Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 1, pp. 83–93, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Press Ltd.

Analysis for penicillins and cefoperazone by HPLC–photolysis–electrochemical detection (HPLC–hv–EC)

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Abstract: Continuous, post-column, on-line, real-time photolytic derivatization or degradation can now be used following HPLC separation of various penicillin derivatives prior to conventional thin-layer, amperometric electrochemical detection using oxidative working potentials. Beta-lactam derivatives are separated by conventional reversed-phase HPLC, and each separated penicillin is then photolytically degraded to form, it is presumed, stable anionic species, which are then conveyed to the on-line electrochemical detector for qualitative and quantitative determinations. These methods of trace drug analysis have been applied to four separate penicillins or prodrugs, as well as one typical cephalosporin, *viz.* cefoperazone. Analytical parameters of the analysis have been determined, including dual electrode response ratios, sensitivity, minimum detection limits, linearity of calibration plots and the range of linear calibration. Finally, the analysis of cefoperazone-spiked saline solutions for i.v. administration has been performed in a single-blind study, as well as the determination of bacampicillin HCl in formulations obtained from a drug manufacturer in the United States. The overall method of analysis for these drugs has been demonstrated as being reproducible, accurate, and precise for at least five *beta*-lactam analogs. It is suggested that other *beta*-lactams will be amenable to these newer methods of analysis in a wide variety of sample matrices, including solid or liquid formulations, aqueous infusion solutions, and biological media, such as blood and urine.

Keywords: *Ampicillin; bacampicillin HCl; penicillin G; penicillin V; cefoperazone; HPLC; post-column derivatization; photolysis; electrochemical detection.*

Introduction

Penicillins and cephalosporins form a very large class of *beta*-lactam antibiotics that have been synthesized, analysed, and studied in depth for many decades [1–21]. Despite this long-standing and sustained interest in the analytical chemistry of various beta-lactams, the current HPLC-based approaches generally suffer from a number of deficiencies.

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Virtually all of these methods use either UV-VIS or fluorescence (FL) detection, with or without pre- or post-column derivatizations, but the final sensitivity and detection limits may be less than adequate for biological fluid and tissue work. In addition, all of the common detection methods, with the exception of liquid chromatography–mass spectrometry (LC–MS), lack a high degree of analyte selectivity or specificity. Even those methods which rely on post-column derivatization are not uniquely selective to *beta*-lactams [10]. Electrochemical (EC) detection approaches have not been extensively studied in either the HPLC (LCEC) or flow injection analysis (FIA) of these compounds, and this would relate to the general lack of suitable oxidative and/or reductive properties of most of these antibiotics [13, 22–24]. In addition, even for those methods wherein the use of electrochemical detection has been described for a limited number of beta-lactams, dual LCEC approaches have not, as yet, been used.

For about the past year and a half, the present authors have been directly involved in the research and development of post-column photolytic derivatizations (*viz.* degradation) for LCEC [25–28, 42]. Though continuous, post-column, on-line photochemistry has been used in HPLC-UV and HPLC-FL in the past [29–41], only a few methods have been described for EC detection. Even the work of Snider and Johnson did not utilize continuous, on-line, real-time photolysis of the HPLC eluants prior to the final EC detection step [34]. At times, chemical visualization reactions of photolytically generated products derived from chromatographically separated analytes have also been used [40]. Photolytic derivatizations in LCEC (HPLC–hv–EC) have been applied for the determination of a number of organic thiophosphate agricultural chemicals, such as malathion and parathion [25]. These same approaches have also been used for the trace analysis of organic nitro compounds, including explosives, drugs and environmental pollutants [26]. The novel HPLC–hv–EC methodology used for sensitive and selective detection of common antibiotics is reported here (see Fig. 1). Results include minimum detection limits (MDLs), linearity of calibration plots, optimal oxidative working potentials, dual electrode response ratios, analyses of spiked samples and assays of drug formulations.

