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High-performance liquid chromatographic separation and electrochemical detection of cephalosporins

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Abstract

Pulsed amperometric detection (PAD) is useful for detection of cephalosporins following separation on a C_{18} column using an acetate buffer solvent with a small percentage of organic modifier. Under these conditions, the indirect PAD mode worked better than direct PAD, with IPAD outperforming both. A gradient program was demonstrated that allowed separation and sensitive electrochemical detection of eleven different cephalosporins with widely differing side chain structures. The cephalosporins could be detected to sub-micromolar levels with this separation. Applications of the method for quantitation of pharmaceutical formulations and for monitoring cephalexin in porcine serum were demonstrated. To improve the detectability of cephalexin, an on-column concentration scheme using separate concentration and elution solvents was applied to porcine serum.

Keywords: Cephalosporins

1. Introduction

Cephalosporins are antibiotics from the β-lactam family, which inhibit bacterial cell wall synthesis of different Gram-positive and Gram-negative bacteria, often prescribed to fight organisms that have become penicillin resistant. They are used to treat a wide variety of infections throughout the body, and also find common use as prophylaxis for surgical procedures where infections in the operating area could pose a serious risk [1]. Cephalosporins also have veterinary applications, notably for shipping fever, pneumonia, and mastitis in beef and dairy animals; they are also used in dogs [2]. Cephalosporin C was first isolated in 1948 from the fungus *Cephalosporium acremonium*. Chemical modification of cephalosporin C allowed production of a whole series of semisynthetic cephalosporins, many of which are currently in use as therapeutics [3].

Numerous situations require quantitative analysis of cephalosporins. Quality control measurements are mandated on each production batch of the pharmaceutical formulations [4]. Manifold pharmacological studies for elucidation of dosage amounts, toxicity and allergic response, and deduction of resistant bacterial strains require quantitative measures. Much like the structurally similar penicillins, hypersensitive reactions to cephalosporins are not uncommon and range from minor rash responses to severe cases of anaphylaxis. This is exacerbated by the fact that no reliable skin test exists to predict whether a patient will undergo an allergic response

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[3]. Due to the veterinary applications of cephalosporins in cattle, unsuspecting consumers risk some degree of accidental exposure from dietary meat and milk.

Microbiological assays for cephalosporins are available but are not specific for particular antibiotic species [5]. To provide this specificity, most chemical methods employ a separation system, with methods including thin-layer chromatography [6,7], pyrolysis gas chromatography [8], an immobilized enzyme reactor [9], and, more recently, capillary electrophoresis [10]. However, the vast majority of methods for analysis of cephalosporin employ HPLC to allow analyte speciation in the face of the multitude of cephalosporins and structurally related degradation products. Reversed-phase separations predominate, with C_{18} stationary phases being the most common [11], although other resins have seen limited use [12–14]. These separations have generally employed aqueous buffer mobile phases modified with a small percentage of either methanol or acetonitrile, with UV absorbance detection in the 220–280 nm range [11]. All of the cephalosporins and most of their degradation products absorb in this region, and the sensitivity is reasonable, with limits of detection (LODs) often being quoted in the vicinity of 0.1 ppm [12,13].

UV detection for cephalosporins has been stated to lack sufficient selectivity for work with biological fluids [15]. For this reason and to pursue greater sensitivity, other detection systems have been examined for these compounds. Blanchin et al. [15] were able to label cephalosporins containing a primary amine group on their side chain postcolumn with fluorescamine, followed by fluorescence detection. Similar procedures with fluorescamine have been presented [16], and others have described production of fluorescent cephalosporin derivatives by corrosive degradation of the parent structure [17–19]. Selavka et al. [20] demonstrated photolytic derivatization of cefoperazone with an intense UV source following HPLC, allowing amperometric sensing at a downstream glassy carbon electrode.

