirosin A and B, represent a complex of closely related fermentation products. Others are derivatives of fermentation products, e.g. dihydrostreptomycin and amikacin are derivatives of streptomycin and kanamycin A, respectively. Under certain circumstances, determination of product purity may be necessary.

Because most of these antibiotics lack chromophores, US Pharmacopeia (USP) required the comparison of either IR spectra or thin-layer chromatographic banding patterns between a standard and the pharmaceutical preparation for identification. Quantification or potency determination typically requires the utilization of time-consuming microbial assays. Unfortunately, none of these approaches allows the ready visualization of minor impurities. Alternative methods of analysis have included liquid chromatography (LC) with either pre-column derivatization [1–6] or electrochemical detection [7], ion-pair chromatography [8–10], agarose gel electrophoresis [11],

SSDI 0731-7085(95)01502-7

immunoassay [12,13], and mass spectrometry [14,15].

The first major work on the use of capillary electrophoresis (CE) in the study of aminoglycoside antibiotics utilized indirect detection at low pH under reversed polarity conditions [16]. The results indicated that good sensitivity and selectivity could be achieved, but that some of the closely related species could not be separated. Studies conducted by Hoffstetter-Kuhn et al. [17] utilized the formation of negatively charged complexes between carbohydrates and borate for direct UV detection. The magnitude of the negative charge, and thus the electrophoretic behavior of the complex, was dependent on the stability of the complex. Based on the equilibria involved, and a constant amount of carbohydrate, the concentration of the complex and the detection sensitivity increased with the borate concentration [17]. The stability of the complex depended on the structure of the carbohydrate, the number of hydroxyl groups, and the presence of substituents [17].

The work presented in this paper utilized borate buffers which allowed the direct detection of aminoglycoside antibiotics, and in some cases emphasized minor differences among the components of a particular compound, through complexation with their hydroxyl moieties. These studies illustrate the use of CE for the identification and quantification of selected aminoglycosides, and suggest potential alternative methodologies to those required by current USP protocols.

2. Experimental

2.1. Reagents

The sulfate salts of amikacin, bekanamycin, butirosin, dibekacin, dihydrostreptomycin, gentamicin, kanamycin, paromomycin, ribostamycin, sisomicin, streptomycin, and tobramycin, as well as individual components butirosin A and butirosin B, were obtained from Sigma (St. Louis, MO, USA). Representative structures are shown in Fig. 1. Additional standards of amikacin, bekanamycin, dihydrostreptomycin, kanamycin A, streptomycin, and tobramycin were obtained from Calbiochem (La Jolla, CA, USA), Fluka Chemical Corp. (Ronkonkoma, New York, USA), ICN Biomedicals, Inc. (Costa Mesa, CA, USA), and/or United States Biochemical (Cleveland, OH, USA). Myristyltrimethylammonium bromide (TTAB) was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade. Distilled, deionized water was obtained in the laboratory from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Buffers were filtered through 0.2 μm Nylon 66 filters (Alltech Associates, Inc., Deerfield, IL, USA), and were degassed under aspirator vacuum. Uncoated fused silica capillaries of 50 μm i.d. and 360 μm o.d. were purchased from Polymicro Technologies (Phoenix, AZ, USA), and Isco, Inc. (Lincoln, NE, USA).

2.2. Instrumentation

The Isco Model 3140 Electropherograph was utilized in these studies, and data collection and processing were accomplished with the Isco Capillary Electrophoresis software package. Peak areas were normalized with respect to their migration times during data processing. Samples were introduced into the capillary by vacuum injection at 25.0 kPa s. Separations were accomplished at 34 °C in capillaries with lengths of either 90 cm (65 cm to detector) or 70 cm (45 cm to detector), with direct UV detection at 195 nm. The capillary was rinsed with the appropriate buffer for 15 min at the start of the day, and 4 min before sample injection.

2.3. Sample analyses

Stock solutions of antibiotics were prepared in distilled water, and were diluted as required with either distilled water or sodium tetraborate decahydrate (borate) buffer.

Butirosin A and B

Injected sample solutions were prepared in 75 mM borate. Separation was accomplished in 150 mM borate at +18 kV, using a 70 cm capillary.

Streptomycins

The separation of streptomycin and dihydrostreptomycin required a 90 cm capillary. Stock solutions were diluted with distilled water. Analyses in the anodic mode (detector at cathode) required a 160 mM borate buffer and a voltage of +18 kV. Separations in the cathodic mode were accomplished in a borate (75 mM)–TTAB (0.5 mM) buffer at −18 kV.

