ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
19 (1999) 239–252

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Pulsed electrochemical detection of sulfur-containing antibiotics following high performance liquid chromatography

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Received 27 January 1998; accepted 17 April 1998

Abstract

Pulsed electrochemical detection (PED) following reversed-phase chromatography has been applied to the direct detection of sulfur-containing antibiotics, specifically, penicillins, cephalosporins, and lincomycin. The compounds are detected sensitively and selectively without the need for derivatization. Integrated pulsed amperometric detection (IPAD) yields limits of detection lower than UV detection for these compounds. Detection limits using an optimized IPAD waveform are typically 10 ppb or less. The high selectivity of PED for thiocompounds reduces sample preparation. This work is applied to the determination of penicillin and related analogues in various pharmaceutical formulations/preparations, including a chicken feed.

Keywords: Cephalosporins; Integrated pulsed amperometric detection; Penicillins; Pulsed electrochemical detection; Reversed-phase chromatography; Sulfur-containing antibiotics

1. Introduction

Sulfur-containing antibiotics are some of the most effective agents against bacterial diseases and infections. For instance, β-lactams, which include penicillins and cephalosporins, exploit the unique bacterial biochemistry that is without a counterpart in animals—they block bacterial cell-wall synthesis. The development of simple, rugged, and reliable techniques for the detection of sulfur-containing antibiotics in many different matrices is an on-going analytical quest. Their detection at low levels is not trivial in many cases, as these compounds often have a weak chromophore. As a consequence, detection of β-lactam antibiotics in liquid chromatographic analysis often relies on low wavelength UV detection [1–5] and therefore requires difficult sample preparation protocols or post-column derivatization [6,7] in order to obtain adequate selectivity and/or sensitivity. Concerns of adequate detection are further amplified for sulfur-containing aminoglycosidic antibiotics (e.g. lincomycin), which have little or no chromophore, fluorophore, and/or constant applied (dc) potential electrophore.

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0731-7085/99/$ - see front matter
PII S0731-7085(98)00089-2

Pulsed electrochemical detection (PED) has been shown to be effective for the detection of sulfur-containing compounds following HPLC [8–11]. PED denotes several techniques which couple a detection step (e.g. amperometric, coulometric) with pulsed potential cleaning to maintain uniform and reproducible electrode activity. Sulfur-containing compounds are detected via an oxide-catalyzed mechanism, in that oxide formation at the electrode surface occurs simultaneously and is required for the detection process. The formation of the surface oxide is associated with large background signals, reduced analyte signal, and unstable baselines. These problems are essentially eliminated with integrated pulsed amperometric detection (IPAD) [10,12–14]. In IPAD, a linear cyclic scan between two potentials is performed during the detection step. The charge from the forward and reverse scans is summed to electronically subtract the oxide formation and dissolution charges (i.e. $\Sigma = 0$, ideally) from the total signal. This waveform increases analyte signal and improves baseline stability resulting in better reproducibility and lower limits of detection for sulfur-containing compounds [13,15]. In addition, IPAD at a gold electrode under mildly acidic conditions has been shown to be selective for sulfur-containing compounds [8,10,11]. The use of PED following HPLC for the detection of sulfur-compounds, including antibiotics, has been reviewed [16,17].

A recent application of HPLC-PED focused upon an assay for ampicillin and cephapirin in milk [11]. This work highlighted the power of this approach over UV detection in dealing with complex matrices. Here the application of IPAD following reversed-phase chromatography is extended generally to sulfur-containing antibiotics, specifically penicillins, cephalosporins, and lincomycin. Electrochemical characterization via cyclic voltammetry is the basis of optimization of the IPAD waveform, and mechanistic information is used to improve the choice of the chromatographic solvent system. In addition to determining analytical figures of merit for standard compounds, chromatographic assays of three penicillin-based formulations underscore the high sensitivity and selectivity of PED.

2. Experimental

2.1. Materials

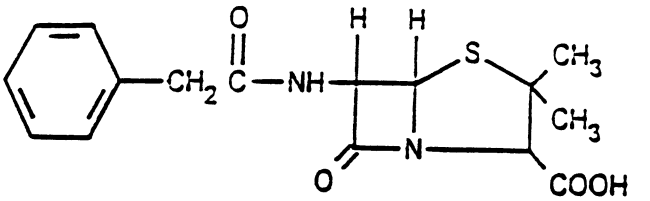
All solutions were prepared from reagent grade chemicals. All solvents were HPLC grade. Mobile phases were filtered with 0.2-μm Nylon-66 filters (Rainin, Woburn, MA) and a solvent filtration apparatus (Microfiltration Systems; Rainin). All antibiotic standards were purchased from Sigma (St. Louis, MO) and prepared in water. If not used immediately, standard solutions were either stored in a refrigerator or freezer overnight. Water was purified using a reverse osmosis system coupled with multi-tank/ultraviolet/ultrafiltration stations (US Filter/IONPURE, Lowell, MA).

2.2. Voltammetry

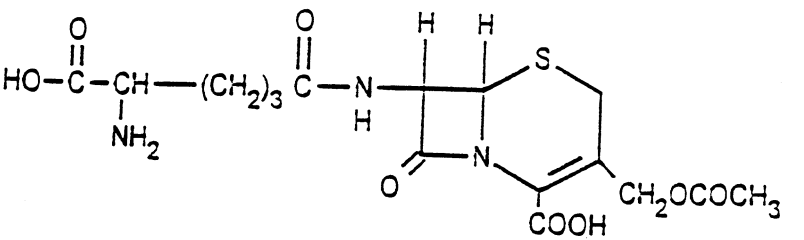
Cyclic voltammetric data were collected at a gold rotating disk electrode (RDE) using a model BAS RDE-1 rotator and a BAS 100-B electrochemical workstation (Bioanalytical Systems, West Lafayette, IN) with a 75-MHz Pentium computer (Gateway 2000, North Sioux City, SD). The reference electrode was Ag/AgCl (BAS), and the auxiliary electrode was a Pt wire. Cyclic voltammograms were taken using a scan rate of 250 mV/s and a rotation speed of 1600 rpm.