Although a significant number of studies have examined the electrochemistry of various cephalosporins, very few of these have been utilized as part of a chromatographic detection scheme. One electrochemical procedure that has wedded well with HPLC for pharmaceutical detection is pulsed amperometric detection (PAD), considered a subset of pulsed electrochemical detection (PED) [21,22]. In particular, the penicillins, which are structurally similar to the cephalosporins, have been determined using PAD [23–25]. Recent work in this laboratory [26], has shown that cephalosporins can be determined in a flowing stream to trace levels by direct PAD, indirect PAD, and integrated pulsed amperometric detection (IPAD). The goal of this work was to adapt the PAD detection to render it compatible with a reversed-phase HPLC separation, which could then be applied for cephalosporin determinations.

2. Experimental

2.1. Materials and reagents

All cephalosporins were purchased from Sigma (St. Louis, MO, USA). Ceclor tablets were manufactured by Eli Lilly (Indianapolis, IN, USA), and contained 250 mg of cefaclor. Keftab tablets were manufactured by Dista (Indianapolis, IN, USA), and contained 500 mg of cephalexin. 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) 0.20-μm nylon filter and sonicated before use. Samples were filtered through nylon 0.45-μm syringe tip filters before injection into the HPLC. Standard solutions were prepared in either the chromatographic solvent or water. Serum samples were obtained from the supernatant of fresh porcine blood after centrifuging for 10 min at 2600 *g*. Prior to injection, 10.0 ml of serum was mixed with an equal volume of acetonitrile, vortexed for 20 s, then centrifuged for 10 min at 2040 *g*. The supernatant recovered from this procedure was diluted to a volume of 50 ml with DI water before injection.

2.2. Chromatographic apparatus

A Spectra Physics (San Jose, CA, USA) IsoChrom HPLC pump coupled with an Isco (Lincoln, NE, USA) Model 2368 gradient programmer was used for flowing stream studies. The standard flow-rate was 1.0 ml/min unless noted otherwise. A Rheodyne (Cotati, CA, USA) model 7010 injection loop fitted with a 50-μl injection loop was used for the injection of samples. The dual pump trials employed an SSI (State College, PA, USA) Model 300 LC Pump and with an SSI Model 210 Guardian module for delivering the sample solution. A Waters (Milford, MA, USA) Nova-Pak C_{18} Radial-Pak cartridge (100×8 mm, particle size 4 μm) was utilized in a Waters 8×10 radial compression module. All separations were done at ambient laboratory temperature (~20±2°C). The Dionex (Sunnyvale, CA, USA) pulsed electrochemical detector was utilized with a Ag/AgCl reference electrode and a stainless steel counter electrode. The working electrode was a circular gold model with a diameter of 2.0 mm. Data from the Dionex potentiostat was acquired using a 386SX IBM compatible computer using a Keithley (Rochester, NY, USA) Chrom1-AT interface card and Galactic (Salem, NH, USA) LAB CALC software. The 'multiple point baseline correction option' of LAB CALC was undertaken where noted, using a 30-point correction. All LODs are quoted at $S/N=3$.

For single-pump on-column concentration procedures, a single chromatographic pump pushed solvent through the column. The experiment was initiated by pumping the concentration solvent through the chromatographic column to the detector. Upon switching to the elution solvent, a solution change was made without alteration to the flow path. For the dual-pump approach, during the concentration phase the concentration solvent was propelled by one pump through the chromatographic column and then out to waste, while the elution solvent was pushed by the other pump through the detector cell to waste without going through the chromatographic column. At the end of the concentration phase, a valve switch reroutes the flow such that the pump propelling the elution solvent forces this solution through the chromatographic column and then to the detector cell, while the concentration solvent is pumped directly to waste (and is often shut off during this phase).

3. Results and discussion

The flow injection analysis results showing that direct PAD, indirect PAD, and IPAD all detected the cephalosporins with excellent sensitivity [26] opened possibilities, as it was likely that one of these modes would adapt well with whatever chromatographic solvent program was necessary, as long as sufficient electrolyte were present. Using the Nova-Pak C_{18} column, pure aqueous buffer had insufficient eluting power to remove the cephalosporins from the stationary phase. Both acetonitrile and methanol performed well as organic modifiers, reducing retention of the cephalosporins as their percentage content was increased. As is illustrated in Fig. 1 for cephalexin, the eluting power of acetonitrile was greater than that of methanol on the Nova-Pak C_{18}. The advantage of acetonitrile was not strictly based on polarity, but was more a function of the relative adsorptive strengths with regard to the stationary phase.

