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High-performance liquid chromatographic separation and electrochemical detection of penicillins

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ABSTRACT

A reversed-phase high-performance liquid chromatography method used to determine penicillins is described. A C_{18} stationary phase is used in conjunction with mixed solvent systems containing acetate buffer, methanol, and acetonitrile in various proportions. An isocratic separation is illustrated, but usage of a gradient program varying the composition of the organic modifier gives better performance. Detection is accomplished using pulsed amperometric detection to indirectly monitor the penicillins. The detection limit for ampicillin is $4 \cdot 10^{-7}$ *M*.

INTRODUCTION

Penicillins are the most popular class of antimicrobial agents, used to fight off a wide range of bacterial infections [1]. Even though they are widely used, the incidence of oversensitive reactions may be as high as 10%, with symptoms ranging from skin rashes to, in rare cases, fatal episodes of anaphylaxis [2]. Because of their widespread use in fields such as pharmaceuticals, health care, research, and regulation, the analysis of penicillins is relevant and has many practical applications. Veterinarians find penicillins to be useful for fighting bacterial infections in various domesticated animals [3,4]. This can lead to the transfer of penicillins to milk and meat products by and from the animals. Although the amounts are small, this is a serious matter due to the aforementioned potential for severe allergic response, even in small doses. This hypersensitivity insures that methods for monitoring penicillins in food products must have very low detection limits [5]. The Food and Drug Admninistration has recently unveiled plans for a nationwide screening of milk for several drugs, including penicillins, so there is a demand for this type of methodology [6].

Most of the current methods use high-performance liquid chromatography (HPLC) coupled with a variety of detectors. Taking advantage of the carboxylate group of the penicillins, some early separations employed anion-exchange HPLC in alkaline solution [7,8]. Recent work has focused on reversed-phase HPLC on C_8 [9–12] and C_{18} columns [1,5,13–15]. The chromatographic efficiency of the reversed phase separations are superior, and the more moderate pH values minimize penicillin degradation. Others have used ion pair separations on C_{18} columns [13,16].

The most common detection mode is absorbance spectrophotometry in the ultraviolet (UV) region [1,5,13–16]. The absorbance maxima are just outside the vacuum UV range, resulting in excitation wavelengths in the range of 200–230 nm. Detection limits have been measured as low as 0.1 to 0.5 μg/ml [1,10,15] for direct determinations. Due to the large number of compounds displaying absorbance at these same wavelengths, peak overlap can be problematic for complex biological samples. Application

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of higher excitation wavelengths (254 nm typically) will improve selectivity, but limited sensitivity is displayed due to lower absorptivity [7,8,17].

Various indirect methods and derivatization schemes have been examined to improve detection. Besada and Tadros [18] oxidized the penicillins with potassium iodate, then monitored the amount of iodine formed colorimetrically. Morelli [19] oxidized the penicillins with ammonium vanadate, monitoring the amount of blue vanadium(IV) produced spectrophotometrically. A similar procedure using ammonium molybdate has also been published [20]. Kok *et al.* [21] monitored the penicillins indirectly by oxidizing them with electrogenerated bromine followed by subsequent measurement of the amount of Br_2 in excess. Several studies have formed mercuric mercaptides of penicillins by pre- [22,23] or post-column [24] addition of imidazole and mercury(II) chloride coupled with a reversed-phase separation. Derivatization of the penicillins with *o*-phthaldialdehyde [23,25] can produce fluorescent derivatives, although this is limited to only the species that have a primary amine group on their side chain. Reported detection limits range from 0.5 to 1 μg/ml. Amoxicillin can be determined with fluorescence spectroscopy following electrochemical oxidation [26] to 50 ng/ml levels. This method is limited solely to amoxicillin, which has an electroactive phenol group in its side chain.

Voltammetric detection methods have been investigated in several previous studies, leading one group to conclude that the penicillins, "do not contain a polarographically reducible or oxidizable functional group" [27]. Due to, "the general lack of suitable oxidative and/or reductive properties," another group [28] has photolytically derivatized the penicillins with a UV flashlamp to allow electrochemical detection. Musch *et al.* [29] have reported penicillin detection using constant potential (d.c.) amperometric detection at a glassy carbon working electrode. Recent work in this research group [30,31] has shown that penicillins can be monitored with good sensitivity on gold and platinum electrodes using pulsed amperometric detection [32,33]. Electrode fouling problems were minimized due to the built-in cleaning mechanism of pulsed amperometric detection. This work sought to couple this detection scheme with a chromatographic separation that could speciate the various penicillins.