2.3. Chromatographic system

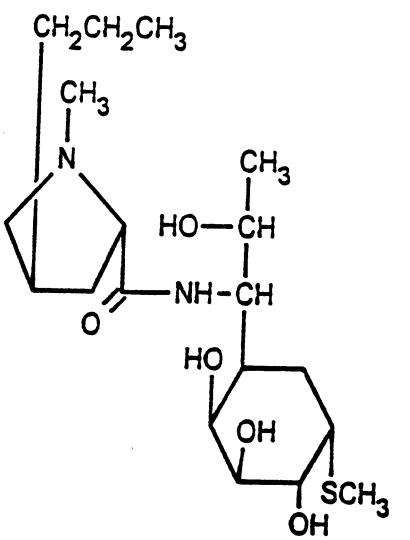
The chromatographic system consisted of a GP-40 solvent delivery pump (Dionex, Sunnyvale, CA) with an ED-40 electrochemical detector (Dionex) equipped with a gold working electrode, a combination pH and Ag/AgCl reference electrode, and a titanium auxiliary electrode. A UV detector (Dionex VDM-2) was also used for online comparisons and was placed in series after the electrochemical detector. The separations were accomplished with a Luna C-8, 5 μm particle size, 150×4.6 mm analytical column (Phenomenex, Torrance, CA) and a Symmetry C-8 guard column (Waters, Milford, MA). The columns and the electrochemical cell were temperature controlled at 30°C with an LC-30 chromatography oven (Dionex). Data was collected via computer



PENICILLIN G



CEPHALOSPORIN C



LINCOMYCIN

Fig. 1. Structures of model sulfur-containing antibiotics.

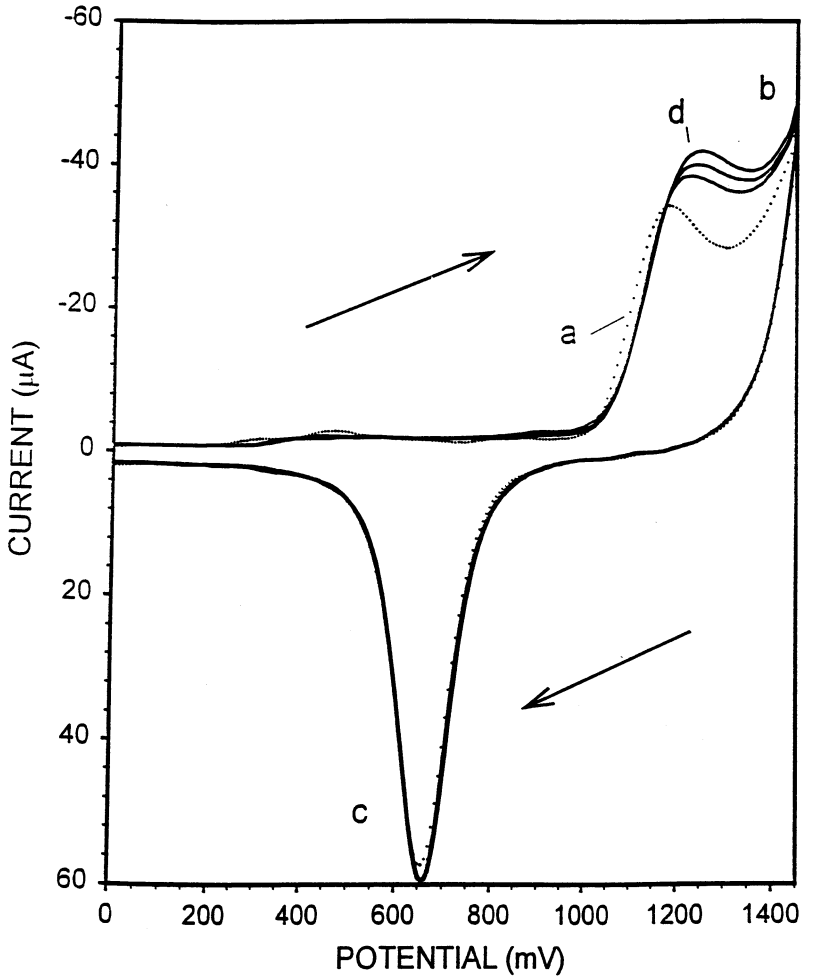


Fig. 2. Cyclic voltammetry of (——) penicillin G at 5.2, 10.4, and 15.9 μM at a gold RDE in (· · ·) 100 mM NaAc buffer (pH 3.75)/CH_3CN/MeOH (80:10:10, v/v/v). Residual and sample voltammograms collected in the absence of dissolved O_2. Conditions: scan rate, 250 mV/s; rotation speed, 1600 rpm. Arrows denote direction of scan.

with PeakNet software (Dionex). The data from the electrochemical and UV detection were smoothed using a Savitsky–Golay algorithm with a filter size of 11 points and two iterations. Unless otherwise specified, the mobile phase solvents were A = methanol (MeOH)/water (90:10, v/v); B = sodium acetate (NaAC) buffer (pH 3.75, 0.5 M); C = water; and D = acetonitrile (CH_3CN)/water (90:10, v/v).

