Singleton for technical assistance.

Registry No. Hg, 7439-97-6; Pt, 7440-06-4; NaBr, 7647-15-6; NaCl, 7647-14-5; $Na_2C_2O_4$, 62-76-0; NaOH, 1310-73-2; EDTA, 60-00-4; NaSCN, 540-72-7; $NaNO_3$, 7631-99-4; H_2NSNH_2, 62-56-6; $HClO_4$, 7601-90-3.

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Determination of Cephalosporins and Decomposition Products by Liquid Chromatography with Indirect Electrochemical Detection

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Indirect electrochemical detection, using in-line electrochemically generated bromine as oxidizing agent, has been used for the determination of cephalosporins with liquid chromatography. The method presents a general detection principle for cephalosporins since it is related to their basic structure. The detection limits obtained, which were between 0.4 and 3 ng, and the repeatability with a relative standard deviation below 1.5% comply with the requirements for drug stability studies in vitro. Application to the determination of cefotaxime and its main metabolite in serum and urine showed an improved selectivity compared to that obtained with UV detection. The method is also of interest for the detection of decomposition products resulting from the cleavage of the β-lactam ring, which cannot be detected by UV.

Cephalosporins are generally determined by microbiological techniques or by high-performance liquid chromatography (HPLC). As often outlined, the major drawback of microbiological procedures is their lack of specificity in stability studies when the decomposition products are microbiologically active (*1*). In that case HPLC with ultraviolet absorption detection is the method of choice, although some potential decomposition products that do not absorb UV light cannot be detected. For pharmaceutical preparations, the selectivity of reversed-phase separation and the sensitivity of UV absorption detection—due to the strong absorbance of the β-lactam moiety—allow the parent compound and trace levels of its main degradation products to be determined simultaneously (*2*). In most formulations the added ingredients do not generally interfere with the determination. In contrast, when cephalosporins have to be determined in biological samples at low levels, the interference of the matrix is a limiting factor for the detectability. The high aqueous solubility of cephalosporins does not allow their extraction with organic solvents. The difficulties that can be encountered are typically shown in the simultaneous determination of cefotaxime and its highly polar metabolite desacetylcefotaxime. Numerous extraction and HPLC procedures have been proposed to overcome overcome the interference problems (*3*, *4*).

In the search for sensitive and selective methods for the determination of cephalosporins (*5*, *6*), we have investigated the possibilities of HPLC with oxidative amperometric de-

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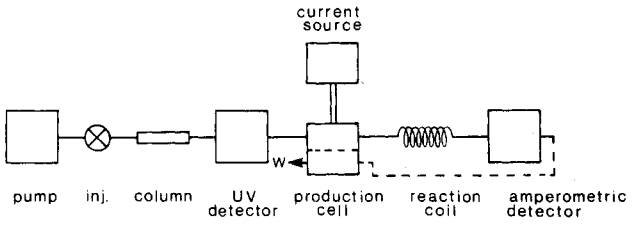


Figure 1. Scheme of the experimental setup.

tection. Despite the high detection potential that had to be used (+0.95 V vs a calomel electrode at a pH of 7.6), the detection limits obtained with standard solutions of the test compounds, cefotaxime and two of its metabolites, were about 1 ng. However, direct electrochemical detection in the oxidative mode cannot be used as a general method for cephalosporins, since the electroactivity presented by some of them is not related to the β-lactam structure but to the presence of an oxidizable substituent on their side-chain (*6*, *7*). For this reason, and to avoid possible electrode passivation phenomena with biological samples, we have studied the use of indirect electrochemical detection. In this in-line coulometric titration technique, an oxidizing reagent is produced electrochemically in the column effluent from a precursor in the mobile phase. After the passage through a short reaction capillary, the excess of reagent is monitored with an amperometric detector in the reductive mode. Details on the characteristics and performance of this technique have been published (*8*). Indirect electrochemical detection has been shown to be advantageous in the determination of compounds with slow electrode kinetics in direct oxidation (*9*). Besides, electrode passivation is avoided by the moderate potential that can be applied in the reductive detection. Applications to the determination of sulfur compounds in biological samples with iodine and bromine as oxidizing reagents have been published (*9*, *10*). Since cephalosporins react with iodine only after the opening of their β-lactam ring (*11*, *12*), bromine was used as reagent in this work.