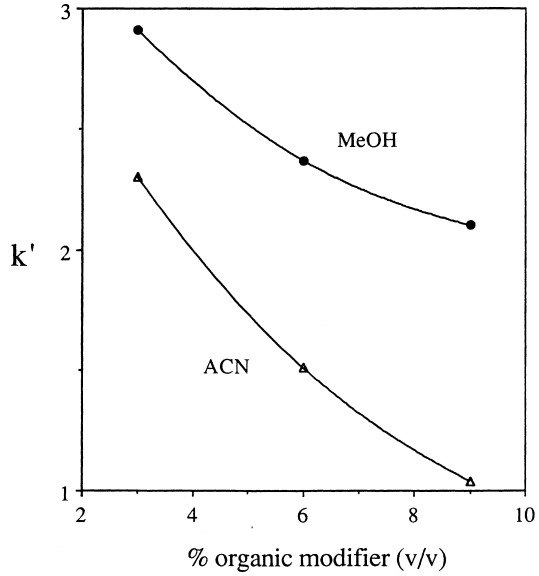


Fig. 1. Effect of organic modifier content on k' value of cephalexin; Nova-Pak C_{18} column. The unspecified fraction of the solvent is 0.01 *M* acetate buffer (pH 4.7).

Chromatographic trials using the NovaPak C_{18} column led to the adaptation of mobile phases that in general contained about 87% 0.01 *M* acetate buffer (pH 4.7) with the balance being organic modifier as either acetonitrile, methanol, or a mixture of the two. Detector studies showed that indirect PAD was preferable to direct PAD using the gold working electrode with the aforementioned solvent systems. Selecting a specific solvent containing 0.01 *M* acetate buffer (pH 4.7)–methanol–acetonitrile (87:11:2, v/v/v), both indirect PAD and IPAD waveforms were optimized for detection of cephalexin. The optimized waveforms are given in Table 1A and B respectively. The cephalexin limits of detection were $8 \cdot 10^{-8}$ *M* (30 ppb) for indirect PAD and $3 \cdot 10^{-8}$ *M* (10 ppb) for IPAD.