2.4. Sample preparation procedures

For the assay of the amoxicillin formulation, a 250-mg capsule was dissolved in 1.00 l of water and the resulting 250 ppm solution was diluted to 100 ppb in water. This solution was filtered through a 0.45-μm filter and injected onto the chromatographic system. A similar procedure was followed for the PenVee K tablets, except that the final solution was 300 ppb with water.

All feed samples were prepared using an Accelerated Solvent Extraction (ASE) system (Dionex). An 11-ml ASE cell was loaded with a 1-μm glass fiber filter (Whatman, Clifton, NJ), 50–70 mesh sand (Aldrich, St. Louis, MO), and 1 g of feed. Any excess volume in the cell was filled with sand.

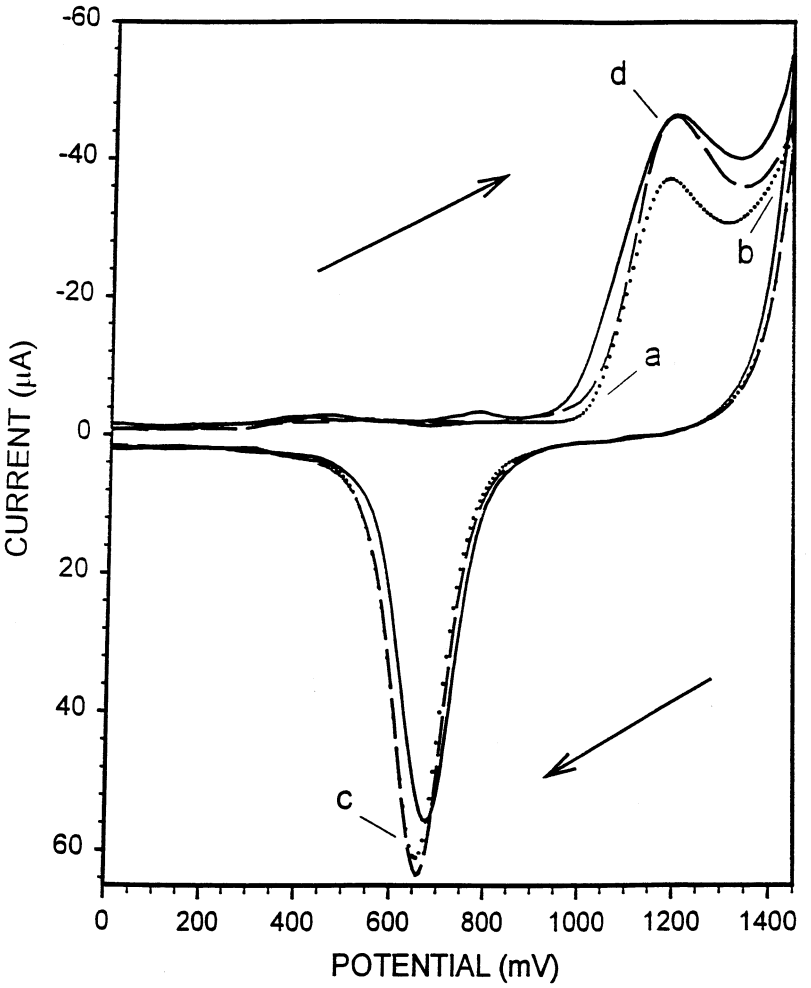


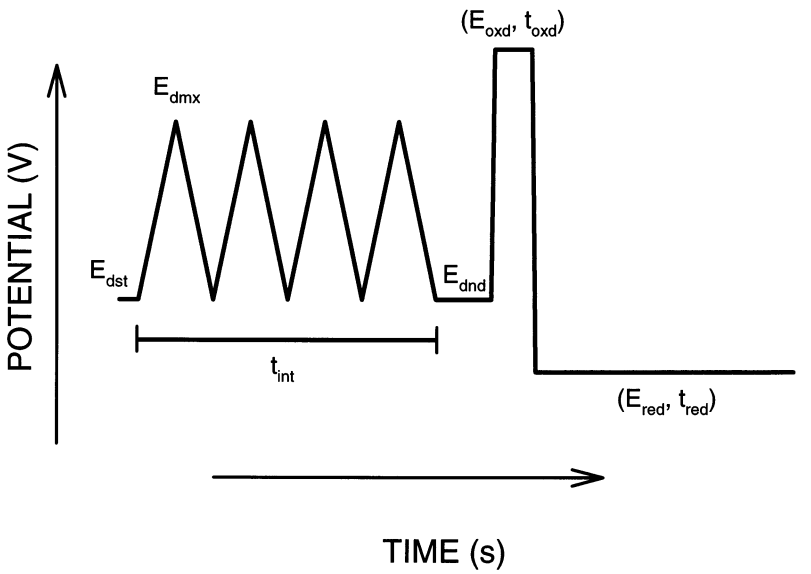
Fig. 3. Cyclic voltammetry of (——) lincomycin and (- - -) cephalosporin C at 24.8 and 27.6 μM, respectively, at a gold RDE in (· · ·) 100 mM NaAc buffer (pH 3.75)/CH_3CN/MeOH (80:10:10, v/v/v). Conditions are the same as in Fig. 2.

The sample was extracted with water for three cycles each of 3 min duration at 60°C and 1500 psi. The resulting solution was diluted to 25 ml with water. A 200 mg C-18 solid phase extraction (SPE) cartridge (Waters, Milford, MA) was conditioned consecutively with 5 ml methanol, 5 ml water, 5 ml 2% $NaNO_3$, and 5 ml of phosphate buffer (pH 8.5, 0.1 M). One milliliter of the extracted sample solution was then added to the cartridge, and the cartridge was rinsed with 5 ml water and 3 ml of 5% CH_3CN. The sample was eluted with 1.0 ml CH_3CN into a 5-ml flask. The eluent was diluted to 5 ml with water.