The reactivity and stoichiometry of the reaction were studied for cefotaxime (C) and five of its decomposition products: desacetylcefotaxime (D), desacetylcefotaxime lactone (L), desacetoxycefotaxime (Do), 7-aminocephalosporanic acid (ACA), and thiazoximic acid (Th). For comparison 7-aminodesacetoxycephalosporanic acid (ADCA) was also investigated (see Table I).

Signal-to-noise ratios were measured in order to optimize the operating conditions. The linearity range, the repeatability and the detection limits were compared to those obtained with UV detection. The applicability of the method to biological samples is shown in the simultaneous determination of cefotaxime and its active metabolite in serum and urine. The interest of the method for the monitoring of decomposition products of cephalosporins in raw materials and drugs is also shown. The extension of the method to other compounds was studied with cephalosporins (I–VIII) bearing various substituents.

EXPERIMENTAL SECTION

Apparatus. The liquid chromatograph consisted of a Gynkotek (Germering, FRG) Model 300 C pump, a Rheodyne injection valve with a loop volume that was determined to be 24 μL, a Waters Model 440 fixed-wavelength (254-nm) absorbance detector, and, in series, a production cell, reaction coil, and amperometric detector. Figure 1 shows the experimental set-up schematically. The KOBRA cell used for in-line generation of bromine (*8*) was obtained from the Chemistry Department Workshop of the Free University (Amsterdam, The Netherlands). The constant-current source was an Elektronica Micrometer (Amsterdam, The Netherlands). The reaction coil was a 1 m × 0.5 mm i.d. poly(tetrafluoroethylene) (PTFE) capillary with a 6.6-s hold-up time at the

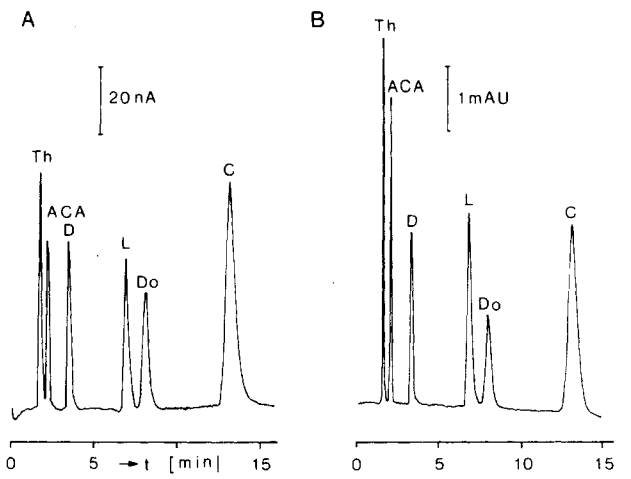


Figure 2. Separation of cefotaxime and five of its decomposition products: (A) indirect electrochemical detection, i_G = 25 μA; (B) UV detection. Amount injected: 92 ng of C, 25 ng of D, 47 ng of L, 24 ng of Do, 11.5 ng of Th, and 24 ng of ACA.

flow rate used. A Metrohm (Herisau, Switzerland) 656 detector cell was used with a glassy carbon working electrode at a potential of +0.4 V vs a Ag/AgCl/KCl (Saturated) reference electrode. The cell was connected to a BAS (West Lafayette, MA) potentiostat. The efficiency of the detector cell, measured as the ratio of the detector current to the generating current applied, was about 1.5%. A 200 × 4.6 mm i.d. column was used, slurry packed with Hypersil ODS (5 μm). The flow rate was 1.8 mL min^{-1}, and the pressure was about 120 bars. All experiments were conducted at ambient temperature (20 ± 2 °C).

Chemicals and Solutions. Cephalosporins were obtained as gifts: C, D, Do, L, ACA, and Th from Roussel UCLAF Laboratories (Romainville, France); ADCA and VI from Bristol Laboratories (Paris, France); III from Glaxo Laboratories (Greenford, UK); I from Boehringer (Mannheim, FRG); II, IV, and VII from Cassenne Takeda (Paris, France); VIII from Allard (Paris, France), and V from MSD-Chibret (Paris, France).