EXPERIMENTAL

Materials and reagents

All penicillins were purchased from Sigma (St. Louis, MO, USA). Reagent-grade acetic acid from Fisher (Pittsburgh, PA, USA) and sodium acetate from Baker (Phillipsburg, NJ, USA) were used to produce acetate buffer solutions. HPLC-grade acetonitrile and methanol from Fisher were used as mobile phase organic modifiers. Water was distilled and deionized before use as a solvent. All mobile phases were vacuum filtered through an Alltech (Deerfield, Ill, USA) 0.2-μm nylon filter and sonicated before use. Samples were filtered through nylon 0.45-μm syringe tip filters before injection into the HPLC system. Standard solutions were prepared in either the chromatographic solvent or water.

Chromatographic apparatus

A Waters (Milford, MA, USA) 625 Gradient LC System was used for all HPLC work. A standard flow-rate of 2.0 ml/min was used for most applications. The pump was run in "silk mode", a Waters feature designed to reduce pump noise. The injection loop had a volume of 50 μm.

A 10-μm μBondapak C_{18} Radial-Pak cartridge (Waters) was employed as the chromatographic stationary phase. The cartridge had an internal diameter of 8 mm and a length of 100 mm. The cartridge was housed in a Waters 8 × 10 Radial-Pak compression module. All separations were done at ambient laboratory temperature (*ca.* 20 ± 2°C).

Electrochemical detection used the Waters 464 pulsed electrochemical detector. For all experiments, a thin-layer cell was utilized that had a block with dual gold electrodes in the series configuration. The upstream element was used as the working electrode, while the downstream electrode served as the counter electrode for a majority of this work. The stainless-steel block opposing the dual gold electrodes was tested as an alternative counter electrode; no significant difference in performance was noted. The detector was configured in the floating ground mode throughout this project to minimize detector noise. This served to ground the detector at a virtual point rather than to the chassis of the instrument. The thin-layer cell used a Ag/AgCl reference electrode. A time constant of 0.5 s was used for

d.c. amperometric detection; 1250 mV was the optimum potential setting. Many different pulsed amperometric detection waveforms were used; details are given in the following section. A majority of the data was collected with a Gateway (N. Sioux City, SD, USA) 386SX computer using a Keithley MetraByte (Taunton, MA, USA) Chrom-1AT interface board.

RESULTS AND DISCUSSION

Previous work [30] has demonstrated the applicability of pulsed amperometric detection for penicillin quantitation in a flowing liquid stream. By adapting an HPLC scheme followed by pulsed amperometric detection we sought to allow separation and speciation of the common penicillins without interference from other sample matrix components. Upon addition of a C_{18} HPLC column to the flow injection analysis system used previously, some modification was needed. The prior solvent, 0.2 *M* acetate buffer (pH 4.7), provided a proper pH to insure the chemical stability of the penicillins, was compatible with a silica-based C_{18} resin, and provided electrolyte for electrochemical detection.

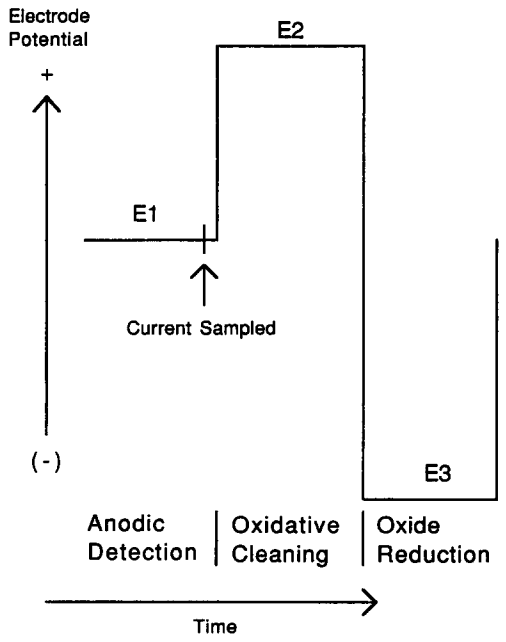

Fig. 1. Pulsed amperometric detection waveform schematic.

However, when penicillin solutes were dissolved in the acetate mobile phase and passed through a μBondapak C_{18} column, the penicillins were not recovered from the column.