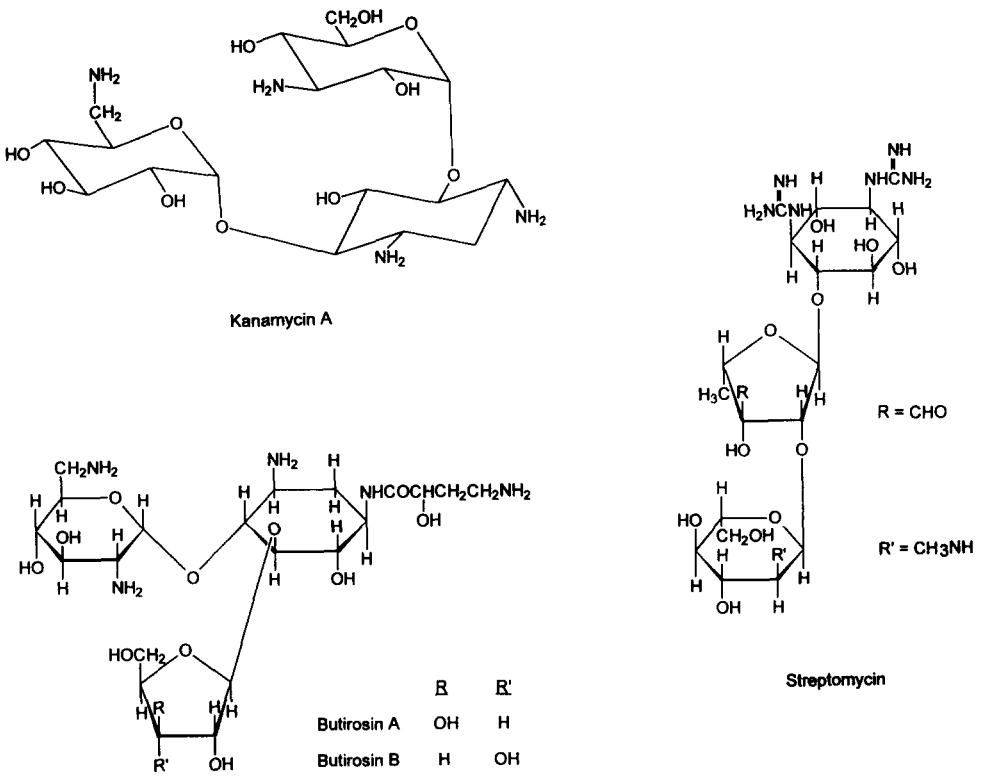

Fig. 1. Structures of representative aminoglycoside antibiotics.

Calibration standards were prepared by diluting a stock mixture of streptomycin and dihydrostreptomycin to produce five standard solutions of 1.0, 0.50, 0.25, 0.10, and 0.050 mg ml^{-1}. Four repetitions were performed at each concentration level. The declared potencies of the standards used were 766 units of base mg^{-1} (streptomycin) and 744 $\mu g\ mg^{-1}$ (dihydrostreptomycin). Solute concentrations were converted to $\mu g\ ml^{-1}$ according to potencies and were plotted against normalized peak area.

In order to determine the residual quantity of streptomycin present in bulk dihydrostreptomycin preparations, a stock solution of dihydrostreptomycin was diluted to 1.0 mg ml^{-1} and was analyzed as a 0% (w/w) spike. Standard additions of a 1.0 mg ml^{-1} streptomycin solution were made to produce approximately 1%, 2%, 4%, and 6% (w/w) streptomycin spikes, and duplicate injections were done. The concentration of added streptomycin was calculated in terms of its potency, and plotted against the average normalized peak area of the corresponding peak. The x-intercept, in $\mu g\ ml^{-1}$, was used in the calculation of the (w/w) percentage of residual streptomycin in the dihydrostreptomycin preparation.

Kanamycins

Analyses were accomplished at +15 kV in a 70 cm capillary, which permitted run times of 15 min or less. The buffers used in potency determinations were 185 mM borate for tobramycin and 175 mM borate for amikacin, bekanamycin, and kanamycin A. Final concentrations of the analytes were 0.50 mg ml^{-1} amikacin and 2.0 mg ml^{-1} for bekanamycin, kanamycin A, and tobramycin.