The compounds were used as received. Stock solutions (0.2–0.4 g L^{-1}) were prepared daily and suitably diluted in water or the mobile phase before use. Other chemicals were of analytical grade purity. The mobile phase consisted of 0.1 M acetate buffer, pH 4.1, and methanol (83:17) with 0.01 M NaBr.

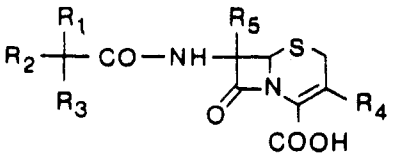
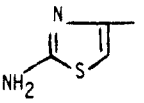
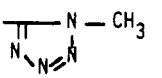
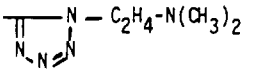
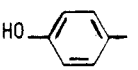
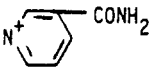
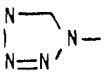
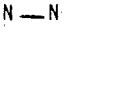
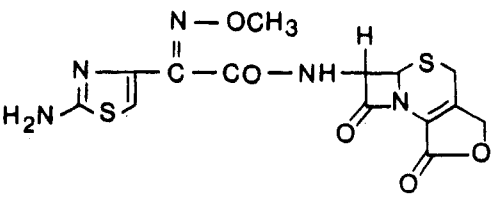
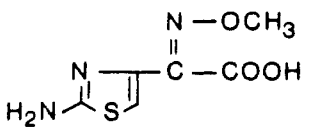
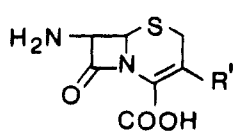
Sample Preparation. For recovery studies in serum, samples were spiked with 26.6 μg mL^{-1} of C and 11.5 μg mL^{-1} of D or with 5.3 μg mL^{-1} of C and 2.3 μg mL^{-1} of D, respectively. Deproteination in acidic methanol was carried out according to the procedure described by Lecaillon et al. (*13*). After this treatment the samples were diluted with water (1:4).

For recovery studies in urine, samples were spiked with 0.20 mg mL^{-1} of C and 0.97 mg mL^{-1} of D, and diluted with water (1:49).

RESULTS AND DISCUSSION

Optimization of the Experimental Parameters. The separation was optimized both for the determination of cefotaxime and its deacetylated metabolite in biological samples and for a stability indicating assay in vitro. The mobile phase previously used (*2*), with a pH of 7.6, is incompatible with the electrochemical generation of bromine. Among the different mobile phases investigated in the pH range of 2–5, the best separation was obtained when using a 0.1 M acetate buffer, pH 4.1, and methanol in the ratio 83:17. A further decrease of the pH of the mobile phase resulted in an overlapping of the peaks of D and L, which are the major decomposition products of C. The values of k' of D and L were 1.1 and 3.5 at pH 4.1, while, for instance, at pH 3.5 they were 1.3 and 1.5, respectively. Chromatograms of a standard solution of cefotaxime and five of its decomposition products are shown in Figure 2. Since the electrochemical detection system was positioned in series after the UV detector, the resolution of early eluting peaks was slightly decreased. However, when

Table I

Compound	Structure			
				
	R_1	R_2, R_3	R_4	R_5
Cefotaxime (C)		= N - OCH_3	- CH_2OCOCH_3	- H
Desacetylcefotaxime (D)	id	id	- CH_2OH	- H
Desacetoxycefotaxime (Do)	id	id	- CH_3	- H
Ceftizoxime (I)	id	id	- H	- H
Cefmenoxime (II)	id	id	- CH_2 - S 	- H
Ceftazidime (III)	id	= N-$OC(CH_3)_2$ COOH	- CH_2 - 	- H
Cefotiam (IV)	id	- H, - H	- CH_2 - S 	- H
Cefoxitin (V)		- H, - H	- CH_2 - O-CO - NH_2	- OCH_3
Cefadroxil (VI)		- NH_2, - H	- CH_3	- H
Cefsulodin (VII)		- SO_3H,-H	- CH_2 - 	- H
Cefazolin (VIII)		- H, - H	- CH_2 - S 	- H
Desacetylcefotaxime lactone (L)				
Thiazoximic acid (Th)				
7-aminocephalosporanic acid (ACA)			R' = - CH_2OCOCH_3	
7-aminodesacetoxycephalosporanic acid (ADCA)			R' = - CH_3	