Experimental

Reagents, chemicals and standards

The *beta*-lactam antibiotic standards (ampicillin trihydrate, penicillin V potassium, penicillin G potassium, and cefoperazone sodium, dihydrate and the free acid) and drug formulations (experimental formulations similar to Spectrobid powder for oral suspension) were all provided by the Analytical Research Department, Pfizer Central Research, Pfizer, Inc. (Groton, CT). Inorganic salts added to the mobile phase were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Aldrich Chemical Co. (Milwaukee, WI). HPLC solvents were from Waters Associates (Milford, MA) or MCB Chemicals Co. (Gibbstown, NJ), the latter as the Omnisolv brand distributed by EM Science, Inc. (Gibbstown, NJ).

Instrumentation and equipment

Figure 2 illustrates the HPLC–hv–EC instrumentation and arrangement of the parts used. The HPLC portion utilized: a Rheodyne Model 7125 syringe loading injection valve with a 200-μl sample loop (Rheodyne Corp, Cotati, CA), a Laboratory Data Control (LDC) Constametric II solvent delivery system (Laboratory Data Control,

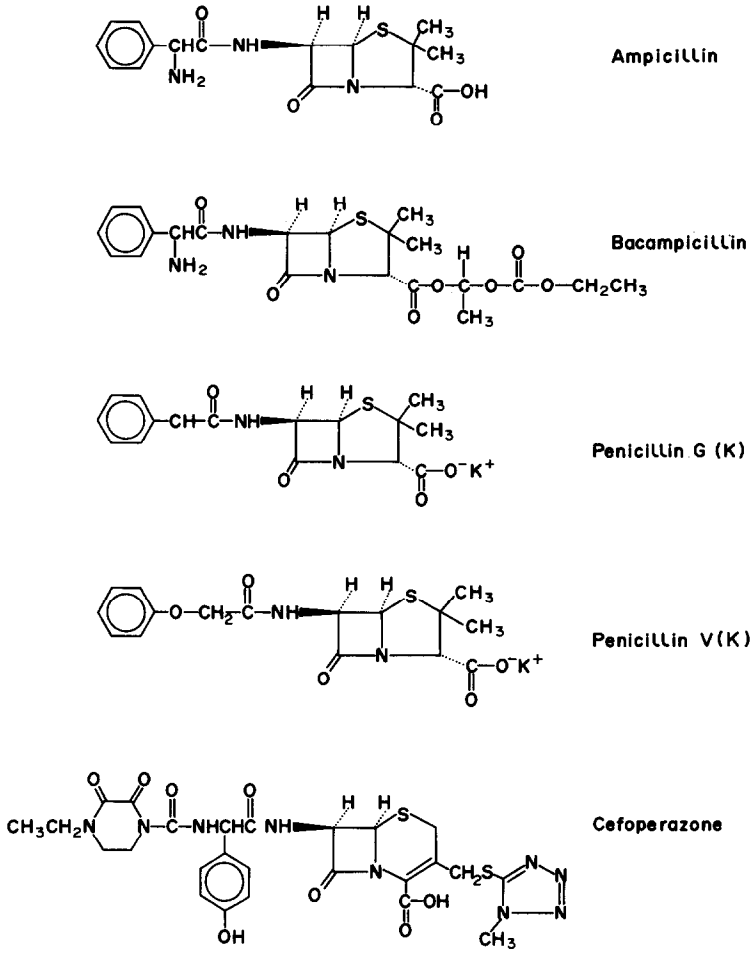

Figure 1
Chemical structures for the *beta*-lactam antibiotics used in this study.

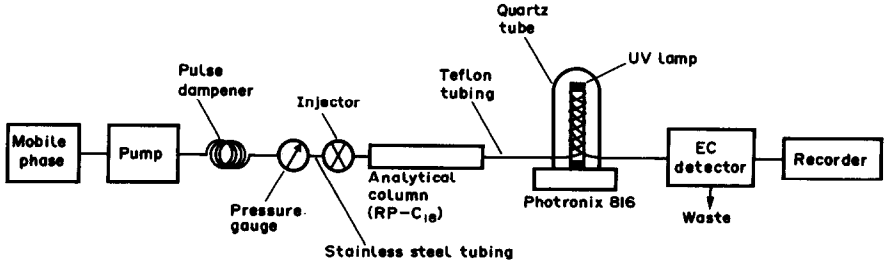

Figure 2
Schematic diagram of the on-line approach for performing HPLC–hv–EC analysis. After reversed-phase separation, photolytic derivatization by UV irradiation generates oxidatively electroactive products which are detected downstream at the electrochemical detector.

