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LIQUID CHROMATOGRAPHIC SEPARATION OF AMINOGLYCOSIDES WITH PULSED AMPEROMETRIC DETECTION

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SUMMARY

The absence of strong chromophoric groups in aminoglycoside antibiotics virtually eliminates the possibility of their photometric detection in analytical liquid chromatography. Pulsed amperometric detection is described for the sensitive detection of several nebramycin factors following separation on a neutral polystyrene column. The alkaline eluent (0.25 *M* sodium hydroxide) is appropriate as the electrolyte for the electrochemical detection, and the need for post-column addition of reagents is avoided. The use of a coupled fore-column is illustrated for on-line preconcentration when concentrations are below *ca.* 1 ppm in 50-μl samples (*i.e.*, *ca.* 50 ng).

INTRODUCTION

Previous publications have demonstrated the application of anodic pulsed amperometric detection (PAD) for alcohols, polyalcohols and carbohydrates[1–4] at Pt and Au electrodes; and amino acids[5] and organic sulfur compounds[7] at Pt electrodes in alkaline solutions in miniature flow-through detector cells. Anodic amperometric detection of these aliphatic organic compounds at constant (d.c.) potentials is usually accompanied by severe loss of electrode activity resulting from the accumulation of strongly adsorbed reaction products (*i.e.*, free radicals). PAD effectively avoids this problem through use of a triple-step potential waveform in which the analytical signal is measured within a few milliseconds after application of the detection potential. The potential is then usually pulsed to a large positive value, at which the electrode surface is oxidatively cleaned; the subsequent application of a large negative potential pulse causes reduction of the electrode surface oxide and allows adsorption of analyte prior to the next detection cycle. PAD is demonstrated here to be applicable for detection of aminoglycosides, as exemplified for some of the nebramycin factors.

Nebramycin factors are a group of closely related aminoglycosides which are produced by fermentation of *Streptomyces tenebrarius*[6]. The structures of the nebra-

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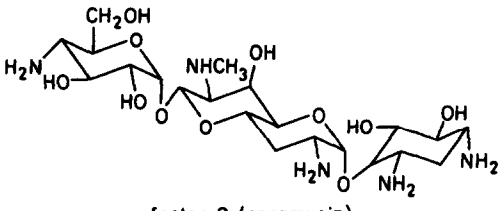
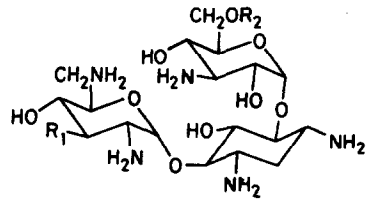
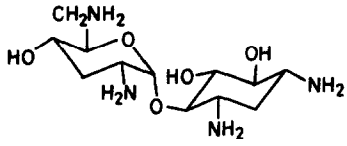
mycin factors used in this work are illustrated in Fig. 1. The nebramycin factors 6 and 2, also known as tobramycin and apramycin, respectively, are important antibiotics. Their production by fermentation usually results in a complex mixture of the various nebramycin factors plus a variety of products commonly associated with growth of the producing microorganism. The ability to monitor the nebramycin factors in the fermentation broth as well as their determinations in biological fluids is desirable. Single-column and coupled-column chromatography combined with PAD are demonstrated here for the separation of these compounds, as well as the determination of tobramycin and apramycin in blood serum. Novel chromatographic conditions employ the alkaline electrolyte (0.25 *M* sodium hydroxide) necessary for the sensitive detection as the chromatographic eluent. This allows for direct monitoring of the chromatographic effluent by the PAD, eliminating the need for cumbersome post-column addition of reagents. The coupled-column technique allows for on-line sample preconcentration and pretreatment, if necessary. In this manner, the blood serum was analyzed with deproteination and filtration as the only pre-injection steps.



factor 2 (apramycin)



factor 4, R_1=OH, R_2=$CONH_2$
factor 5 (kanamycin B), R_1=OH, R_2=H
factor 5′, R_1=H, R_2=$CONH_2$
factor 6 (tobramycin), R_1=H, R_2=H



factor 8 (nebramine)

Fig. 1. Structures of nebramycin factors.