By the addition of an organic modifier and by shifting from 0.2 to 0.02 *M* acetate buffer, the mobile phase was made less polar, which enabled penicillin recovery from the C_{18} column. Methanol and acetonitrile were chosen as organic modifiers since both had been used previously for penicillin separations on C_{18} columns [5,34] and both had been used successfully with pulsed amperometric detection [35–38]. Finding the proper mobile phase composition involved compromising detector performance, which was best at high concentrations of aqueous buffer, for the sake of optimizing HPLC performance, as increased organic modifier concentrations improved peak shapes and reduced retention times. The best combination was in the range of 70% (v/v) aqueous buffer and 30% organic modifier (hereafter designated 70:30).

Detector performance had been optimized previously [30] for use with the 0.2 *M* acetate buffer solvent. With the modification in solvent composition for use as a chromatographic mobile phase, reoptimization of the pulsed amperometric detection waveform was necessary. In particular, the addition of organic modifier causes a significant alteration of electrochemical response. As was discussed previously [30], either "direct" or "indirect" detector response can be obtained depending on the pulsed amperometric detection waveform that was chosen. In brief, direct detection refers to an increase in the anodic current resulting from oxidation of the analyte. Indirect detection refers to a suppression of the anodic background current in the absence of significant current from analyte oxidation. The adsorbed analyte occupies space on the electrode surface, attenuating the signal from the surface-dependent residual formation of gold oxide. The analyte will not be significantly oxidized during indirect detection due to slow kinetics. By adjusting the pulsed amperometric detection waveform (Fig. 1) one can convert negative response from indirect detection to positive response from direct detection. Increasing the overpotential (*i.e.* increasing *E*1) can accelerate the reaction rate of penicillin oxidation [30] to produce positive peaks. In some cases, by sampling the current after a long time delay at the *E*1 potential, it

TABLE I

PULSED AMPEROMETRIC DETECTION WAVEFORM VARIATION WITH SOLVENT COMPOSITION

Indirect pulsed amperometric detection, 0.2 *M* acetate buffer	Indirect pulsed amperometric detection, 0.02 *M* acetate–organic modifier (70:30)	Direct pulsed amperometric detection, 0.2 *M* acetate buffer	Direct pulsed amperometric detection, 0.02 *M* acetate–organic modifier
*E*1 = 1100 mV	*E*1 = 1300 mV	*E*1 = 1500 mV	*E*1 = 1500 mV
*T*1 = 0.200 s	*T*1 = 0.167 s	*T*1 = 0.333 s	*T*1 = 1.333 s
*E*2 = 1600 mV	*E*2 = 1500 mV	*E*2 = 1600 mV	*E*2 = 1600 mV
*T*2 = 0.167 s	*T*2 = 0.167 s	*T*2 = 0.167 s	*T*2 = 0.167 s
*E*3 = −200 mV	*E*2 = −200 mV	*E*3 = −200 mV	*E*2 = −200 mV
*T*3 = 0.167 s	*T*3 = 0.333 s	*T*3 = 0.167 s	*T*3 = 0.333 s

is possible to observe positive peaks at potentials where penicillin oxidation occurs very slowly.

Both direct and indirect detection were observed in 0.2 *M* acetate buffer solvent [30], but the *S/N* ratio was vastly superior using direct pulsed amperometric detection. However, adapting a 70:30 acetate buffer–organic modifier solvent led to much slower penicillin oxidation kinetics than seen with the 0.2 *M* acetate buffer solvent. A much greater overpotential was needed to produce positive response with a 70:30 solvent, and the noise present under these conditions hindered the performance of direct pulsed amperometric detection waveforms. The *S/N* response of the indirect pulsed amperometric detection waveforms was better for mobile phases containing organic modifier. Optimization of both waveforms with 70:30 solvents and subsequent comparison clearly showed that indirect pulsed amperometric detection was superior to direct pulsed amperometric detection. Hence, indirect detection was used throughout the balance of this work when pulsed amperometric detection was employed. D.c. amperometric waveforms were also investigated and found to be clearly inferior to pulsed amperometric detection in all cases. See Table I for a compilation of pulsed amperometric detection waveforms.

Nine common penicillins (Table II) were obtained with the intention of resolving each using HPLC. Various different aqueous–organic (70:30) phases were examined for the job. For further studies of penicillin retention times two of the best 70:30 mobile phases were selected: acetonitrile–methanol–0.02 *M* acetate buffer) (20:10:70) and (25:5:70).