Riviera Beach, FL), a LiChromaDamp II pulse dampener (Alltech Assocs., Inc., Deerfield, IL), a Bioanalytical Systems (BAS) pulse dampening column (Bioanalytical Systems, Inc., West Lafayette, IN), a BAS Model LC-4A single-electrode amperometric controller or dual BAS Model LC-4B controllers for dual electrode work, BAS single or dual glassy carbon electrochemical cells with a stainless steel auxiliary electrode cell half and an Ag/AgCl reference electrode, and a Linear Instruments Model 585 dual-pen strip chart recorder (Linear Instruments, Inc., Reno, NV). At times, a Honeywell dual-pen strip chart recorder was used (Honeywell Instruments, Inc., Minneapolis, MN), as well as a Hewlett–Packard Model 3380A Integrator. HPLC injections were made with a 250 μl flat-tipped Hamilton HPLC syringe (Hamilton Corp., Reno, NV). HPLC mobile phases were filtered and degassed prior to use with a 0.45-μm solvent filtration kit (Millipore Corp., Bedford, MA). The photolysis apparatus was a Photronix Corp., model 816 UV batch irradiator (Photronix Corp., Medway, MA), which is normally used to eradicate the organic content of distilled water such that this water can then be used for HPLC with low-wavelength UV detection. The apparatus is supplied with a medium pressure Hg vapor discharge lamp, a 5 gallon stainless steel bucket to hold the distilled water being irradiated, a quartz finger to isolate the lamp from the solution being irradiated, and accompanying electronics for the lamp. Studies in the authors' laboratories have shown that the output from this lamp consists of an intense band at 254 nm and several relatively weak bands at 313, 365, 404 and 435 nm. The irradiation finger was maintained at 0–5°C with a constant-temperature water bath (Forma Scientific, Model 2095, VWR Scientific Co., Boston, MA) or with an ice-water bath. Irradiation of the HPLC effluent took place within woven Teflon PTFE tubing, ⅛ in o.d. × 0.5 or 0.8 mm i.d. (Rainin Instruments Co., Woburn, MA). The principle of using a woven mesh arrangement, as opposed to a simple Teflon winding around the irradiation finger, has been described previously for reduction of band-broadening in post-column chemical reaction and irradiation chambers [36]. Although configured Teflon tubing reactors are commercially available (Kratos Analytical Instruments, Ramsey, NJ), the specific weave design utilized in these reported studies was developed in the authors' laboratories. Swagelok stainless steel fittings and ferrules were used for all connections (Cambridge Valve and Fitting Co., Billerica, MA), except where the EC cell required its own fittings. Reversed phase HPLC separations were performed using an Alltech 10-μm, C-18, 25 cm × 4.6 mm i.d. column (Alltech Associates). Distilled water was obtained from a Corning Mega-Pure distillation apparatus (Corning Corp., Corning, NY).

Analytical procedures

The specific approaches for optimization of analyte response by HPLC–hv–EC have been described in previous publications [25, 26, 41], and these steps, briefly discussed below, have now been used for selected antibiotics. Basically, this involves injecting a constant amount or concentration of a representative analyte and systematically varying one experimental variable of the analytical system (flow rate, salt concentration, residence time in the photolysis chamber, ratio of organic:aqueous constituents of the mobile phase, etc.) at a time. Linear Hydrodynamic Voltammograms (HDVs) were derived using flow injection analysis (FIA)–hv–EC methods by measuring EC response as a function of the working electrode potential. After determining the optimum mobile phase compositon needed for the reversed phase separation of the compounds of interest, it was necessary to determine the optimum amount of UV-irradiation needed to produce the largest current response for each of the antibiotics. Using a constant-length