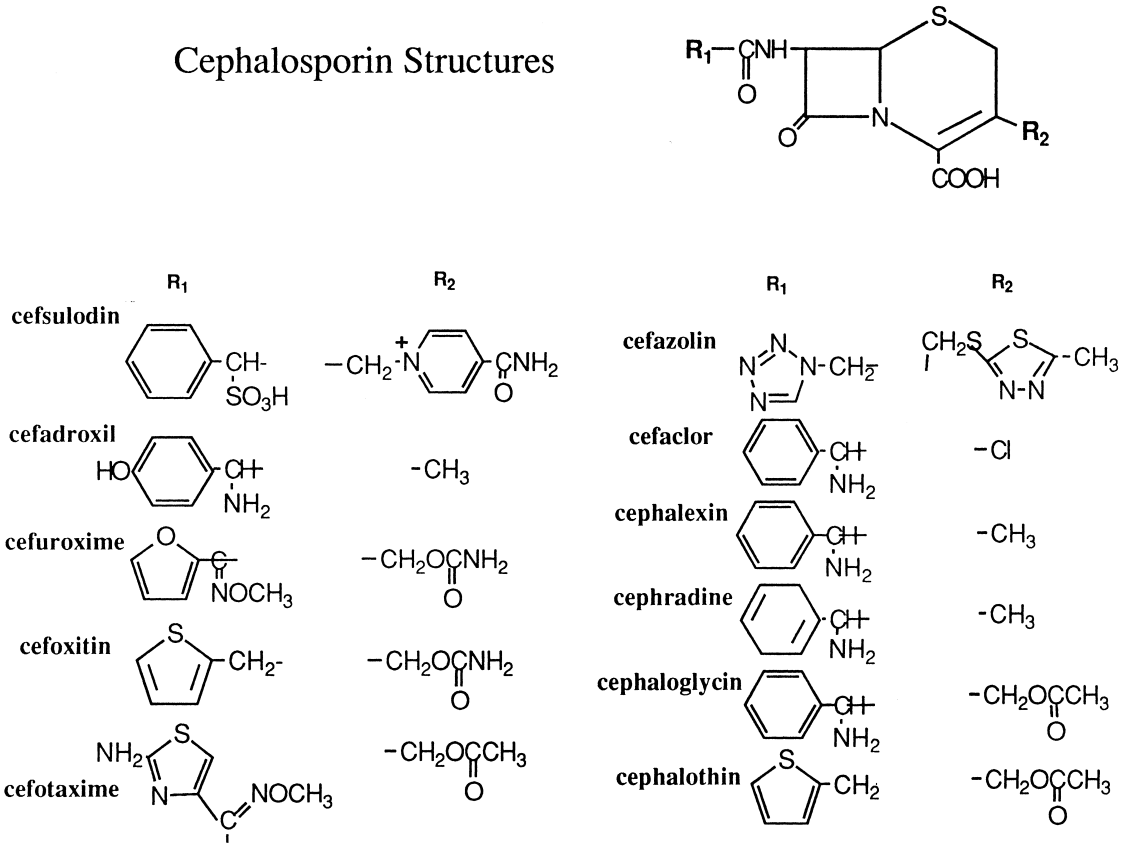
Chromatographic studies were initiated with the goal of speciating a mixture of eleven cephalosporins (see Table 2 for the formulae of the cephalosporins). This was made difficult by the wide variety of side chain groups present, yielding a broad range of polarities and the expected broad range of k' values under a given set of chromatographic conditions. Isocratic elution was unable to achieve a successful separation of the entire mixture, so gradient elution was investigated. Preliminary trials indicated that an increase in the amount of organic modifier over time would be helpful in producing a good separation, but there were concerns about the impact of this type of gradient on the PAD response. A number of prior reports have described grappling with this conundrum [24,27,28]. Detection via indirect PAD seemed certain to be altered, although the affect on IPAD was harder to predict. An early report on IPAD predicted that its baseline was virtually unaffected by solvent programs [29], although other applications have noted significant shifts in response to gradients [30,31]. Early gradient trials where the percentage of organic modifier was increased produced baseline shifts with both indirect PAD and IPAD. One strategy that cut back on the baseline shift was utilizing a ternary buffer–methanol–acetonitrile solvent and keeping the total fraction of organic modifier constant. Given the difference in elution strength of methanol and acetonitrile seen in Fig. 1, solvent eluting power could be increased by exchanging methanol content for acetonitrile, maintaining the same overall percentage of organic modifier.

The optimized solvent program, described in Table 3, still produced a shift in baseline. A comparison of the separation with indirect PAD (Fig. 2A) and IPAD (Fig. 2B) modes of detection shows the difference in response. The IPAD actually produces a greater baseline shift upon gradient application than indirect PAD. The IPAD shift is much smaller in a relative sense compared to the typical peak heights, as the indirect PAD produces a less sensitive response to the cephalosporins. Given that all of the cephalosporins are present at identical concentrations, the indirect PAD response is remarkably

Table 1
Description of PAD waveforms

A		B	
Indirect PAD		IPAD	
Time (ms)	Potential (*V* vs. Ag/AgCl)	Time (ms)	Potential (*V* vs. Ag/AgCl)
0 to 150	1.48	0 to 220	0.04
150 to 320	1.85	220 to 420	0.04 to 1.30
320 to 470	−.28	420 to 720	1.30
		720 to 1020	1.30 to 0.04
		1020 to 1370	0.04
		1370 to 1380	0.04 to 1.50
		1380 to 1580	1.50
		1580 to 1590	1.50 to −0.20
		1590 to 1790	−0.20
		1790 to 1800	−0.20 to 0.04
Integrate: 100 to 150 ms		Integrate: 220 to 1370 ms	