TABLE II

RETENTION TIMES FOR PENICILLINS IN SELECTED MOBILE PHASES

Flow-rate: 2 ml/min. Mobile phase: acetonitrile–methanol–acetate buffer.

Penicillin	Retention time at 20:10:70 (min)	*k'* Value 20:10:70	Retention time at 25:5:70 (min)	*k'* Value 25:5:70
Amoxicillin (Amox)	1.97	0.15	1.78	0.11
Amphicillin (Amp)	2.38	0.40	2.03	0.26
Methicillin (Meth)	4.58	1.66	3.18	0.98
Penicillin (Pen) G	6.35	2.73	3.94	1.43
Penicillin V	9.62	4.62	5.35	2.34
Oxacillin (Oxa)	12.57	6.35	6.62	3.11
Cloxacillin (Cloxa)	19.27	10.25	9.80	5.07
Nafcillin (Naf)	25.03	13.64	12.28	6.62
Dicloxacillin (Dicloxa)	38.02	21.30	18.11	10.32

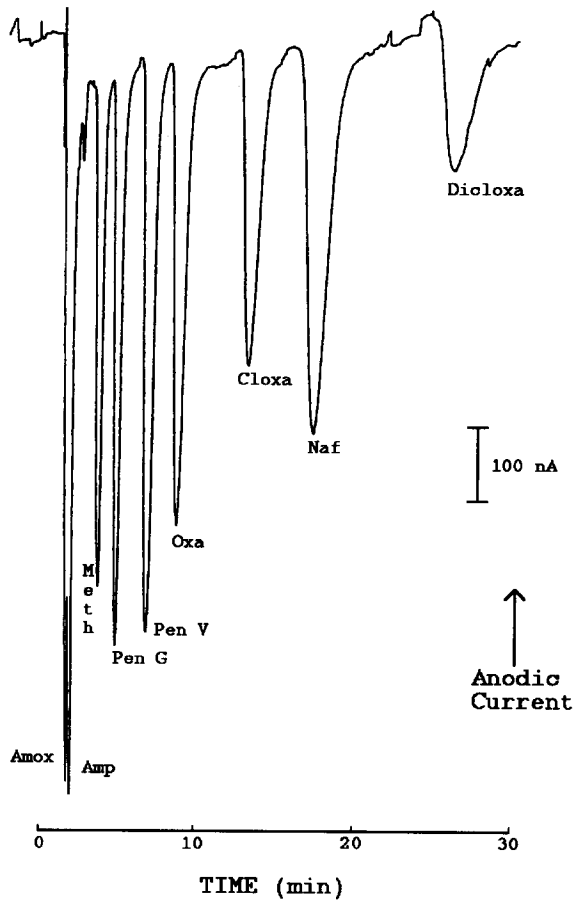

Fig. 2. Isocratic separation of nine penicillins. Mobile phase was acetonitrile–methanol–0.02 *M* acetate buffer (22.5:7.5:70), 2 ml/min. Indirect waveform (Table I). Penicillin concentrations = 1 m*M*.

Trials with the two organic modifiers had shown that acetonitrile had greater eluting strength than methanol, so it was expected that retention times would be shorter with 25:5:70. Retention times and k' values for the penicillins with the two solvents are given in Table II. The data in Table II might suggest that the 25:5:70 solvent should function well, with k' values all differing and under 10.5. However, the early eluting peaks are not well resolved. Amoxicillin and ampicillin (1.78 and 2.03 min, respectively) are so close together that they practically coelute. In 20:10:70, the resolution of the early peaks was improved. Unfortunately, the separation took over 35 min at a 2 ml/min flow-rate. Thus, 25:5:70 mobile phase is too strong, while 20:10:70 is too weak. For an isocratic separation, an intermediate strength mobile phase was needed.

To produce a mobile phase intermediate in strength to the previous two, a 22.5:7.5:70 solvent was chosen for an isocratic separation (see Fig. 2). This separation gave k' values better than the other two mobile phases used in the retention time study. All nine penicillins were eluted in less than 30 min, and the earliest peaks were less cramped than the 25:5:70 retention times. Despite the improvement, amoxicillin and ampicillin were still not adequately resolved (resolution = 0.29). To improve upon their separation, gradient elution was the obvious choice. This would allow usage of a weaker solvent early in the separation to allow improved resolution of the low k' peaks, with a gradient to a stronger solvent to minimize retention of the later-eluting peaks.