woven 'irradiation chamber', the flow rate was varied in order to produce different 'residence times' for the analytes in this irradiation chamber. The areas of the peaks arising from these various residence times were then used as a measure to determine the optimum photolysis time needed for the maximum formation of an EC active species from the parent compound and the minimum concomitant photolytic destruction of these newly-formed EC-active moieties. Once this optimum residence time was determined for each of the compounds, and with an understanding of the flow rate needed for chromatographic separations in the study, a new irradiation mesh was produced which had the proper length of 0.5-mm i.d. tubing to ensure this residence time under these chromatographic flow rate conditions.

A number of inorganic salts were studied for their compatibility with the system and their usefulness as electrolytes for the EC detector (e.g. Na_2SO_4, NaBr, $NaClO_4$) but NaCl was chosen because it offered comparatively low background currents. The mobile phase compositions used throughout these studies varied between 35:65 and 65:35 MeOH:0.2 M NaCl, depending on the particular separation involved, and all of the antibiotics showed some degree of retention within these composition extremes when a C-8 or C-18 column and flow rates between 1.5 and 2.2 ml min^{-1} were used.

MDLs were determined by standard HPLC techniques, and all MDLs reported correspond to 200-μl injections of standards and a signal-to-noise (S/N) ratio of 3:1. Calibration plots and detector linearity studies were performed using injections of standards at five different concentrations, and measuring current responses as a function of these concentrations injected.

Sample preparation for the bacampicillin HCl for oral suspension involved initial dissolution of the contents of the sample bottle in 75 ml of water. A 5 ml portion of this sample was then shaken with a mixed ethanol/phosphoric acid solvent to fully extract all of the drug from its matrix, and after a centrifugation step to clarify the solution, a small volume of this clarified solution was diluted in the mobile phase and injected onto the chromatograph. At least three separate injections of both standard and sample were performed under the same HPLC–hv–EC conditions, and the three quantitative determinations were then used to calculate the mean concentrations and standard deviations (S.D.) for bacampicillin HCl in these drug formulations (i.e. $n = 3$ or more).

The analysis for the cefoperazone Na salt commercial sample was performed in a single-blind study, in which one analyst prepared three separate solutions in 0.9 M NaCl infusion solution (saline solution) at varying levels of concentration near the 20 mg/ml^{-1} level normally used for human i.v. administration. These solutions were then given to a second analyst for quantitation, who filtered the solutions and, after diluting them by a factor of 10 000 in mobile phase, used them directly for the quantitative determinations of cefoperazone. The external standards used throughout these studies were prepared daily, as needed, in fresh mobile phase.

Results and Discussion

Figure 3 illustrates typical dual electrode chromatograms for the HPLC–hv–EC analysis of four standard *beta*-lactam derivatives; the conditions of analysis and amounts injected are indicated (note: the small box included in Figs 3 and 4 denotes the fact that *dual* electrodes in the parallel orientation (with respect to the flowing stream) are simultaneously monitoring the electrochemical response at two different working potentials). Although baseline resolution of these compounds is not portrayed in this