3. Results and discussion

Penicillin G, cephalosporin C, and lincomycin (Fig. 1) were chosen to represent penicillins, cephalosporins, and sulfur-based aminoglycosides, respectively. The thioether group of penicillin G, cephalosporin C, and lincomycin is considered to be the active site of electrocatalytic detection under the conditions presented here. The electrochemical responses of these antibiotics are similar to related compounds within each compound's class and reflect a wide range of adsorption characteristics.

Table 1
Optimized IPAD waveform



Time (ms)	Potential, mV (Ag/AgCl reference)	Potential, mV (pH reference)	Integration period
0.00	240	50	
0.05	240	50	Begin
0.15	1340	1150	
0.25	240	50	
0.35	1340	1150	
0.45	240	50	
0.55	1340	1150	
0.65	240	50	
0.75	1340	1150	
0.85	240	50	End
1.00	240	50	
1.01	1790	1600	
1.11	1790	1600	
1.12	−210	−400	
1.82	−210	−400	

3.1. Voltammetry and waveform optimization

Cyclic voltammetry (CV) is an effective tool to evaluate the electrochemical properties of compounds without the need of a chromatographic separation. In addition, the cyclic waveform used in CV mimics the potential scan in the detection step of the IPAD waveform, and, as a consequence, it is an effective tool for optimizing potential parameters of the waveform. The current–potential (i–E) response is shown in Fig. 2 for a Au RDE in 100 mM NaAc buffer (pH 3.75)/CH_3CN/MeOH (80:10:10, v/v/v) with (——) and without (· · ·) penicillin G in the absence of dissolved O_2. The residual response for the supporting electrolyte (· · ·) exhibits anodic waves

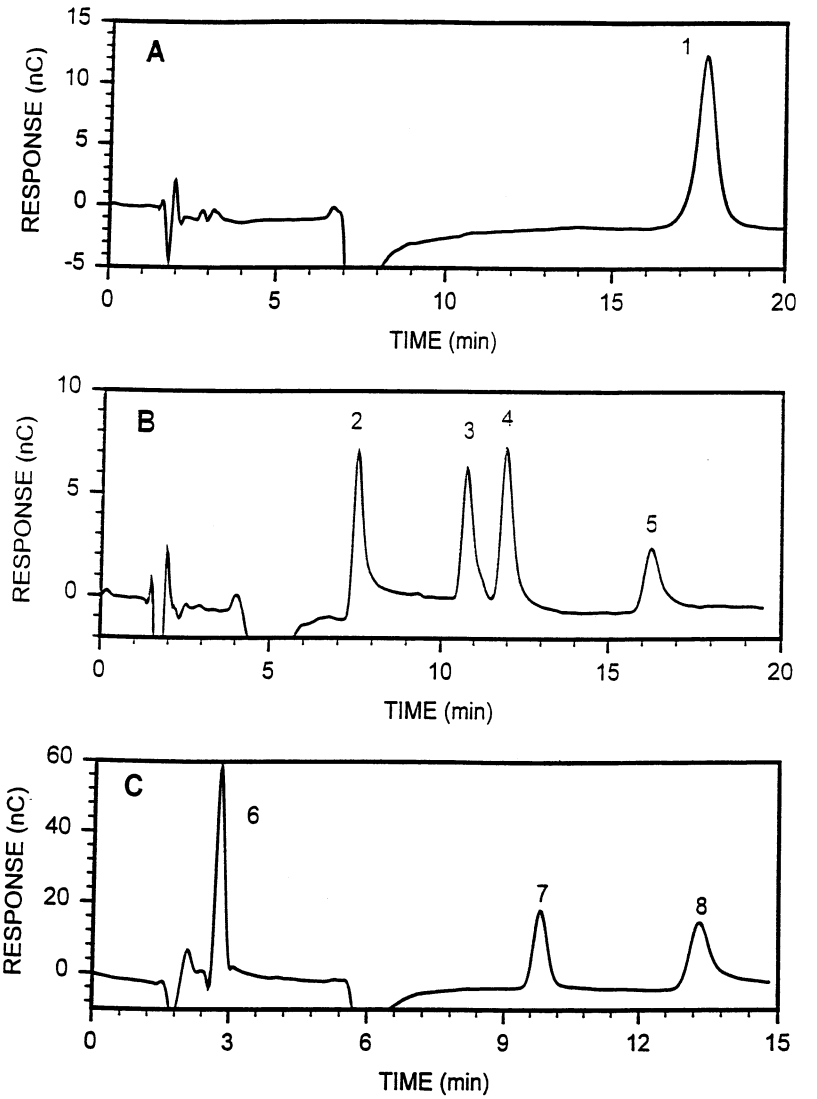


Fig. 4. Chromatograms of standards of (A) lincomycin, (B) penicillins, and (C) cephalosporins. Mobile phase conditions: (A) 20% B, 71% C, 9% D; (B) 36% A, 20% B, 34% C, 10% D; and (C) 15% A, 20% B, 55% C, 10% D. Peaks: (1) lincomycin, 450 ppb; (2) penicillin G, 500 ppb; (3) penicillin V, 500 ppb; (4) oxacillin, 500 ppb; (5) cloxacillin, 500 ppb; (6) cephalosporin C, 1 ppm; (7) cephalexin, 1 ppm; and (8) cefazolin C, 1 ppm.

on the positive scan in the regions of ca. +1000 to +1450 mV (wave *a*) for oxide formation and $E > +1450$ mV (wave *b*) for O_2 evolution. A cathodic peak is obtained on the negative scan in the region of ca. +900 to +400 mV (wave *c*) corresponding to the dissolution of the oxide formed on the positive scan. When penicillin G is added, an anodic wave (——) is observed on the positive scan beginning at ca. +1050 mV (wave *d*). This wave corresponds to oxidation of the sulfur group of the preadsorbed analyte. Wave *d* increases as the concentration of penicillin G increases (Fig. 2). The lack of signal on the reverse scan in the region of +1450 to +800 mV is indicative of the nonreactivity of the oxide-covered surface for thioether oxidation, which sug-

