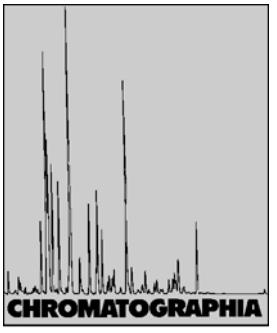
Capillary Electrophoresis for the Direct Determination of Cephalosporins in Clinical Samples



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Key Words

Capillary electrophoresis
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Summary

The possibility of determination of four cephalosporin antibiotics in clinical samples by capillary electrophoresis has been investigated. The separation conditions for capillary zone electrophoresis (CZE) were studied in detail. The precision of migration times measured by use of the optimized method was satisfactory (RSD < 1%) and response was linearly dependent on concentration over the approximate range 2 – 150 mg L^{-1} for all the compounds studied (cefuroxime, cefotaxime, ceftriaxone, and ceftazidime). Complete separation could be achieved within 5 min. The CZE method was found to be highly suitable for direct determination of the antibiotics in clinical samples such as wound drainage, cerebrospinal fluid, and urine; for serum, however, the use of micellar electrokinetic capillary chromatograpy (MECC) was more advantageous.

Introduction

The cephalosporins are regarded as one of the most important and most frequently used groups of antibiotics used in medicine. Because many cephalosporins cannot be absorbed orally and must be administered intravenously or intramuscularly, interest has focused on study of their bioavailability.

In recent decades the use of the high-performance liquid chromatography (HPLC) has dominated the analysis of pharmaceuticals, although capillary electrophoresis (CE) has also proven to be a powerful technique for the analysis of these compounds [1]. In the last few years methods for the determination of cephalosporins by capillary zone electrophoresis (CZE) [2–4] and micellar electrokinetic capillary chromatograpy (MECC) [5–8] have been published.

CE is a promising technique (compared with, e. g., immunoassay) for clinical analysis because of its excellent separation efficiency, high versatility, and low cost. Because untreated biofluid samples can be injected directly into CE capillaries, considerable savings in analysis time and cost of consumables can be achieved. Direct injection of clinical samples can be advantageously performed by CZE only if the sample does not contain very large amounts of protein (e. g. plasma or serum). Because biofluid samples containing large amounts of proteins can generate interfering peaks and lead to disadvantageous matrix effects, most direct-injection analyses are performed by MECC. Because the SDS micelles interact with the sample proteins, giving then a net negative charge, the proteins are eluted after the analyte peak of interest. Direct injection of serum in MECC has been used for the analysis of drugs [9]. It has also been shown that the extent of binding between the serum proteins and different antibiotics affects peak shape after direct injection [9, 10].

In our work the possibility of direct analysis of four frequently used cephalosporins (ceftazidime, cefotaxime, cefuroxime, and ceftriaxone) in clinical samples obtained after neurosurgery (wound drainage, cerebrospinal fluid, serum, and urine) was investigated. The quantitative determination of cephalosporins in these samples is important in neurosurgery because the concentration of the analytes in serum can be different from that in wound drainage or cerebrospinal fluid. In our work CZE was usually used for the determination of cephalosporins; MECC was used only for direct injection of clinical samples with a high protein content.

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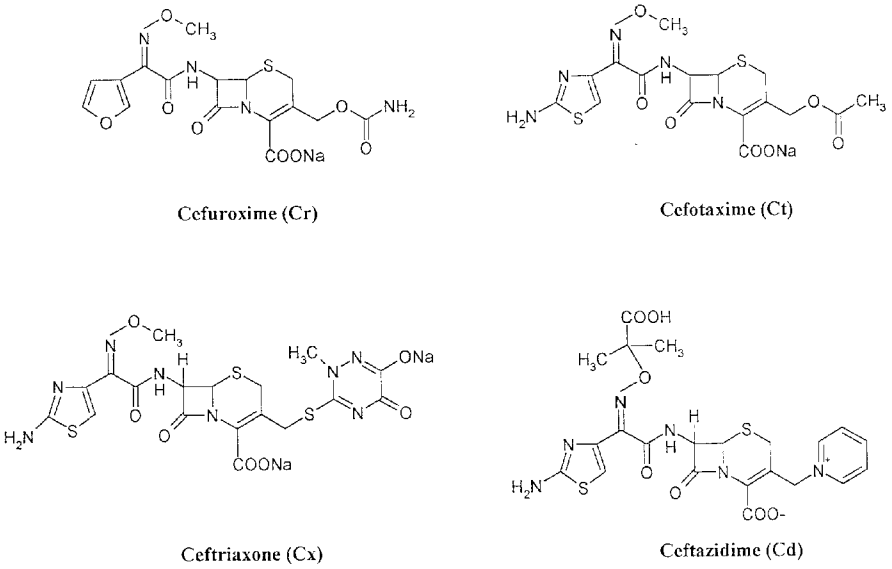

Figure 1. The structures of the four cephalosporin antibiotics.