Injected samples were prepared in 140 mM borate, using sisomicin at a concentration of 0.48 mg ml^{-1} as an internal standard. Four repetitions each of standard and solute determinations were done per set, with four to eight sets of determinations per solute. Each set of analyses utilized fresh buffer solution, standard–sisomicin, and sample–sisomicin dilutions. Potencies were calculated by comparing the ratios of the normalized peak areas of the standard–sisomicin to the solute–sisomicin. The standard for one solute was chosen from one of the five suppliers listed above, and the remaining solutes were analyzed in order to compare the calculated potencies with those declared by the suppliers. Day-to-day method reproducibility was evaluated by averaging the

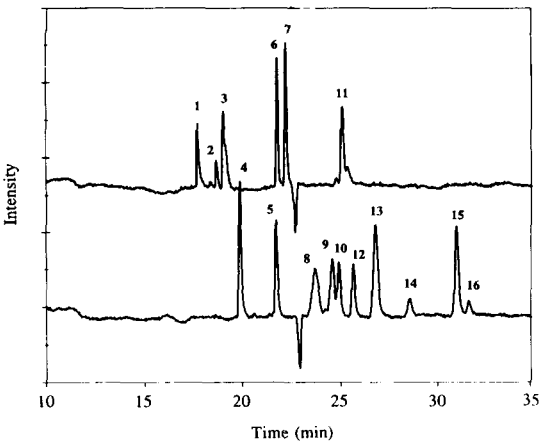

Fig. 2. Electrophoresis separation of aminoglycoside antibiotic standards. Peak identification, component concentrations and migration times are found in Table 1. Running buffer, 185 mM borate (pH 9); sample buffer, 140 mM borate (pH 9). Other conditions are given in the text.

potency values of all sets of analyses for each solute.

3. Results and discussion

Fig. 2 shows the separation of 12 antibiotics. As will be discussed, the separation conditions chosen were not optimal for all compounds, but were meant to demonstrate the feasibility of general aminoglycoside screening. As was the case with gentamicin (peaks 1–3) [18] and butirosin (peaks 13 and 14), the utilization of the borate buffer system permitted the resolution of individual components within a complex. This separation also suggested that other compounds are present in the paromomycin (peak 11) and ribostamycin (peaks 15 and 16) standards used.

One drawback concerning the use of borate complexation for UV detection is that absorptivity is dependent upon the strength of the complex. In the work presented by Ackermans et al. [16] utilizing indirect detection with imidazole, all solutes yielded similar detector responses. The concentrations of solutes in Fig. 2 varied from 2.0 mg ml^{-1} for the gentamicin complex to 0.10 mg ml^{-1} dihydrostreptomycin, as given in Table 1. Table 1 also lists average migration times for six determinations, with relative standard deviations (RSDs) between 0.21 and 0.44%.

Neomycin was also studied in these borate buffer systems. Under the conditions used, it migrated at or near the electroosmotic flow (EOF) marker and could not be analyzed reliably. Therefore, no further experiments were conducted with neomycin.

3.1. Butirosin

Although this antibiotic is not marketed in the United States, it was an interesting demonstration of the ability to separate *trans*- versus *cis*-1,2-diols (Fig. 1) based on differences in their complexation equilibria with the borate ion [17]. Fig. 3 is an example of a butirosin standard that is supplied as a mixture of approximately 85% A and 15% B. The relative

Table 1
Migration times of aminoglycoside antibiotics

Peak	Component	Conc. (mg ml^{-1})	Migration time (min)
1	Gentamicin C_1		17.88 ± 0.04
2	Gentamicin C_{1a}	2.0	18.88 ± 0.04
3	Gentamicin $C_2 + C_{2a}$		19.22 ± 0.05
4	Sisomicin	0.30	19.90 ± 0.07
5	Dibekacin	0.79	21.75 ± 0.08
6	Dihydrostreptomycin	0.10	21.98 ± 0.05
7	Streptomycin	0.10	22.44 ± 0.06
8	Tobramycin	0.97	23.8 ± 0.1
9	Amikacin	0.20	24.6 ± 0.1
10	Bekanamycin	0.50	25.0 ± 0.1
11	Paromomycin	1.0	25.4 ± 0.1
12	Kanamycin A	0.48	25.7 ± 0.1
13	Butirosin A	0.33	26.9 ± 0.1
14	Butirosin B	0.06	28.7 ± 0.1
15 + 16	Ribostamycin	0.97	31.1 ± 0.1
			31.7 ± 0.1