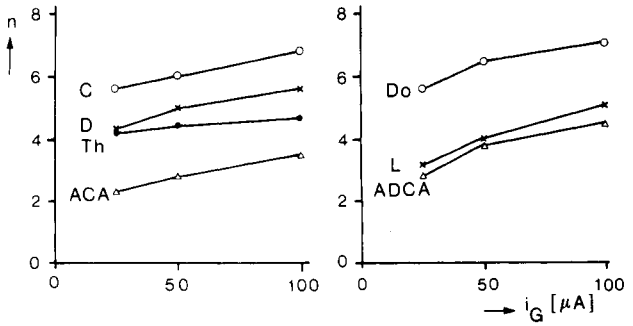


Figure 3. Dependency of the bromine consumption by cefotaxime and decomposition products on the generating current. Number *n* of bromine molecules consumed by one molecule of the compound.

Table II. Sensitivities and Detection Limits with UV and Electrochemical Detection

	UV		electrochemical	
compd	sensitivity, mAU ng^{-1}	LOD,[a] ng	sensitivity, nA ng^{-1}	LOD, ng
C	0.031	3.3	0.74	3.0
D	0.104	1.0	2.04	1.1
L	0.062	1.7	0.98	2.2
Do	0.056	2.0	1.50	1.4
ACA	0.192	0.5	2.12	1.0
Th	0.488	0.2	6.16	0.4

[a] LOD: limit of detection, signal-to-noise ratio of 2.

the column was connected directly to the reagent production cell, no significant differences in peak widths were observed compared to those observed with UV detection.

In the indirect electrochemical detection mode employed, the decrease of the reagent concentration is measured. The amount of bromine consumed by the analytes in the column effluent depends on the hold-up time in the reaction coil and the concentration of bromine produced. The latter parameter is easily controlled, since the reagent concentration is directly proportional to the generating current i_G. To investigate the rate and stoichiometry of the reactions, the compounds were injected at different generating currents. Care was taken to keep the peak concentrations of the compounds well below the reagent concentration. The number of bromine molecules reacting with one molecule of a compound was calculated from the peak areas by applying Faraday's law, taking into account the efficiency of the detector cell. Results are shown in Figure 3. A fast reaction with bromine is observed not only with the β-lactam moiety but also with the aminothiazole side chain in the 7-position. The amount of bromine consumed by one molecule is about equal to the sum of that of its component parts (see C vs ACA and Th or Do vs ADCA and Th). A comparison of the reactivity of C, D, and Do or of ACA and ADCA shows that the reactivity of the β-lactam structure is influenced by the substituent in the 3-position.

With a generating current of 25 μA, the reaction is already almost complete for the compounds investigated. This implies that the highest signal-to-noise ratios will be obtained at low generating currents. The high number of bromine molecules consumed indicates that low detection limits can be obtained.

Sensitivity, Linearity, and Repeatability. The sensitivity, defined as the slope of the plot of peak height vs amount injected, increases slightly with the generating current. However, since the noise increases proportionally with it, the lowest limits of detection are obtained at a low generating current. In Table II sensitivities and detection limits at 25-μA generating current are compared with those obtained with UV detector. With respect to detectability, the two methods give comparable results.

Table III. Recovery of C and D from Spiked Serum, Determined with UV and Electrochemical Detection

		% recovered[a]	
compd	concn, μg mL^{-1}	UV	electrochemical
C	1.8	167 ± 4	91 ± 7
	5.3	109 ± 6	102 ± 3
	26.6	107 ± 4	103 ± 4
D	2.3	106 ± 1	101 ± 3
	11.5	104 ± 5	103 ± 4

[a] Mean ± SD, four separate extractions.

Since the amount of bromine available for the reaction with the analyte is limited, the upper limit of linearity increases with the generating current. Evidently, the linear range is also related to the retention time of a compound due to the peak dilution. With a generating current of 50 μA, upper limits of 350 (C), 100 (D), 150 (L), 150 (Do), 40 (Th), and 75 ng (ACA) were found. Within the linear range, coefficients of correlation for the calibration plots were better than 0.9997 for all compounds. The upper limits of linearity obtained when using a generating current of 25 μA were about half of those obtained at 50 μA.