EXPERIMENTAL

Current–potential (*i–E*) curves were obtained by triangular sweep voltammetry at a Pt rotated disc electrode (RDE, 0.46 cm^2; Pine Instrument, Grove City, PA, U.S.A.) using a PIR rotator and a RDE3 potentiostat (Pine Instrument). The *i–E* curves were recorded on a RE0074 *X–Y* recorder (EG&G Princeton Applied Research, Princeton, NJ, U.S.A.). A CMA-1 chromatographic module and a PMA-1 pumping module (Dionex, Sunnyvale, CA, U.S.A.) were used also. The separator column was a MPIC-NS1 neutral polystyrene column (25 cm × 4 mm, Dionex),

normally recommended for paired-ion chromatography. Direct injection of the sample onto the separator column is referred to here as the "single-column technique". The preconcentrator column was a HPIC-CS1 cation-exchange guard column (25 cm × 4 mm, Dionex). The valving of the CMA-1 was configured such that the sample could be injected onto the preconcentrator column by a phosphate buffer (10 m*M*) adjusted to pH 5.2 and subsequently backflushed to the separator column by the eluent (0.25 *M* sodium hydroxide). Separations using the coupled preconcentrator column are referred to here as the "dual-column technique". The Pt flow- through detector cell was potentiostated by a microprocessor-controlled UEM PAD (Dionex). All electrode potentials are reported in volts (V) *vs.* the silver–silver chloride electrode (SSCE) reference.

The nebramycin factors and fermentation broth were from Eli Lilly and Company (Indianapolis, IN, U.S.A.); other chemicals were reagent grade. Water had been distilled, deionized, and filtered through charcoal. Serum samples were deproteinated and centrifuged. The clear solutions of serum and broth were filtered through a Millipore (Bedford, MA, U.S.A.) PTGC 013 10 filter attached to the sample syringe by a Luer adaptor.

RESULTS

Current–potential (*i–E*) curves for tobramycin at the Pt RDE in 0.25 *M* sodium hydroxide are shown in Fig. 2. These curves adequately represent the voltammetric response of all the nebramycin factors tested and we conclude that all the amino-

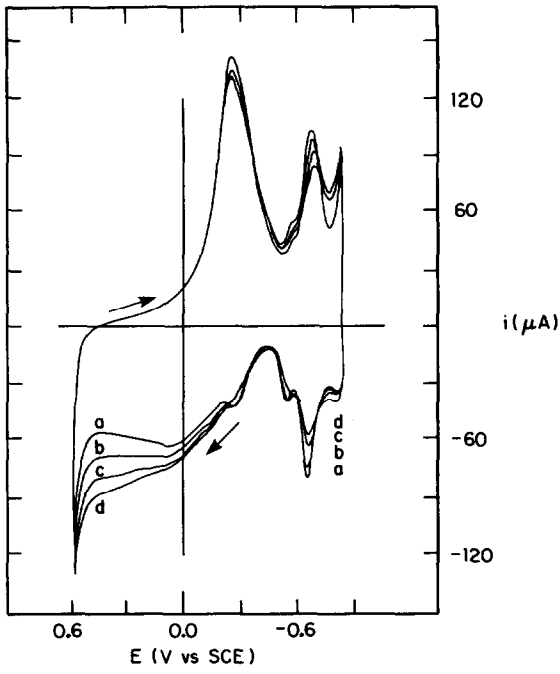


Fig. 2. Current–potential curves for tobramycin at a Pt RDE in 0.25 *M* sodium hydroxide. Conditions: φ = 6.0 V min^{-1}, W = 900 rev. min^{-1}. Concentrations (ppm): (a) 0.0, (b) 1.0, (c) 5.0, (d) 20.0.