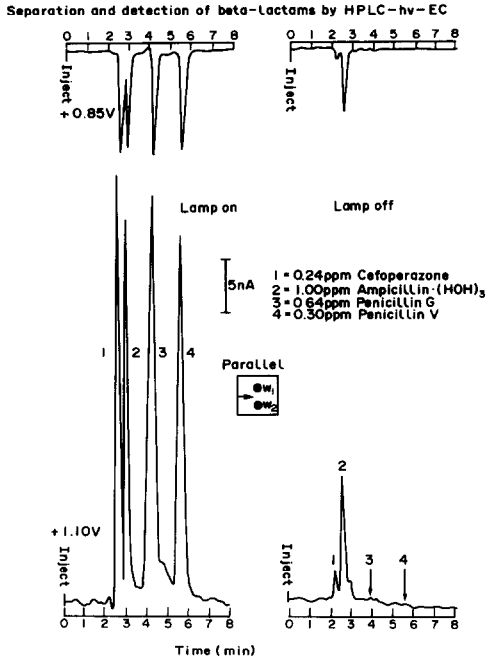

Figure 3
Typical dual-electrode chromatogram for the analysis of a mixture of standards using HPLC–hv–EC. The left trace is obtained with the lamp on, the right trace is obtained when the lamp is extinguished. Conditions: 45:55 MeOH:0.2 M NaCl, flow rate = 2.05 ml min^{-1}, Alltech C-18, 10-μm, 4.6-mm i.d. × 25-cm length.

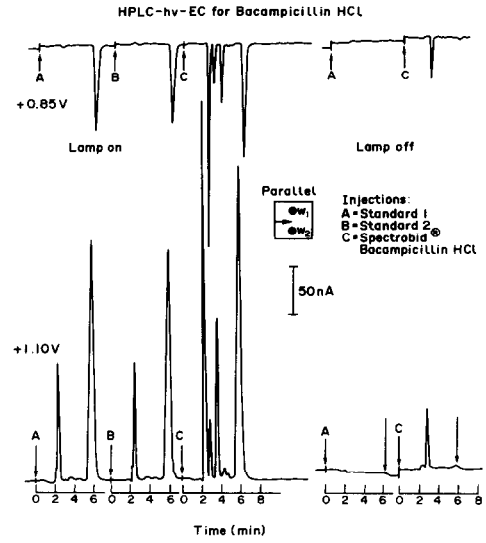

Figure 4
HPLC–hv–EC analysis of commercial formulation containing bacampicillin HCl. Injections with the *lamp on*: (A) bacampicillin HCl standard 1, (B) bacampicillin HCl standard 2, (C) commercial formulation for oral suspension containing bacampicillin HCl and eight other ingredients. Identical injections with the *lamp off*: (A) bacampicillin HCl standard 1, (C) commercial formulation. Conditions: 60:40 MeOH:0.2 M NaCl, flow rate = 2.15 ml min^{-1}, Alltech C-18, 10-μm, 4.6-mm i.d. × 25-cm length.

demonstrative chromatogram, it is highly unlikely that one would ever have to analyse a real sample which contained mixtures of these derivatives. Rather, this chromatogram displays the three unique 'modes' of selectivity inherent in the use of this novel method for the detection of these compounds. The first mode of selectivity is normally the only selective feature of most HPLC detection methods, and that is the retention time or capacity factor of the compound on the column. Using dual-electrode LCEC

approaches, it is possible to gain a second mode of selectivity by simultaneously monitoring the eluent at two working electrode potentials, and then ratioing the current responses generated as a function of these two potentials. This 'response ratio' is reproducible and is different for compounds having different electrochemical behavior, and thus may be used to assist in the qualitative identification of an unknown peak in the chromatogram. The third mode of selectivity, which is unique to a system which utilizes photolytic derivatization for generation of an electroactive species from a nonelectroactive parent, is the qualitative response obtained when the lamp is on or off. When combined, the qualitative information gained in each of these three modes of selectivity enables the analyst to make a more definite identification of an unknown in a sample mixture. Table 1 summarizes the response ratios and response factors (current generated per unit mass of analyte injected) for the *beta*-lactams and ampicillin prodrug, bacampicillin HCl, under both lamp-on and lamp-off conditions. The table reveals the qualitative responses evidenced in Fig. 3, in that it is clear that when no photolysis is occurring, penicillin V, penicillin G and bacampicillin HCl have no inherent oxidative electroactivity and thus cannot be detected. Ampicillin and cefoperazone both exhibit some degree of inherent electroactivity at these potentials, but their response factors are lower when the lamp is extinguished than when under photolytic conditions. Also, the response ratios under both lamp-on and lamp-off conditions for these two compounds are easily distinguishable and may even be used to confirm an assigned identity in the chromatographic analysis of a mixture of unknown composition. Furthermore, all of the results reported here were obtained using glassy carbon working electrodes. It should be possible to change the selectivity presently observed via dual electrode response ratios by substituting one or more of the glassy carbon working electrodes with other electrode materials (e.g. platinum or silver).