The repeatability was assessed by seven successive injections of separate solutions of each compound at i_G = 50 μA and also of C and D at i_G = 25 μA. The injected amounts were between 20 and 200 ng. The relative standard deviation of the peak height was always better than 1.5% for both detection methods, except for the lactone L for which a variation of ±4.2% was observed with electrochemical detection and of ±4.7% with UV detection.

Application to Biological Samples. For most applications to biological samples the highest sensitivity that can be obtained at low generating currents is not required. Still, to prolong the lifetime of the column we have used the 25-μA current and diluted the samples after pretreatment to match the linear range. For serum samples a simple deproteination procedure with acidified methanol was applied (*13*). Individual and pooled serum samples were investigated. In most individual serum samples less interfering peaks were found with electrochemical detection. Typical chromatograms of blank serum and serum spiked with C and D are shown in Figure 4. Table III gives the recoveries found in the simultaneous determination of C and D in spiked, pooled serum when using the two detection methods. The selectivity of the electrochemical detection is better than that of UV detection. In the blank pooled serum a compound was present that caused an interfering peak. The limit of determination (an accuracy better than ±10%) for C in serum is about 5 μg/mL with UV detection and about 1.5 μg/mL with indirect electrochemical detection.

For the determination of C and D in urine, the sample was diluted (1:49) with water. Typical chromatograms of blank urine and urine spiked with C and D are given in Figure 5. Recoveries were 111% (UV) or 96% (electrochemical) for D and 95% (UV) or 94% (electrochemical) for C. The superior selectivity of the electrochemical method in the determination of D is clearly seen in Figure 5. D can be determined reliably in urine at levels of 100 and 20 μg/mL with UV and electrochemical detection, respectively.

Stability Studies in Vitro. As shown for cefotaxime and its decomposition products (Figure 2), indirect electrochemical detection can be an alternative method for carrying out the stability-indicating assays necessary for licensing medicinal products. The sensitivity and repeatability are comparable to those obtained with UV detection for these decomposition products. However, in addition to the products resulting from the breakdown of the side chain, a nonnegligible amount of