glycosides react by a commion mechanism. The residual i–E response in the absence of aminoglycosides, shown also in Fig. 2, is characterized by an anodic wave on the positive potential scan ($-0.3 < E < 0.6$ V) corresponding to formation of surface oxides (*i.e.*, PtOH followed by PtO). The vigorous evolution of O_2(g) occurs for $E > 0.6$ V. The surface oxides are reduced on the negative scan to produce the cathodic peak (E_p = *ca.* -0.3 V). The cathodic production and anodic dissolution of adsorbed atomic hydrogen are responsible for the peaks at moderately large negative potentials ($-0.6 > E > -0.9$ V). The vigorous evolution of H_2(g) occurs for $E < -0.9$ V. The presence of the aminoglycoside results in an increased anodic signal during the positive scan in the region of PtO production ($E > 0.0$ V). This signal exhibits no dependence on electrode rotation speed (W), but varies linearly with potential scan rate (φ). This behavior is characteristic of surface-controlled processes and we conclude that the anodic reaction is the surface-catalyzed oxidation of aminoglycoside which has been adsorbed in the potential region where surface oxide does not exist (*i.e.*, $E < -0.2$ V). Direct evidence for the presence of adsorbed aminoglycoside is the suppression of the peaks for adsorbed hydrogen.

Two triple-step potential waveforms (Table I) for pulsed amperometric detection of aminoglycosides were designed on the basis of the $i-E$ curves in Fig. 2. Waveform A was established in the customary manner with the step for oxiditive cleaning to a potential greater than for detection ($E_2 > E_1$), followed by the negative step for reduction with adsorption. There is significant flexibility in the design of waveforms which are suitable for PAD, as is illustrated by waveform B. In this case, the detection potential (E_1) was chosen to be sufficiently large so that significant oxidative cleaning occurred during the detection period; hence, a more energetic oxidative pulse was not needed subsequent to E_1. The value of E_2 caused very rapid reduction of the surface oxide and E_3 allowed adsorption of analyte for the next detection cycle. The detectability with waveform B was slightly greater because of the longer adsorption period $t_2 + t_3$. The frequency of the waveforms (*ca.* 1 Hz) was sufficiently rapid to allow the detector to faithfully monitor the chromatographic elution peaks. PAD is stable with minimal decay of sensitivity over long time period (*e.g.*, < 10% R.S.D. h^{-1} for 40 ppm tobramycin).

TABLE I

TRIPLE-STEP POTENTIAL WAVEFORMS FOR DETECTION OF AMINOGLYCOSIDES AT Pt ELECTRODES IN 0.25 *M* SODIUM HYDROXIDE

Waveform	*Step*	*Potential (mV)*	*Period (msec)*	*Function*
A	1*	550	250	Anodic detection
	2	700	125	Oxidative cleaning
	3	− 900	425	Reduction of oxide and adsorption of analyte
B	1*	700	125	Anodic detection and oxidative cleaning
	2	−1300	225	Rapid reduction of oxide
	3	− 200	400	Adsorption of analyte

* Sampling of anodic current occurs in the last 50-msec interval of the detection period.

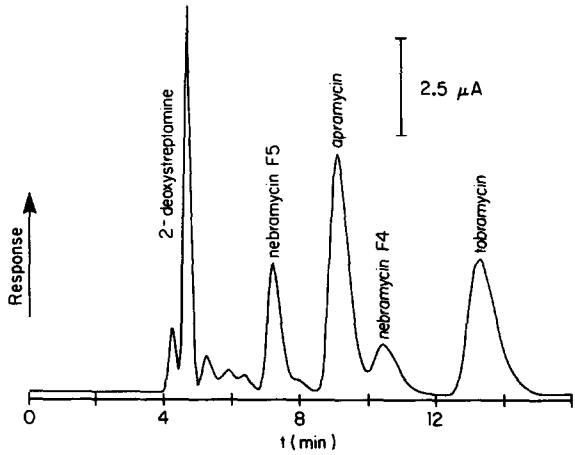


Fig. 3. Chromatogram of mixtures of several nebramycin factors. Conditions: waveform A, single column, flow-rate 0.6 ml min^{-1}. Sample, 50 μl; 100 ppm each compound.