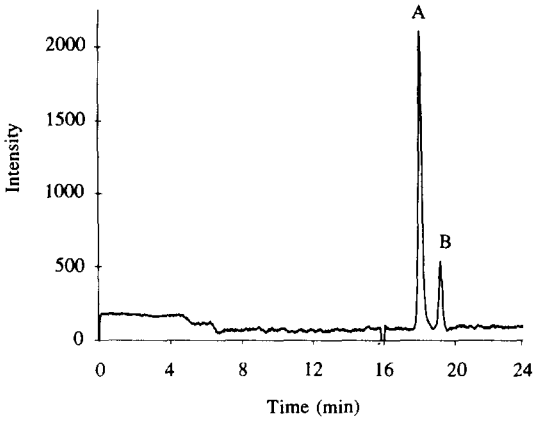

Fig. 3. Analysis of a 1.82 mg ml^{-1} butirosin disulfate standard, labeled as approximately 85% A and 15% B. Experimental conditions are as given in the text.

peak areas of four repetitions gave values of 85.4% A and 14.6% B. Because butirosins A and B are products of the same fermentation process, it is reasonable to assume that cross-contamination of the individual purified standards may occur. Fig. 4(A) is an example of a butirosin A standard that is declared >95% pure; the quantity of butirosin B impurity determined using this CE method was 3.5%. The butirosin B standard shown in Fig. 4(B) was declared 70% pure. Interestingly, no butirosin A was detected, but additional unidentified species were present.

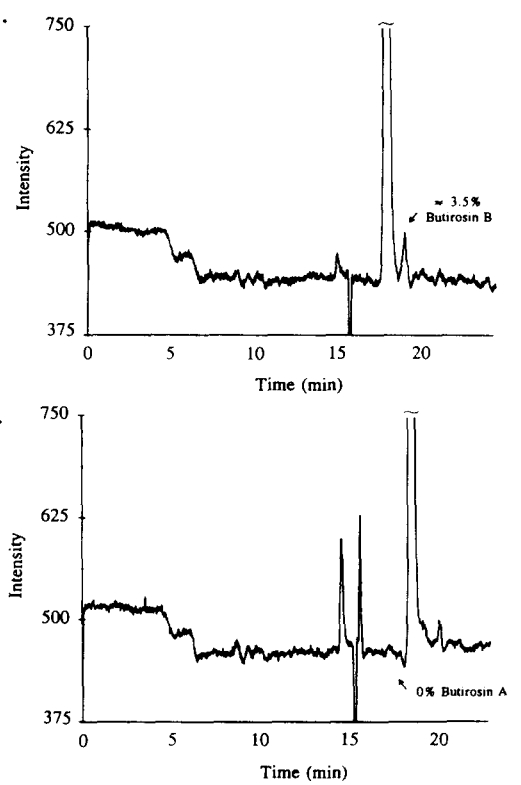

Fig. 4. (A) Analysis of a 2.50 mg ml^{-1} butirosin A standard, labeled as approximately 95% A. (B) Analysis of a 2.50 mg ml^{-1} butirosin B standard, labeled as approximately 70% B. Experimental conditions are as given in the text.

3.2. Streptomycins

Fig. 5 demonstrates the excellent sensitivity that can be achieved in the analysis of both streptomycin and dihydrostreptomycin. The separation shown in Fig. 5(A) was accomplished in the anodic mode, while the separation in Fig. 5(B) utilized the cathodic mode. It was apparent that the reversal of the electroosmotic flow by the addition of a cationic surfactant reversed the order of migration. This became an advantage in the trace component analyses that will be discussed below.

Table 2 gives the calibration data for streptomycin and dihydrostreptomycin in both buffer systems. As can be seen, excellent linearity was achieved. Based on peak response values for the 0.050 mg ml^{-1} solutes, the y-intercepts in the borate buffer system represent 3–5 μg ml^{-1} of analyte, and 20 μg ml^{-1} in the borate–TTAB system. The limit of detection based on signal-to-noise ratio of 3 was approximately 10 μg ml^{-1} in both systems. Although the sensitivity and linearity in the two buffers were comparable, the anodic conditions could be used for quantification, based on the smaller deviation from zero associated with this system.