Table 1
Summary of results of HPLC–hv–EC analysis of *beta*-lactam standards

Compound name	Lamp on		Lamp off	
	Response ratio	Response factor (nA/ng)	Response ratio	Response factor ($nA\ ng^{-1}$)
Ampicillin	3.29 ± 0.02	2.4×10^{-1}	2.06 ± 0.13	1.6×10^{-2}
Bacampicillin	2.92 ± 0.07	3.5×10^{-2}	No response	No response
Penicillin G	4.46 ± 0.02	2.5×10^{-1}	No response	No response
Penicillin V	3.52 ± 0.09	6.8×10^{-1}	No response	No response
Cefoperazone	4.30 ± 0.06	2.1×10^{-2}	1.73 ± 0.06	8.0×10^{-3}

Response ratios based on working potentials of +1.1 and +0.85 V vs Ag/AgCl.

Table 2 summarizes the experimentally determined MDLs and linear dynamic ranges for the penicillins and cephalosporin studied. The correlation coefficients for all of these linearity plots were 0.9996 or better, and, in general, the MDLs obtained are lower by one or more orders of magnitude than the existing literature reports using UV or FL detection, even when post-column derivatizations were used. The linear range for the quantitation of the antibiotics using HPLC–hv–EC was found to be between 2 and 3 orders of magnitude, and these experimentally determined MDLs and linear ranges have been found to be more than adequate in the application of this method to actual samples of drug formulations.

Table 2
Summary of minimum detection limits (MDLs) and linear ranges for *beta*-lactams by HPLC–hv–Ec

Compound name	Minimum detection limits (MDLs)	Linear range
Ampicillin	6 ng (30 ppb)	30 ppb to 10 ppm
Cefoperazone	8 ng (40 ppb)	40 ppb to 10 ppm
Penicillin G	6 ng (30 ppb)	30 ppb to 10 ppm
Penicillin V	8 ng (40 ppb)	40 ppb to 10 ppm

MDLs were determined using 200-μl injections and S/N = 3.

In order to validate these novel methods for *beta*-lactams, two applications involving quality control for antibiotics in drug formulations or saline infusion solutions have been investigated. Current quality control approaches for these antibiotic preparations utilize HPLC–UV, which, though presently adequate, provides significantly less analyte specificity than that now possible by HPLC–hv–EC approaches. Figure 4 illustrates the analysis for bacampicillin HCl, a pro-drug of ampicillin, manufactured by Pfizer, Inc., which is available commercially as a prescription powder for oral suspension. Five injections are depicted in this figure; the first two are injections of two independently-prepared standards of bacampicillin HCl, while the third is an injection of an extract of bacampicillin HCl powder which has been worked-up following the procedure outlined in the experimental section of this manuscript. These three injections demonstrate lamp-on behavior for the standards and sample. The fourth and fifth injections demonstrate the response for one standard and the sample powder extract, respectively, when the photolysis lamp is extinguished. The three modes of selectivity are readily apparent, in that the chromatographic capacity factors of the standards and the unknown peak in the sample chromatogram are identical, the response ratios are similar within experimental error, and, when the lamp is extinguished, the response for the compound eluting at the proper retention time for bacampicillin HCl is no longer present. The samples analysed were virtually identical with those that may be obtained commercially under the name "Spectrobid", but these particular samples were Pfizer experimental lots. Table 3 summarizes the quantitative results for the HPLC–hv–EC analysis of four separate bacampicillin HCl formulations, demonstrating the labelled concentrations of bacampicillin HCl (mg ml^{-1} reconstituted sample), the experimentally determined analytical levels, and the percent difference between these two values. The results demonstrate the inherent precision and accuracy of the HPLC–hv–EC methods, and evidence the feasibility of the use of these methods for the routine analysis of these formulations in an industrial quality control or clinical setting.