Table 2



consistent for the differing structures. IPAD response was consistent except for cefadroxil (peak 2) which was larger, and cefazolin (peak 6) which was smaller than the other peaks. Cefadroxil contains an easily oxidized phenol side chain group, which allows it to give a greater anodic response than the typical cephalosporin on a per mole basis, due to oxidation occurring at both the side chain and the sulfide within the basic cephalosporin structure. Cefazolin is unique among the group studied in having thiadiazole and tetrazole side chain groups. Presumably, sensitivity is lower due to adsorption of one of these groups at the gold surface without oxidation. This would be consistent with the observation that indirect PAD response is standard for cefazolin, although voltammetric studies did not show any obvious evidence to support this conclusion. Despite the lowered sensitivity to cefazolin, IPAD still yields a better LOD than indirect PAD. IPAD was the superior detection method, and by applying a software routine for flattening baselines, the perturbation during the gradient could be virtually eliminated while nearly achieving baseline resolution of the eleven cephalosporins.

Table 3
Gradient separation program

Time (min)	Solvent A (%)	Solvent B (%)
0 to 5	100	0
5 to 15	100 to 70	0 to 30
15 to 27	70 to 0	30 to 100
27 to end	0	100

Solvent A: 0.01 *M* acetate buffer (pH 4.7)–methanol–acetonitrile (87:11:2, v/v/v). Solvent B: 0.01 *M* acetate buffer (pH 4.7)–methanol–acetonitrile (87:2:11, v/v/v).

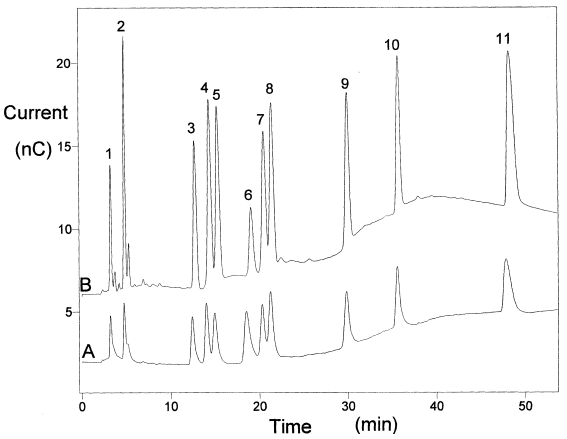


Fig. 2. Comparison of indirect PAD and IPAD response to the solvent gradient; Nova-Pak C_{18} column. Waveforms: given in Table 2. Cephalosporins all present at $2 \cdot 10^{-5}$ *M*. (1) Cefsulodin, (2) cefadroxil, (3) cefuroxime, (4) cefoxitin, (5) cefotaxime, (6) cefazolin, (7) cefaclor, (8) cephalexin, (9) cephradine, (10) cephaloglycin, (11) cephalothin. Solvent program: given in Table 3. Traces: (A) indirect PAD, (B) IPAD.

Two blind studies were undertaken using commercial cephalosporin formulations. A Ceclor capsule (containing cefaclor) and a Keftab tablet (containing cephalexin) were ground to a powder, then a portion removed, weighed, and diluted to volume. It was assumed that the purity of the pharmaceutical formulation was accurately described by the product label. These solutions were injected repeatedly along with a series of premade standard solutions; detection used the optimized IPAD waveform. The concentration was maintained below $1 \cdot 10^{-4}$ *M* for each to avoid potential problems with calibration curve nonlinearity [32]. The calculated percent recovery for the Ceclor was calculated to be 101.8%, for the Keftab 103.3%.

Another test of the PAD methodology was made by applying it to determination of cephalexin in porcine serum. Although cost has inhibited the application of cephalosporins in swine, various clinical studies have been made and the role of cephalosporins is expanding in veterinary medicine. A spiked standard solution of the serum was made by adding a known amount of cephalexin at the same stage as the acetonitrile (see Section 2). A comparison of PAD response to a cephalexin spike solution in serum and a standard of the same final concentration made from DI water yielded the information that 36.0% of the cephalexin was recovered in the serum sample. Using the gradient separation scheme outlined above, an overlay was produced showing the baseline response to a blank serum injection compared with an injection containing a cephalexin spike (Fig. 3). The serum shows a considerable background response, most notably in the first 10 min of the separation. Delectability suffers to a small degree for cephalexin in this complex matrix; the LOD measured was $5 \cdot 10^{-7}$ *M* (200 ppb).