Table 2
Analytical figures of merit

Compound	Mobile phase conditions	LOD, ppb (S/N = 3)	Line equation, nC = $m \cdot$ ppb + b		r^2
			m, nC/ppb	b, nC	
Ampicillin[b]	20% B, 71% C, 5% D	5	0.0087502	0.053800	0.99940
Cephalosporin C	5% A, 20% B, 65% C, 10% D	3	0.027787	0.013272	0.99625
Cephapirin[b]	20% B, 71% C, 5% D	5	0.018303	0.006531	0.99939
Cloxacillin	36% A, 20% B, 34% C, 10% D	8	0.006740	0.020993	0.99905
Lincomycin[a]	20% B, 71% C, 9% D	3	0.014200	0.10870	0.99945
Oxacillin	36% A, 20% B, 34% C, 10% D	6	0.010085	0.020993	0.99867
Penicillin G	36% A, 20% B, 34% C, 10% D	6	0.010772	0.008224	0.99703
Penicillin V	36% A, 20% B, 34% C, 10% D	4	0.008494	0.042873	0.99867

[a] Adapted from Ref. [8].
[b] Adapted from Ref. [11].

gests no signal would be present using dc amperometry.

It is important to note that sulfur-containing compounds are considered to be strongly adsorbed to the electrode surface in comparison to alcohol and amine-based compounds under these conditions. In Fig. 2, thio-oxidation for penicillin G reaches a maximum at ca. 50 mV more positive than the peak response for surface oxide formation. This effect is attributable to penicillin G being strongly adsorbed to the electrode surface. The onset of oxide formation, which was reflected in the shifting of the analyte response also being shifted to more positive potentials, was made more positive in potential due to blockage of active sites on the electrode surface by the adsorbed analyte. Noting that the surface oxide formation process is already partially perturbed by the CH_3CN in the supporting electrolyte, the adsorption effect for penicillin G is particularly strong. Although the shape of the oxide formation wave *a* may be different in the presence of the analyte, the amount of oxide, which corresponds to a monolayer, remains constant as noted by the similar magnitudes of the reduction wave for the surface oxide for all plots. An inference of this observation is that the background subtraction inherent in the IPAD waveform will effectively yield only the charge associated with the analyte.

Fig. 3 shows the i–E plots for cephalosporin C (- - -) and lincomycin (——) in 100 mM NaAc buffer (pH 3.75)/CH_3CN/MeOH (80:10:10, v/v/v) in the absence of dissolved O_2. The residual of the supporting electrolyte ($\cdot\ \cdot\ \cdot$) is also shown. An anodic wave was observed on the positive scan beginning at ca. +900 mV for both compounds corresponding to the oxidation of their respective sulfur groups. The oxidation of thio-groups is classified as an oxide-catalyzed detection, and anodic-oxygen transfer to the analyte is promoted via the labile hydrous oxide intermediates of the forming surface oxide. Note that the onset of activity for the thio-group oxidation occurred at potentials more negative than the apparent wave for the apparent onset of oxide formation in the supporting electrolyte. This observation indicates that the formation of active surface oxide commences to a limited degree in the potential region +800 to +1000 mV. A suppression/shift in the surface oxide formation wave upon addition of these analytes was not observed. This suggests that these compounds are less strongly adsorbed than penicillin G. Had CH_3CN not been present in the mobile phase, a similar effect to that of penicillin G would have been observed for cephalosporin C and lincomycin [8].

Since electrocatalytic detection mechanisms are dictated by the surface state of the electrode, all sulfur-containing compounds are expected to detect at similar potentials. The IPAD waveform was optimized for the analytes of interest using penicillin G (Fig. 2) due to its strong adsorption characteristics. Table 1 shows the optimized wave-