The marriage of gradient elution and electrochemical detection has proven troublesome in previous work [35,36]. After experimentation with many different gradients, it became clear that any gradient that significantly changed the percent organic modifier would cause a large baseline shift. Hence, the simple concept of increasing the percentage of organics over the course of a run was eliminated. An alternative was to produce a solvent program that maintained the same total percentage of organic modifier, but changed its composition. Because acetonitrile was a stronger eluent for these analytes than methanol, it was hypothesized that the overall eluting strength of the mobile phase could be controlled by changing the amounts of the two organics relative to each other. In this manner,

TABLE III

OPTIMIZED GRADIENT PROGRAM FOR PENICILLIN SEPARATION

Flow-rate 2 ml/min.

Time (min)		Solvent (acetonitrile–methanol–acetate buffer)
0		15:10:75
	Linear Ramp	
15		30:0:70
	Isocratic Hold	

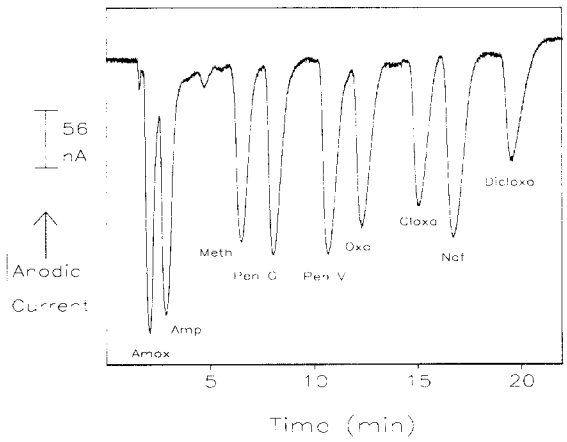

Fig. 3. Gradient separation of nine penicillins. Gradient program is given in Table III. Indirect pulsed amperometric detection waveform (Table I). Penicillin concentrations = 1 m*M*.

solvent strength could be altered while minimizing any baseline offset.

To satisfactorily separate amoxicillin and ampicillin, a slight change in the 70:30 buffer to organic ratio was necessary. The separation of these two penicillins was achieved by starting the gradient at 15:10:75, a 75:25 ratio of buffer to organic. This slight alteration in the buffer to organic ratio was small enough to avoid the baseline shifting problems. Consequently, the gradient ran from 75 to 70% acetate buffer. The exact specifications of the best gradient are detailed in Table III. A chromatogram obtained using this gradient is shown in Fig. 3. From this figure, it can be seen that all nine penicillins were well separated with only a minor baseline perturbation. The problematic amoxicillin–ampicillin duo had a resolution measurement of 1.05 with this gradient, as compared to a value of 0.29 in the isocratic trial from Fig. 2. An additional point to note is that the separation was done in twenty minutes, around a ten minute improvement over the isocratic separation above.

To allow the application of this methodology for quantitative work, the relationship between detector response (anodic current) and concentration must be understood. As was expected from previous work [39,40], pulsed amperometric detection current *vs.* concentration (*I vs. C*) response was non-linear when plotted over a wide concentration range. However, response was linear at low concentrations. The loss of calibration curve linearity can be aptly illustrated and the concentration at which the non-linear behaviour begins shown by a special type of calibration plot. Normalized peak area is plotted *vs.* log concentration, where the normalization process uses statistics from a modified linear regression fit [40] of the linear portion of the *I vs. C* plot.

$$\text{Normalized peak area} = \frac{(\text{peak area} - y\ \text{intercept})}{\text{slope} \cdot \text{concentration}}$$

This plot can be seen in Fig. 4. Ideal linear behavior would result in all normalized peak areas equaling one. All of the points at lower concentrations are within + or − 10% relative deviation from this ideal value. At $3.5 \cdot 10^{-4}$ *M* the plot exits this region; this concentration is designated as the upper limit of linear *I vs. C* behavior. Above this concentration, one must either employ a non-linear calibration curve or dilute samples to reach the linear response region. Using the optimized gradient program on the μBondapak C_{18} column, the detection limit for ampicillin was found to be $4 \cdot 10^{-7}$ *M*. (0.2 μg/ml).