The chromatogram (single-column) for a 50-μl injection of the indicated nebramycin factors at the 100-ppm level is shown in Fig. 3. Resolution is satisfactory. The limit of detection (LOD) for a 50-μl injection was *ca.* 0.8 ppm for tobramycin (signal-to-noise ratio = 2). The detectability was improved significantly by use of the dual-column technique to allow for on-line preconcentration of the aminoglycosides from larger samples. Aminoglycosides are polyvalent in acidic solutions and, as such, are strongly retained on a high capacity cation-exchange column in the manner of Schmidt and Slavin[8] using a pH 5.2 phosphate buffer. This procedure allows also the clean-up of samples by an extended wash period in which weakly adsorbed components of the sample are eluted from the cation column by the buffer. A 20-min wash period was found to be suitable for the deproteinated serum samples. The effect of sample size on peak shape for the dual-column technique is illustrated in Fig. 4. As evidenced, relatively large samples can be concentrated without significant peak broadening, in spite of the long wash period. The chromatogram for a 1.0-ml sample of blood serum spiked with 0.6 ppm each of tobramycin and apramycin is shown in Fig. 5 for the dual-column technique. The first peak is concluded to result from amino acids retained by the cation column but only weakly retained by the separator column. For antibiotic levels exceeding 1 ppm in 50-μl samples (*i.e.*, 50 ng), preconcentration was not needed but was still found desirable for the benefit of sample washing.

Calibration curves for tobramycin and apramycin are shown in Fig. 6. For the short range of dilute concentrations examined, the plot of peak current (I_p) *vs.* concentration (C) is approximately linear for both the single-column technique (SC; tobramycin: slope = 0.0465, s_{xy} = 0.0158, r^2 = 0.9999; apramycin: slope = 0.0623, s_{xy} = 0.0039, r^2 = 0.9999) and the dual-column technique (DC; tobramycin: slope = 0.6630, d_{xy} = 0.0358, r^2 = 0.9892; apramycin: slope = 0.8125, s_{xy} = 0.0388, r^2 = 0.9916). For an extended concentration range to higher values, it has been been shown for other surface-controlled reactions in PAD[7] that the linearity of calibration is improved by plotting $1/I_p$ *vs.* $1/C$. Similar behavior is expected also for amino-

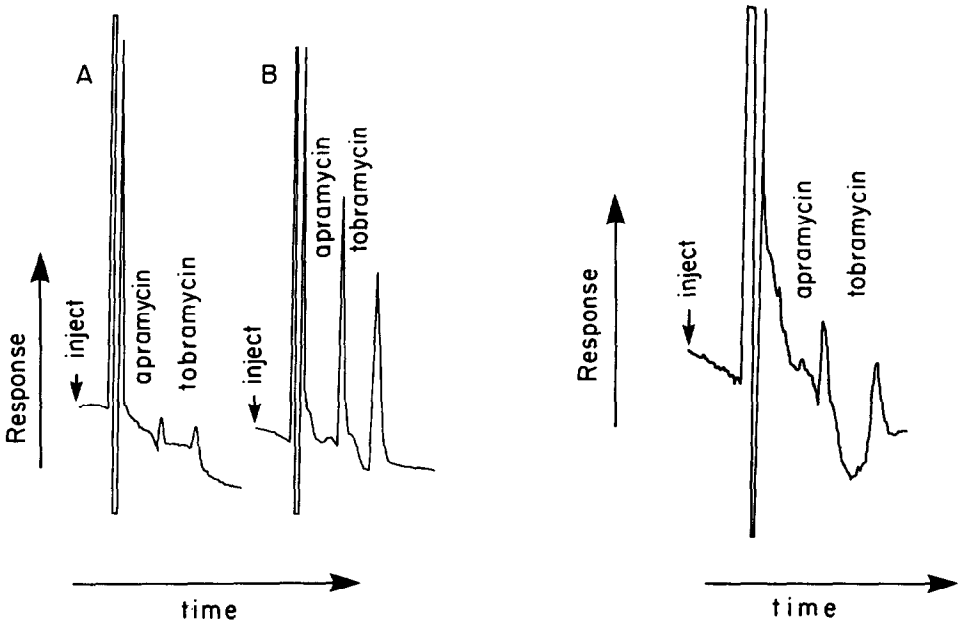


Fig. 4. Effect of sample size in dual-column separation. Conditions: waveform B, 6-min wash, flow-rate 0.6 ml min^{-1}. Samples: (A) 1.0 ml (0.8 ppm), (B) 5.0 ml (0.8 ppm).