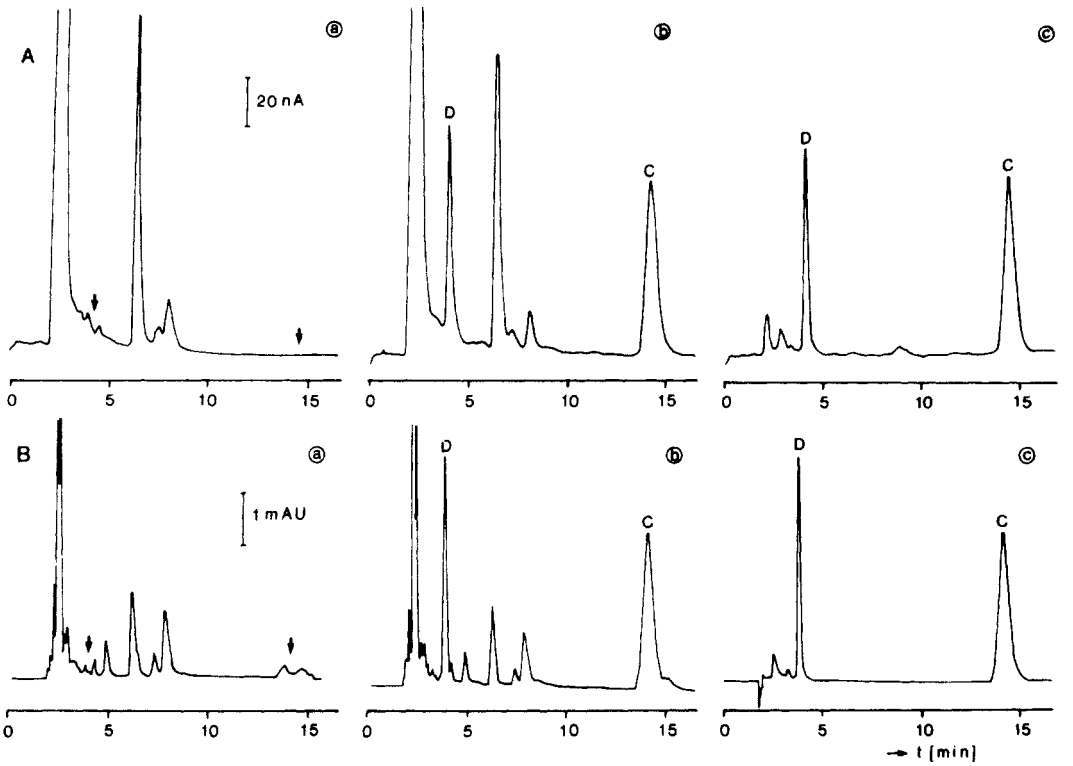


Figure 4. Determination of C and D in serum: (a) blank serum; (b) serum spiked with 26.7 μg mL^{-1} C and 11.5 μg mL^{-1} D; (c) standard solution with the same final concentrations. Samples were diluted with water (1:4) before injection. (A) Indirect electrochemical detection, I_G = 25 μA. (B) UV detection.

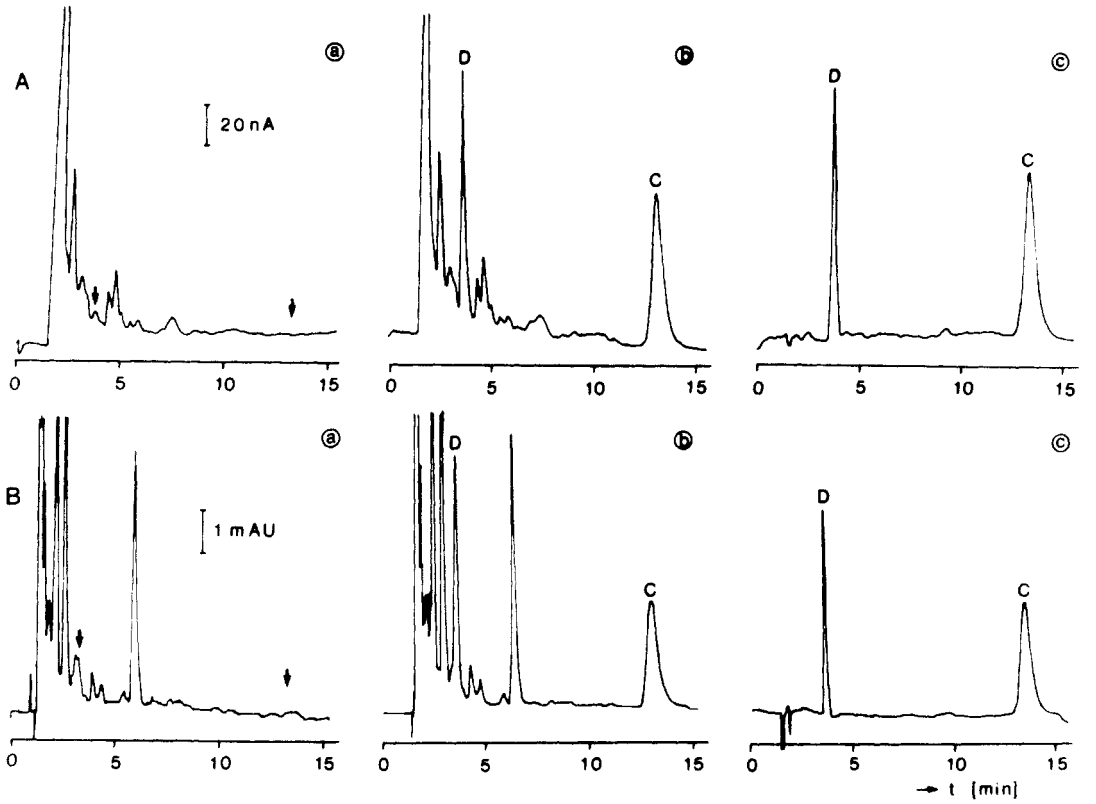


Figure 5. Determination of C and D in urine: (a) blank urine; (b) urine spiked with 204 μg mL^{-1} C and 98 μg mL^{-1} D; (c) standard solution with the same final concentrations. Samples are diluted with water (1:49) before injection. (A) Indirect electrochemical detection, I_G = 25 μA. (B) UV detection.