Table 3
Summary of HPLC–hv–EC analyses for bacampicillin HCl in drug formulations

Sample I.D.	Determined levels (mg ml^{-1}) Stated value	HPLC–hv–EC	Percent difference
39–1	25	25.0 ± 0.6	0.0
39–3	25	24.9 ± 0.5	−0.4
3–1	40	42.0 ± 0.5	+5.0
3–2	40	39.3 ± 0.4	−1.8

Cefoperazone Na salt (see Fig. 1) is a 'third generation' cephalosporin, also manufactured by Pfizer, Inc., which is commonly administered by initial dissolution of the powder in a saline infusion solution (0.9% NaCl). In a single blind study, three separate spiked infusion solutions were prepared, mimicking what might be prepared by a physician for human administration, near the usual dosage level used for intravenous drip (approximately 20 mg ml^{-1}). A single blank sample was prepared at the same time, but the analyst using HPLC–hv–EC had no knowledge of the actual levels spiked or which was the blank solution. All of the quantitative results of the analysis of these spiked saline solutions are presented in Table 4, and the specific analytical conditions are indicated as well. As above, the external standard method of quantitative analysis was used in this application, and, in general, there is good agreement between the stated and determined levels for these cefoperazone-spiked solutions.

Table 4
HPLC–hv–EC analyses for cefoperazone in saline infusion solutions

Sample I.D.	Actual level present (mg 10 ml^{-1})	Level determined (mg 10 ml^{-1})	
38–4–2	208.1	215 ± 1	+3.3
38–5–2	178.8	176 ± 0	−1.6
38–6–2	276.8	293 ± 2	+5.8
38–7–2	Blank	0 ± 0	—

Conclusions

The authors have developed and applied a new HPLC–hv–EC method for the analysis of a number of different *beta*-lactam antibiotics, some of the more widely used and prescribed penicillin derivatives. Post-column, on-line photolytic derivatization for LCEC provides greatly improved analyte identification based on three important parameters: (1) HPLC capacity factors or retention times, (2) photolytic lamp-on and lamp-off qualitative responses, and (3) dual electrode response ratios under both lamp-on and -off conditions. The final applications involving these techniques illustrate an ease and simplicity in the analytical procedures, together with a high degree of qualitative and quantitative agreement between actual and determined levels for two separate antibiotics. The authors further suspect that these methods will prove applicable to a large number of *beta*-lactam derivatives, in a much wider variety of sample matrices than explored in this limited, initial study. Perhaps more importantly, they feel that the greatest utility of the improved sensitivity of this new detection method over those presently incorporated in routine analyses will be in the detection and identification of impurities and degradation products in commercial formulations. It is the hope of the present authors that these methods of analysis will soon be adopted and applied by others interested in *beta*-lactam antibiotics.

Acknowledgements: All of the standards and samples used in these studies were provided by Pfizer, Inc., Groton, CT. This work was supported, in part, by an unrestricted grant from the Analytical Research Department, Pfizer Central Research, Pfizer, Inc., Groton, CT. We are indeed most grateful for the technical, material and financial assistance provided this program at Northeastern University by various individuals within Pfizer, Inc. In addition, we gratefully acknowledge Dr Kissinger and Dr Shoup of Bioanalytical Systems, Inc., West Lafayette, IN for their continued interest in all of the studies involving HPLC–hv–EC. We are also

grateful for the assistance provided by Steve Colgan, a graduate student at Northeastern University, in preparing the cefoperazone Na spiked solutions for use in the single-blind study. Mr X-D. Ding of the Chinese Academy of Sciences, Academia Sinica, Beijing, People's Republic of China, was extremely helpful in earlier research and development work with HPLC–hv–EC, and was involved in perfecting some of the approaches that have now been employed in the current study with drugs and antibiotics. This work would not have been possible without his earlier assistance and collaboration at Northeastern University.

This is contribution number 229 from The Barnett Institute at Northeastern University.

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[*Received for review 20 November 1984; revised manuscript received 7 January 1985*]