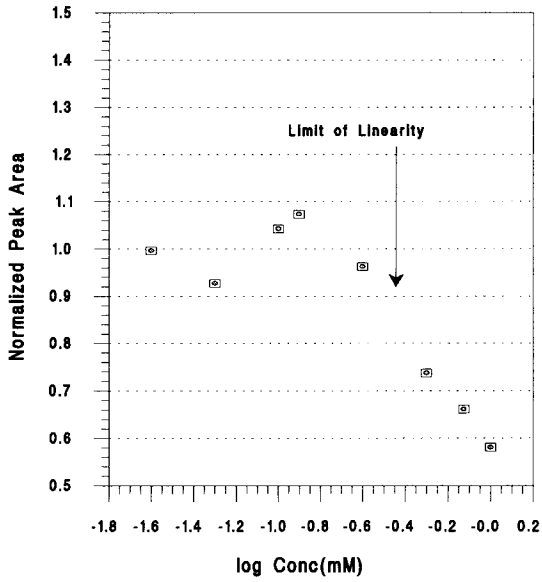

Fig. 4. Normalized calibration curve for ampicillin. 0.9 and 1.1 represent 10% relative deviation from linearity. Mobile phase was acetonitrile–methanol–0.02 *M* acetate buffer (15:10:75), 2 ml/min. Indirect pulsed amperometric detection waveform (Table I).

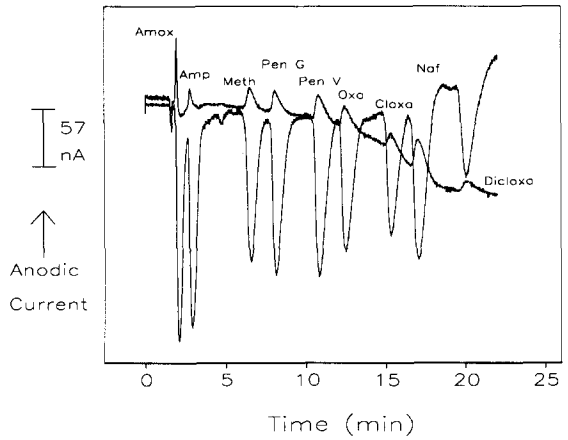

Fig. 5. A comparison of direct and indirect pulsed amperometric detection. Trace with peaks pointing upward is direct pulsed amperometric detection (Table I). Trace with peaks pointing downward is indirect pulsed amperometric detection (Table I). Both were run with 1 m*M* penicillin concentrations and the gradient from Table III.

The response to all of the other penicillins was within a factor of 2 of that for ampicillin.

As noted earlier, the pulsed amperometric detection waveform was optimized for negative response, which was used throughout this study. An alternative waveform optimized to produce positive response will also function for this separation (see Fig. 5). As expected, the *S/N* ratio for the direct pulsed amperometric detection waveform was poorer than with the indirect pulsed amperometric detection waveform. Nevertheless, the selectivities of the two waveforms are different, and it is conceivable that the direct pulsed amperometric detection waveform might be superior in the presence of interfering components during application work.

CONCLUSIONS

Pulsed amperometric detection can provide sensitive detection for penicillins following HPLC as long as one carefully matches the detector waveform with the chromatographic mobile phase. When *ca.* 30% organic modifier was added to an aqueous solvent for chromatographic purposes, slowed electrochemical kinetics required a waveform adjustment. Rather than increasing the detection potential to provide overpotential, better *S/N* was achieved by monitoring the penicillins indirectly by measuring their suppression of the background anodic current from the mobile phase. This type of detection gave non-linear *I vs. C* response just as would be expected for positive pulsed amperometric detection. Despite the overall non-linearity, linear behavior extends nearly three concentration decades above the detection limit.

Although an isocratic separation of the penicillins was feasible, gradient elution produced superior resolution and shorter analysis times. The application of a solvent program that kept the percent organic modifier constant while varying the modifier composition was a major step forward. Attempts to alter solvent strength by the traditional methods of increasing the percentage of the organic modifer all resulted in an unacceptable shift in baseline response. This shift can be attributed to enhanced irreversibility of the electrochemical reactions due to slowed kinetics as the solvent polarity and the ionic strength decreased. By exchanging acetonitrile for methanol, one can keep the ionic strength constant while changing the polarity by only a minimal amount. This served to minimize baseline perturbation as solvent strength was increased.

The detection limits observed for the penicillins were on par with the best values reported using direct UV absorbance. The application of a pulsed coulometric detection system in future studies should provide enhancement of the *S/N* observed with pulsed amperometric detection, giving electrochemical detection a clear edge over direct spectrophotometry. The selectivity of pulsed amperometric detection should also be an advantage that will be borne out in future application work.

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