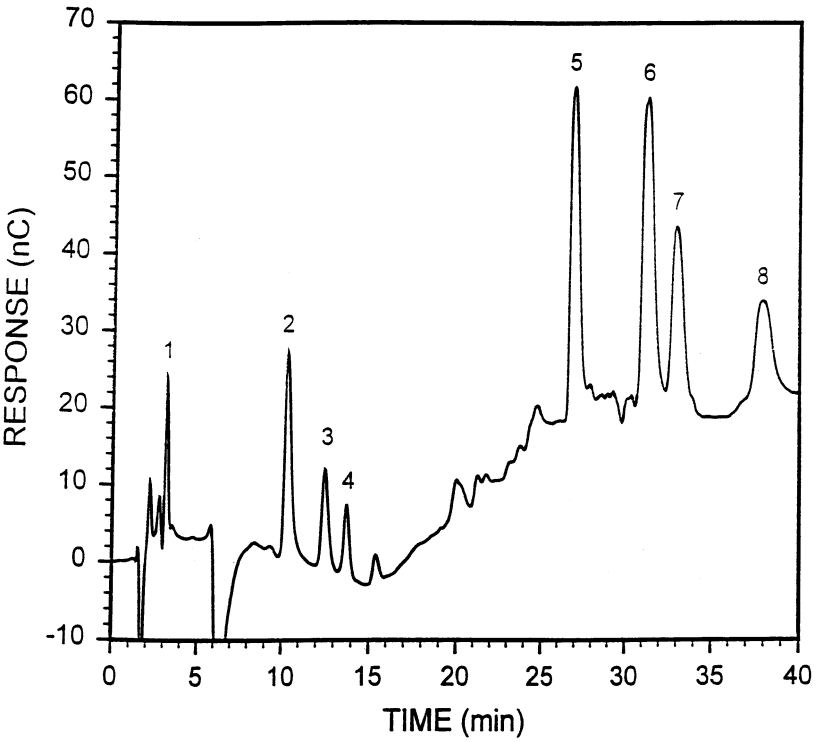


Fig. 5. Gradient separation of a mixture of sulfur-containing antibiotics. Gradient program: 3% A, 20% B, 72% C, 5% D. From 5 to 40 min, A is increased from 3 to 40%. Peaks: (1) cephalosporin C, 500 ppb; (2) cephapirin, 500 ppb; (3) ampicillin, 500 ppb; (4) lincomycin, 500 ppb; (5) penicillin G, 1 ppm; (6) penicillin V, 1 ppm; (7) oxacillin, 1 ppm; and (8) cloxacillin, 1 ppm.

form for the detection of sulfur-containing antibiotics for the Ag/AgCl reference electrode. In this waveform, the initial potential (E_{dst}) of the detection step is held at +240 mV for 50 ms to allow for the preadsorption of the analyte. At this same potential, which is before the formation of surface oxide, the scan is started and it is ramped to a potential (E_{dmx}) of +1340 mV. E_{dmx} is more positive than the start of oxide formation and more negative than the region where anodic solvent breakdown (i.e. O_2 evolution) occurs. The scan is then reversed and ends at a potential (E_{dnd}) of +240 mV, which is more negative than that which is required for cathodic dissolution of the formed oxide. This potential is also more positive than the reduction of dissolved O_2. Hence, the contribution to the overall signal from the reduction of dissolved O_2 is minimized, which is important when working with non-fully deoxygenated solutions, as in chromatography. In this case, the triangular waveform in the detection step is repeated four times to fully exploit the signal from the transient oxide intermediates of this oxide-catalyzed mechanism. The signal-to-noise ratio (S/N) was maximized by integrating over all four cycles. E_{oxd} was selected to be +1790 mV for a period of 100 ms to achieve a fully formed gold oxide layer to allow for complete cleaning of the electrode surface. E_{red} was held at −210 mV for 700 ms. These parameters were empirically determined to be the best and gave stable response characteristics for months of continuous use. When the pH reference electrode is used, all potentials are changed by −190 mV (see Table 1).

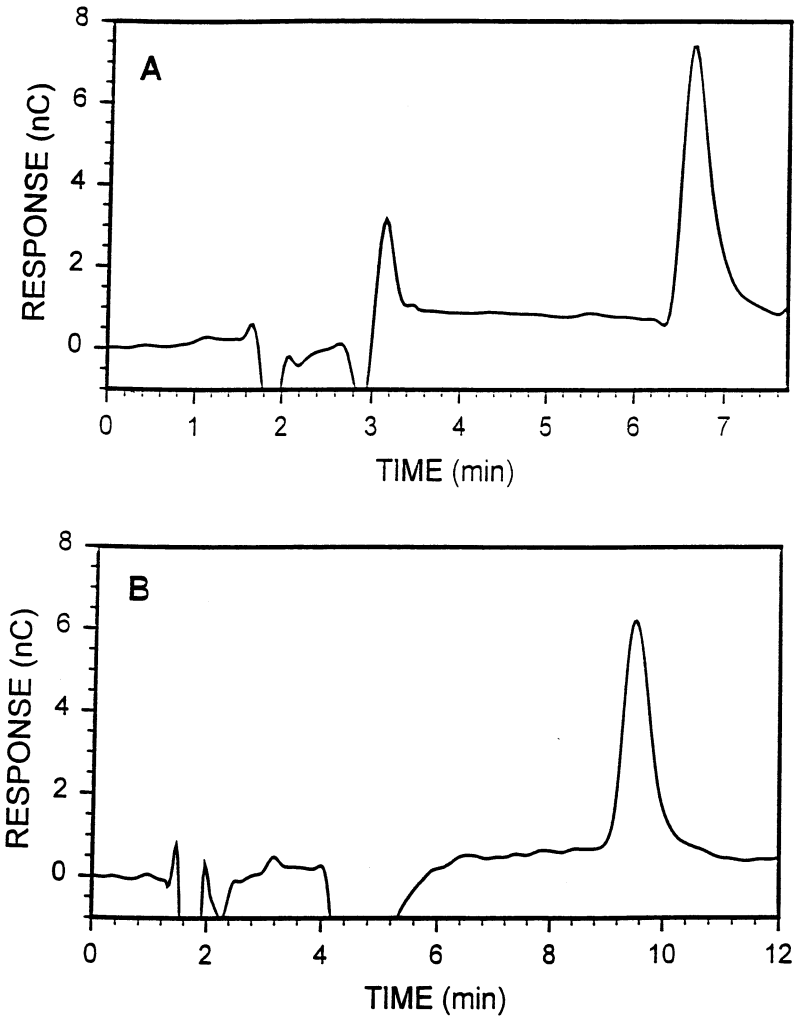


Fig. 6. Chromatograms of pharmaceutical preparation of an (A) amoxicillin capsule and a (B) PenVee K tablet. Mobile phase conditions: (A) 20% B, 77% C, 3% D and (B) 36% A, 20% B, 34% C, 10% D.