decomposition products is formed by the hydrolysis of the β-lactam linkage under nucleophilic attack (*2*). Most of these compounds cannot be detected by UV absorption since the β-lactam ring is responsible for the strong UV absorption of cephalosporins at 250–270 nm (*14*). We have investigated the possibility of using indirect electrochemical detection as a complement to UV detection on degraded solutions of ACA. Degradation was studied on an aqueous solution of ACA (28 μg mL^{-1}) stored for 7 days at 4 °C. In order to separate polar decomposition products a mobile phase containing only 5%

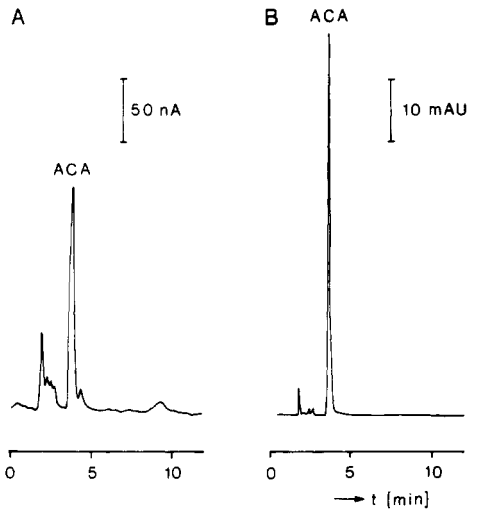


Figure 6. Chromatograms of a degraded ACA solution (28 μg mL^{-1}): (A) indirect electrochemical detection, I_G = 25 μA; (B) UV detection.

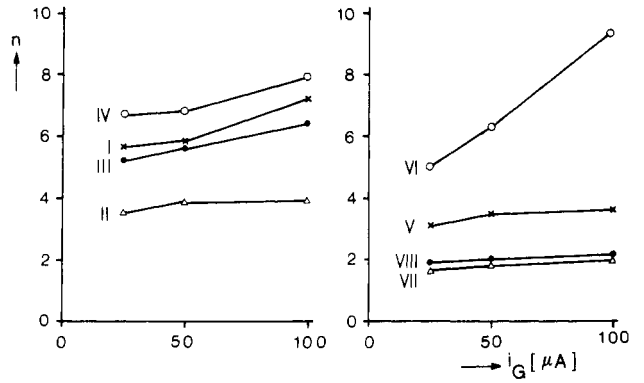


Figure 7. Dependency of the bromine consumption by various cephalosporins on the generating current.

(v/v) methanol was used. Chromatograms recorded with electrochemical detection give evidence of more products than those recorded with UV detection (Figure 6).

Application of the Method to Other Cephalosporins. For comparison the bromine consumption by other cephalosporins was measured under the same conditions as for C. Figure 7 gives the results. Some remarks on the observed reactivity can be made. The aminothiazole group present in I, III, and IV gives these compounds a reactivity similar to C. However, the relatively low consumption of bromine by II, which has the same substituent, is difficult to explain. The high consumption but slow rate noted for VI can be attributed to the presence of a phenolic group (8). The sulfur-containing side chain of VIII is not oxidized by bromine under the conditions used. Although chromatographic optimization has not been carried out, it is clear from these results that indirect electrochemical detection is a promising technique for the determination of all tested cephalosporins in biological fluids.

CONCLUSIONS

The high rate of the reaction of bromine with the basic β-lactam structure of cephalosporins indicates that indirect electrochemical detection can be used as a general method for cephalosporins. The ease of oxidation of the aminothiazole side chain is also of interest since this group is present in most of the cephalosporins recently synthesized.

The selectivity of indirect electrochemical detection has been shown to be superior in the analysis of biological samples in comparison to that of UV detection, even for strongly UV absorbing compounds such as cephalosporins. Therefore, it may be expected that the method is useful for other sulfur-containing compounds as well.

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Registry No. I, 68401-81-0; II, 65085-01-0; III, 72558-82-8; IV, 61622-34-2; V, 35607-66-0; VI, 50370-12-2; VII, 62587-73-9; VIII, 25953-19-9; C, 63527-52-6; D, 66340-28-1; Do, 65052-63-3; L, 66340-33-8; Th, 65872-41-5; ACA, 957-68-6; ADCA, 22252-43-3; cephalosporin, 11111-12-9.

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