Moats [33] stated that antibiotic assays ideally need to be able to detect quantities as low as 10 ppb, which would require an improvement over the serum LOD measured as part of the direct assay, and would be at the limits of the method capability. Alternative sample preparation schemes were pursued that would reduce the cephalexin LOD even further. Kirchmann et al. [25] succeeded in using a solvent-switching concentration method for extending the LOD for penicillins being measured in a milk matrix, and a similar procedure was investigated for use with the cephalosporins. An examination of solvents led to the conclusion that purely aqueous mobile phases containing a high buffer concentration produced essentially infinite retention for cephalexin, stockpiling it on the stationary phase. A solution of 0.2 *M* acetate buffer (pH 4.7) could thus be used as a concentration solvent. By making up the cephalexin

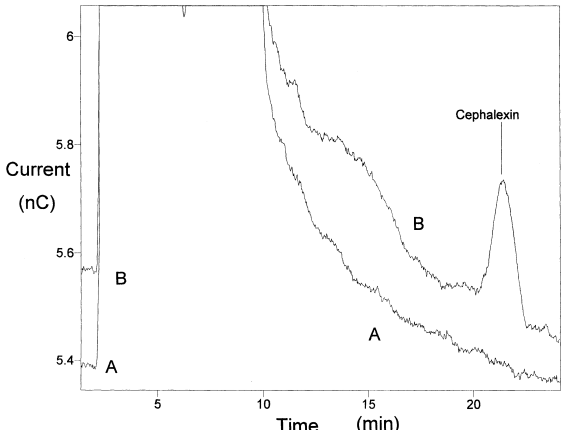


Fig. 3. Detection of cephalexin in serum. Waveform: IPAD, given in Table 2B. Solvent program: given in Table 3. (A) serum blank, (B) serum spiked with $5.6 \cdot 10^{-6}$ *M* cephalexin (after dilution).

sample in the concentration solvent and pumping it for set periods through the Nova-Pak column, very dilute cephalexin solutions, undetectable in a direct assay, could be concentrated on column to detectable levels. By then switching the solvent to the standard chromatographic eluent, the concentrated plug of cephalexin could be separated and washed off the column as done previously with injected sample solutions.

The concentration scheme was done both as a single-pump and a dual-pump method; most of the described results were done with the dual pump approach. One important practical point was that it was advantageous to have the chromatographic solvent pumped through the PAD flow cell at all times, which was only possible with the dual pump approach. If the effluent from the concentration phase was allowed to flow through the detector cell, the changeover to the eluting solvent caused a large perturbation, which was especially problematic when trying to monitor very low cephalexin concentrations. By using the dual pump system, the flow of eluting solvent to the detector could be continued, even during a concentration phase on the column. With this scheme, the only portion of the concentration solvent that reached the column was the volume that was resident within the column upon switching solvents.

Given the nature of the concentration scheme, detectability increased as concentration time increased; LODs ultimately could become a function of the amount of sample and time available. Increasing concentration time reached the point of diminishing returns somewhere of the order of 30–40 min concentration time for spiked serum samples, at which point baseline noise problems began to override the increase in time. The best detectability seen for cephalexin was $2 \cdot 10^{-9}$ M (0.7 ppb), using a concentration time of 30 min and IPAD detection (Table 1B waveform).

4. Conclusions

A gradient program was demonstrated that allowed separation and sensitive electrochemical detection of eleven different cephalosporins with widely differing side chain structures, suffering only a slight baseline perturbation from the gradient. LODs were of a similar magnitude as those reported for the standard practice of measuring UV absorbance. These limits of detection can be extended to lower values by employing an on-column concentration scheme, which could be completely automated if so desired. Successful application of this concentration scheme does depend on having significant volumes of sample available (>1 ml). Both direct detection of cephalexin and detection after on-column concentration were successful for serum samples. Determination of other later eluting cephalosporins could easily be done in a similar manner. The large background response from the serum would interfere with detection of some of the earlier eluting species; an alternative separation program would be needed to achieve LODs for these compounds that are similar to those reported for cephalexin.

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