3.2. Reversed-phase chromatography with integrated pulsed amperometric detection

Sulfur-containing antibiotics were separated by a reversed-phase mechanism with CH_3CN and/or MeOH as organic modifiers and a NaAc buffer (100 mM, pH 3.75) to maintain the pH for optimum detection conditions. A C-8 column was chosen to allow for the separation while minimizing the amount of CH_3CN needed. Preadsorption of the analyte to the electrode surface increases their sensitivity in PED. Hence, CH_3CN with its affinity for the Au electrode has been shown to attenuate the response of weakly-adsorbed compounds (e.g. carbohydrates and other alcohol-containing compounds) by blocking adsorption of these analytes to the electrode surface. Sulfur moieties are strongly adsorbed to the electrode surface, and, as a consequence, they can effectively compete with CH_3CN for adsorption sites on the electrode surface. No suppression of analyte signal was observed by varying the amount of CH_3CN. Fig. 4A–C show chromatograms for standards of lincomycin, penicillins, and

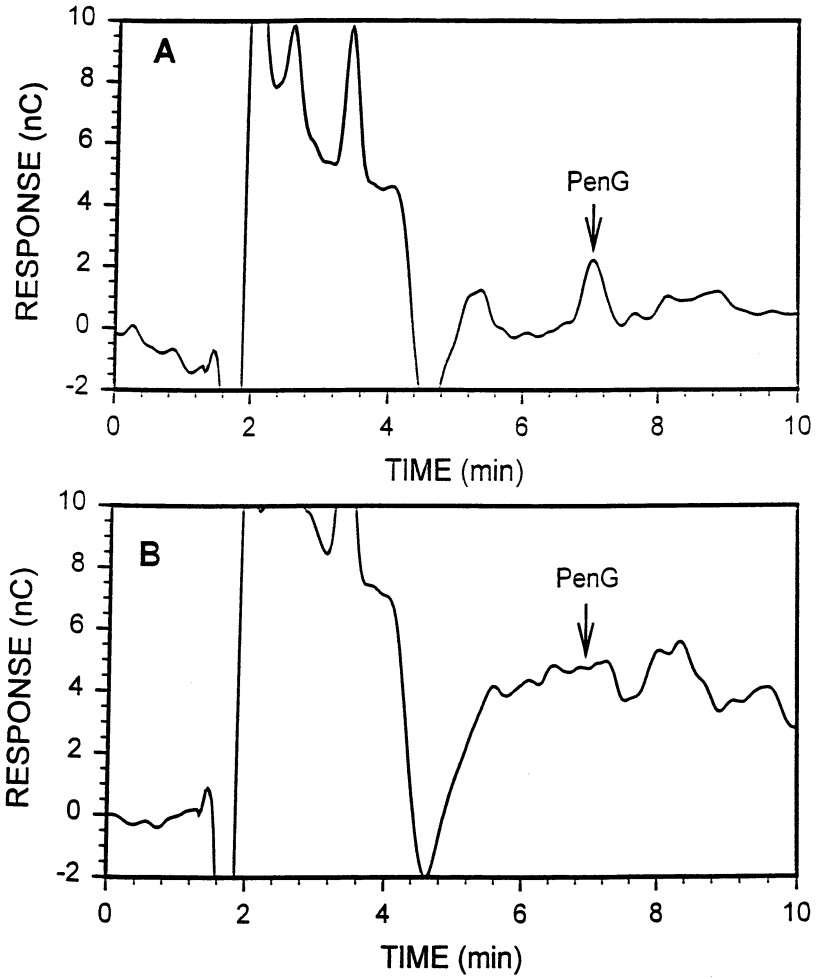


Fig. 7. Chromatograms of (A) medicated feed extract and (B) blank by HPLC-IPAD. Mobile phase conditions are 36% A, 20% B, 34% C, 10% D.

cephalosporins, respectively. The peak shapes for all these compounds are acceptable with asymmetry factors (b/a) of 1.0–1.3. Since the mobile phase is degassed, dissolved oxygen in the sample is always at a greater concentration than in the mobile phase, which is reflected in a negative peak in all chromatograms that is always present. The baseline is quite clean and the response is stable over long periods of time.

The analytical figures of merit for a variety of compounds are given in Table 2. These compounds were separated using similar conditions to those in Fig. 4. All compounds are linear over two to three decades with correlation coefficients ranging from 0.99625 to 0.99945. Under their respective conditions, limits of detection ranged from 3 to 8 ppb at a $S/N = 3$. These analytical figures of merit are typical for virtually all sulfur-containing antibiotics by IPAD.

As expected for a reversed-phase mechanism, changes in the concentration of the organic modifier controls the retention of all these compounds. Although varying the amount of CH_3CN does not affect the detection of sulfur-containing antibiotics, the oxide formation process is perturbed by the CH_3CN, which is reflected in shift-