Because dihydrostreptomycin is produced by the reduction of the aldehyde moiety of streptomycin, detection of residual streptomycin in a bulk dihydrostreptomycin sample might be expected. In the anodic borate buffer system, the streptomycin impurity migrated on the trailing edge of dihydrostreptomycin at the higher concentrations used. By adding the cationic surfactant TTAB, the migration order was reversed, which allowed streptomycin to be visualized more readily and quantified more efficiently. Fig. 6 shows the increase in the peak corresponding to streptomycin when standard additions were made. By plotting streptomycin peak area as a function of the concentration of added streptomycin, the following equation was obtained:

$$\text{Area} = (30.86)(\text{conc.}/\mu\text{g ml}^{-1}) + 129.3$$

$$r = 0.9984 \quad (1)$$

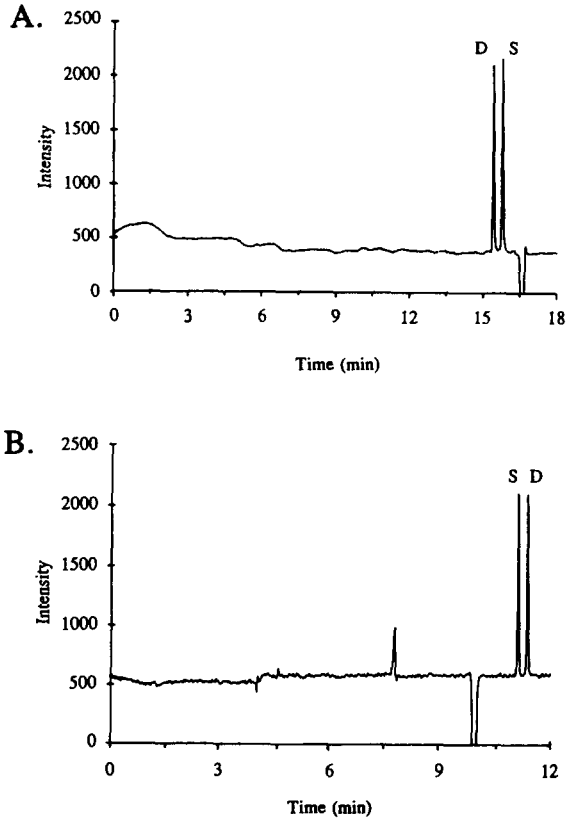

Fig. 5. Separation of dihydrostreptomycin (D) and streptomycin (S). (A) Running buffer, 160 mM borate (pH 9); applied voltage, +18 kV. Concentration of solutes, 0.17 mg ml^{-1} each. (B) Running buffer, 75 mM borate, 0.5 mM TTAB (pH 9); applied voltage, −18 kV. Concentration of solutes 0.10 mg ml^{-1} each.

The x-intercept, or no added streptomycin, was (−)4.2 μg ml^{-1}. This value is equivalent to 0.54% (w/w) of an impurity in the 774 μg mg^{-1} dihydrostreptomycin standard. A similar set of experiments with a 667 μg mg^{-1} dihydrostreptomycin standard from a second manufacturer yielded (−)2.9 μg ml^{-1} streptomycin, or a 0.43% (w/w) impurity level.

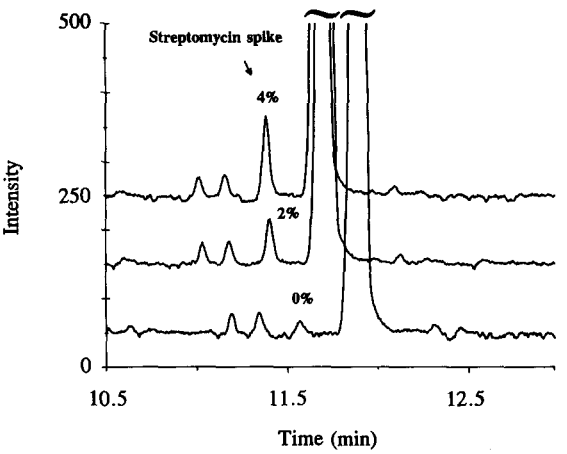

Fig. 6. Determination of residual streptomycin in dihydrostreptomycin standard. Experimental conditions are as given in Fig. 5(B); a 92 cm capillary was used. The migration shift that occurred from the 0% spike to the 2% and 4% spikes is due to the amount of time that elapsed between analyses.

3.3. Kanamycins

Typically, samples for capillary electrophoretic analyses are prepared in either distilled water or a low ionic strength buffer to permit solute stacking. Unfortunately, for the kanamycin family in particular, the use of distilled water led to very broad, and in some cases split, peaks at the concentration levels used for potency determinations.