Fig. 5. Chromatogram of spiked blood serum. Conditions: waveform B, 20-min wash, flow-rate 0.6 ml min^{-1}. Sample: 1.0 ml, 0.60 ppm each tobramycin and apramycin.

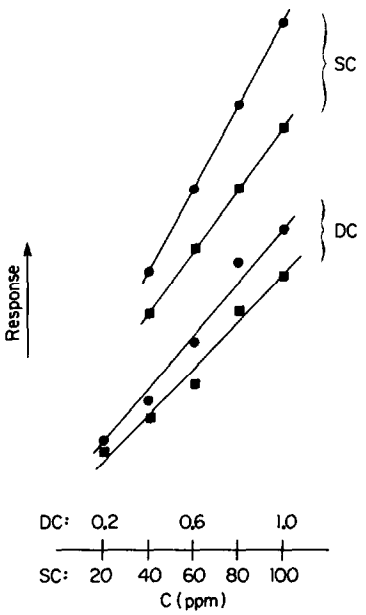


Fig. 6. Calibration curves. Conditions: waveform B, flow-rate 0.6 ml min^{-1}. Curves: (●) apramycin, (■) tobramycin. SC = single column, sample volume 1.0 ml. DC = dual-column, sample volume 10.0 ml.

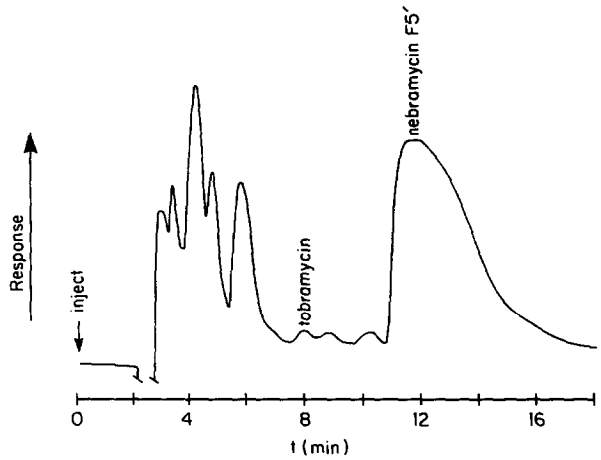


Fig. 7. Chromatogram of fermentation broth. Conditions: waveform B, dual-column, flow-rate 1.0 ml min^{-1}. Sample: 21:1 dilution, 50 µl.

glycosides. The explanation for this phenomenon is that sensitivity is controlled by adsorption and the adsorption isotherm is a non-linear function of the concentration at high concentrations where there is a significant fraction of the electrode surface covered by adsorbed analyte.

The chromatogram for a sample of fermentation broth is shown in Fig. 7 using the single-column technique. The peak with the longest retention (nebramycin F5') is well resolved from its hydrolysis product, tobramycin. This is of particular interest since nebramycin F5' is a main component of tobramycin fermentation. Addition development of the chromatography is needed to obtain satisfactory resolution of all components of this complex sample.

DISCUSSION

We conclude that pulsed amperometric detection (PAD) is useful and reliable for detection of aminoglycosides separated by the single-column and dual-column techniques described. The use of the neutral polystyrene separator column is novel in that the 0.25 *M* sodium hydroxide desired for maximum sensitivity of detection can be used as the eluent and post-column addition of reagent is not necessary. However, in cases of more conventional chromatographic systems for which the pH and/or ionic strength of the eluent are not suitable for PAD, post-column addition of electrolyte can be employed successfully[2]. Research is just beginning to extend use of the PAD to mixed solvent systems which are more appropriate to reverse-phase separations.

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