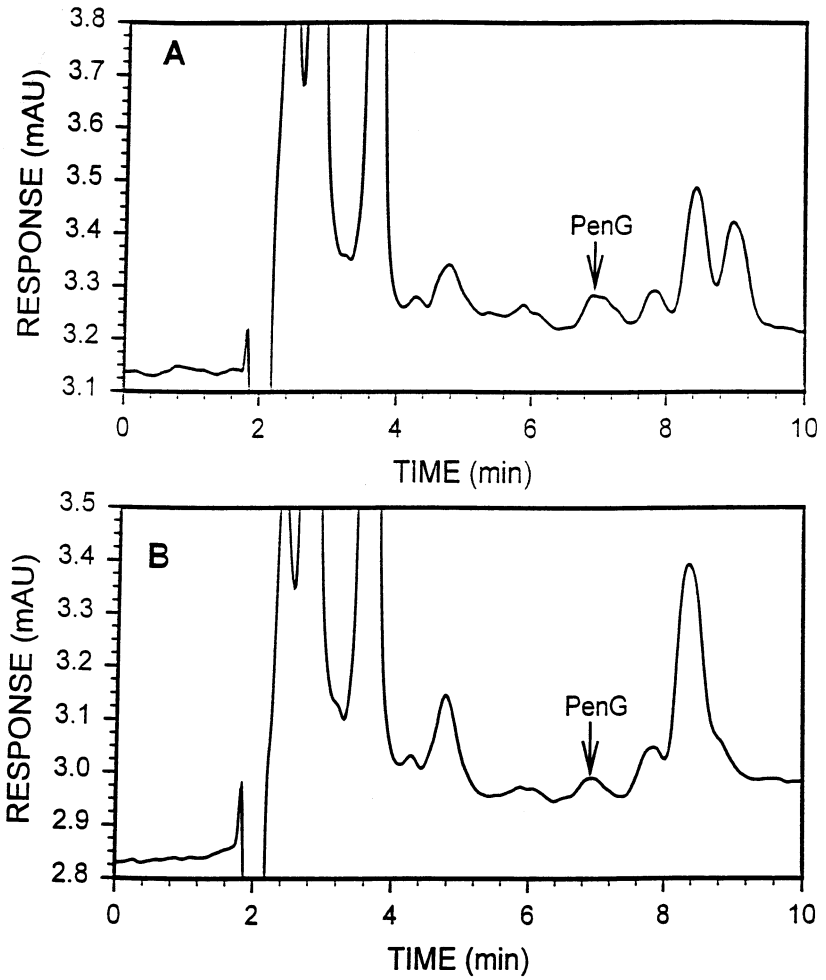


Fig. 8. Chromatograms of (A) medicated feed extract and (B) blank by HPLC-UV at 254 nm. Mobile phase conditions are 36% A, 20% B, 34% C, 10% D.

ing in the background signal. Hence, an organic modifier gradient using CH_3CN often results in major baseline shifts as well. This effect can be dramatically reduced by holding the CH_3CN content of the mobile phase constant and varying the concentration of a weakly-adsorbing organic modifier (e.g. MeOH), which is effectively blocked from the electrode surface by the CH_3CN. Fig. 5 shows a gradient separation of eight sulfur-containing antibiotics which vary greatly in hydrophobicity. Many of the minor peaks are impurities also present in the individual standards. The baseline shift is a result of the gradient.

3.3. Applications

Numerous sulfur-containing compounds can be assayed with the ascribed conditions with simple changes in the mobile phase conditions. The following applications were generated to highlight the utility of HPLC–IPAD and were not intended to be analytically rigorous. Hence, all samples and standards were used 'as is'.

Amoxicillin capsules (250 mg) and PenVee K tablets (250 mg) were assayed using the HPLC-IPAD system with different mobile phase conditions. Typical chromatograms of the amoxicillin (Fig. 6A) and PenVee K (Fig. 6B) solutions were clean with only one major analyte peak, which in each case eluted under 10 min. The amount of amoxicillin in the capsules was determined to be 240 ± 7 mg (3% R.S.D., $n = 3$), which is 96% of the expected dose. The R.S.D. for replicate injections was 0.5% ($n = 3$). A similar assay of the PenVee K tablets resulted in a penicillin V content of 229 ± 9 mg (4% R.S.D., $n = 3$). The PenVee K tablets assayed at 92% of the expected dose. Both results were lower than expected; however, antibiotics degrade with age, and the amoxicillin capsules and PenVee K tablets were 2 and 3 years past their effective dates, respectively. Also no precautions were taken to store the left over prescriptions in any way. HPLC-IPAD has been shown to be very effective for the determination of the purity and degradation analysis of antibiotic preparations, in that IPAD can effectively detect any sulfur-containing degradant that is not fully oxidized.

A true test of the advantages of this technique is for the development of assays for the detection of antibiotics in feeds. Feed samples, used for toxicological studies and veterinarian formulations, require extraction of the analyte of interest from the feed, which often results in complex matrices.

Extraction by ASE was performed on a penicillin G-containing feed medicated at a level of 99.6 g/ton. The extracts were pretreated using SPE to reduce matrix interferents. Extraction efficiencies were determined to be $92 \pm 3\%$. Fig. 7A,B shows chromatograms of penicillin G in the feed extract and a blank, respectively. The coefficient of variation at 100 ppb for this assay was 1%. The feed was found to contain 95.0 ± 5.5 g/ton (6% R.S.D., $n = 3$) of penicillin G by IPAD. Fig. 8A,B shows chromatograms of a typical feed extract and blank, respectively, by UV detection at 254 nm. The peak in the sample corresponds to a similar magnitude peak in the blank. Hence, quantitation was not possible. Detection at lower wavelengths resulted in significantly higher backgrounds.

4. Conclusions

Pulsed electrochemical detection following HPLC allows for the simple and direct detection of numerous sulfur-containing antibiotics, especially penicillins, cephalosporins, and lincomycin. In agreement with past efforts, IPAD is well-suited for the detection of thiocompounds. The IPAD waveform, which needs only to be optimized for a particular pH, allows for the direct detection of the analytes at low levels with limits of detection superior to UV detection at 254 and 214 nm. Through the use of mildly acidic mobile phases under reversed-phase conditions, selectivity is achieved for sulfur-containing compounds. This feature simplifies the resulting chromatograms from complex samples and aids in the easy interpretation of the chromatograms. The system performance was excellent for all pharmaceutical preparations assayed. This method is applicable to most sulfur-containing antibiotics with minor changes to the elution protocol from the SPE cartridge and in the chromatographic conditions. Also, IPAD has been shown to be readily adaptable to capillary electrophoresis, and sulfur-containing antibiotics have been separated and detected using CE-IPAD [18].

Acknowledgements

The authors would like to thank Mary Jo Smith and Fieldale Farms (Baldwin, GA) for the feed samples. Also, the authors would like to acknowledge the use of an ASE unit from Dionex. This work was supported by Grant # FD-R-000903 from the USFDA.

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