When sample stock solutions were diluted with distilled water, both amikacin and bekanamycin exhibited broad peaks in proximity to the EOF marker. As the sample buffer was increased to 35 mM borate, amikacin split into a major peak and a shoulder. The peaks coalesced between 88 and 105 mM borate, and the migration time stabilized at a point well removed from the EOF marker. Peak height,

Table 2
Analytical parameters for dihydrostreptomycin and streptomycin

	Migration time (min)	Calibration plot [a]		
		Slope	y-intercept	r
Borate (160 mM)				
Dihydrostreptomycin	15.40 ± 0.01	268.4	1684	0.99996
Streptomycin	15.76 ± 0.01	268.8	−763	0.9998
Borate (75 mM)–TTAB (0.5 mM)				
Streptomycin	11.03 ± 0.02	309.8	6511	0.9971
Dihydrostreptomycin	11.29 ± 0.02	307.2	6749	0.9969

[a] Normalized peak area is plotted as a function of analyte concentration (μg ml^{-1}). $n = 4$ for each of five standards, from 0.50 to 1.0 mg ml^{-1}.

Table 3
Potency determination of selected antibiotics by capillary electrophoresis [a]

Antibiotic	Potency ($\mu g\ mg^{-1}$) [b]		Average calculated potency per set [c]	Average potency [d]
	Standard	Sample		
Amikacin	715	925	938, 970, 946, 976	958 ± 18 (1.9%)
	715	715	734, 734, 750, 720	734 ± 12 (1.6%)
	925	715	731, 679, 695, 701	702 ± 22 (3.1%)
Bekanamycin	685	705	752, 752, 770, 760	758 ± 8 (1.0%)
	685	Undetermined	742, 740, 731, 728	735 ± 7 (0.95%)
	705	685	646, 657, 664, 660	657 ± 8 (1.2%)
Kanamycin A	774	781	770, 775, 787, 778	778 ± 7 (0.90%)
	774	735	803, 781, 786, 791, 788, 787, 774, 795	788 ± 9 (1.1%)
	774	700	760, 767, 729, 727, 782, 762	754 ± 22 (2.9%)
	735	774	752, 764, 761, 776	763 ± 10 (1.3%)
Tobramycin	658	925	960, 966, 959, 963	962 ± 3 (0.31%)
	658	920–940	1066, 1074, 1059, 1063	1066 ± 6 (0.56%)

[a] Sisomicin used as internal standard. Potency determined by comparing ratios of normalized peak areas of standard and sisomicin to sample and sisomicin: potency (standard)/peak area ratio (standard) = potency (sample)/peak area ratio (sample). An explanation of the column headings is found in Section 2.
[b] Potency declared by manufacturer.
[c] $n = 4$ for each set
[d] Determined by averaging the "average calculated potency" values in the preceding column. Values in parentheses are RSDs.

area, and efficiency continued to increase until maximum values were reached between 140 and 158 mM borate.

The migration time for bekanamycin also increased until it stabilized at a sample buffer strength of 105 mM borate. Peak height, area, and efficiency remained constant between 105 and 140 mM borate, but, in contrast to amikacin, bekanamycin peak shape had deteriorated at 158 mM borate.

This behavior may be explained in terms of the equilibria surrounding the formation of the borate complexes. Equilibrium may not be reached over the time scale of the electrophoresis experiment, so the addition of borate to the sample solutions provides additional time in which the complexes can form and stabilize. Better shapes, resolution and detection sensitivities for the kanamycins were obtained when the samples were diluted with buffer to a range of 130–140 mM borate.

The interest in this group of aminoglycoside antibiotics focused on the ability to utilize CE for potency determinations. Table 3 lists the declared potencies of the standards and "samples", and the potencies determined by CE. RSDs within each set were typically 2–3%. Because it is not known how many determinations were made by the manufacturer to establish potency, the range of values that might be represented by the declared value cannot be determined. Table 3 also gives the average potency over all sets of determinations to represent day-to-day reproducibility of the method for a given solute. These values were determined by averaging the "calculated potency" values from the preceding column, and yielded RSDs that varied from less than 1% to 3%.

4. Conclusions

The experiments presented describe the analyses of aminoglycoside antibiotics by capillary electrophoresis. The utilization of borate buffers allowed the direct detection of these compounds through the formation of UV-absorbing complexes. These analyses permitted the simultaneous identification and quantification of members of this class of antibiotics. As such, CE methodologies may offer alternatives to the procedures currently required in the USP protocols. It is believed that similar potency assays could be developed for other aminoglycosides such as paromomycin and sisomicin, as well as pharmaceutical prepara-

tions, such as injectables and ointments, that require time-consuming microbial assays.

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