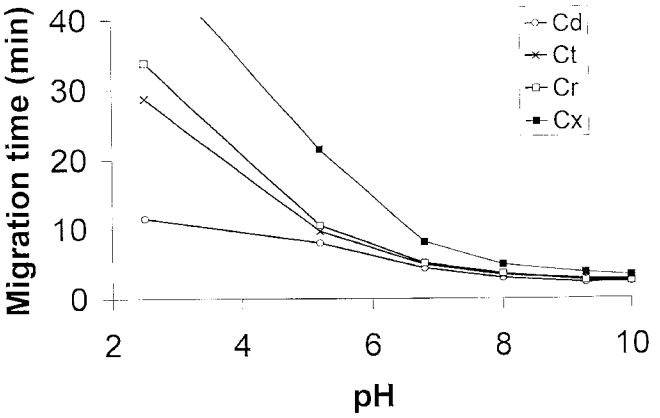

Figure 2. Effect of the pH of 25 mM phosphate buffer on the separation of the cephalosporins.

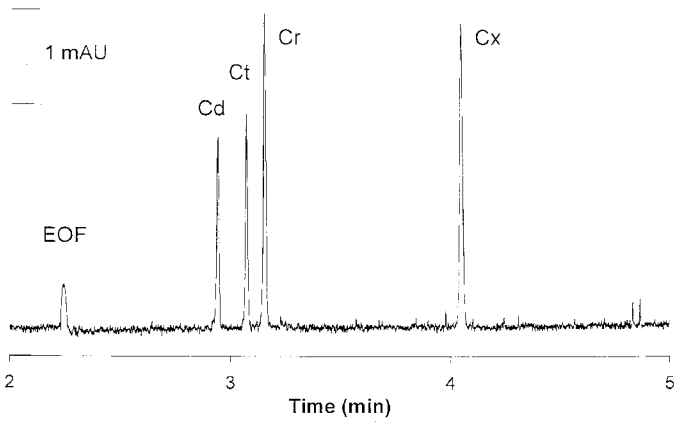

Figure 3. Electropherogram obtained from a mixture of the four cephalosporins investigated. Fused silica capillary, 48.5 cm × 50 μm i. d.; buffer electrolyte 25 mM borate, pH 9.2; applied voltage + 25 kV; detection UV absorption at 270 nm. The concentration of each cephalosporin was 10 μg mL^{-1}.

Experimental

Instrumentation

Capillary electrophoresis was performed with an HP (Hewlett-Packard, Waldbronn, Germany) model 3D. For all analyses hydrodynamic sample introduction (100 mbar s) was used for sample injection. Sample solutions were introduced at the anode end of the capillary. Separations were performed in 48.5 cm long (effective length 40 cm) × 50 μm i. d. fused-silica capillaries coated externally with polyimide (Polymicro Technology, Phoenix, AZ, USA). Before use the capillaries were preconditioned with the buffer electrolyte for 10 min. During analysis of biofluid samples, after each run the capillaries were also post-conditioned by flushing with 0.5 M NaOH (6 min), 0.3 M SDS (2 min), and distilled water (2 min) to remove all the proteinaceous components, which have a high tendency to stick to the capillary walls resulting in variations in the rate of electroosmotic flow (EOF) and thus in migration times [11].

The applied voltage was + 25 kV. The temperature of the capillary holder was kept constant at 25 °C. Detection was achieved by on-column photometric measurement at 270 nm. Use of diode-array detection also enabled recording of UV spectra every 0.2 s throughout the electropherogram. The electropherograms were recorded and processed by ChemStation version 7.01 (Hewlett-Packard).

Chemicals and Samples

Reagents of analytical grade were obtained from a variety of distributors. Sodium tetraborate and sodium dodecyl sulphate (SDS) were purchased from Reanal (Hungary). Benzyl alcohol was used as a marker for electroosmosis. Sample solutions (0.15 mg mL^{-1}) were prepared by dissolving the solid salts of cefuroxime, ceftazidime (Glaxo), cefotaxime (Lek), and ceftriaxone (Roche) in water immediately before analysis. The formulas of, and abbreviations used for, the cephalosporins are summarized in Figure 1. Although the stability of cephalosporin antibiotics in the solid state is usually satisfactory, on dissolution in water they are slowly hydrolyzed to different degradation products. When the conversion rates were studied previously [12] it was found that within 4 h of dissolution degradation (hydrolysis) of these antibiotics was no higher than 2%.

The biofluid samples (wound drainage, cerebrospinal fluid, serum, and urine) were centrifuged and stored at −18 °C. The samples were defrosted immediately before analysis. Fresh human serum was obtained from the Centre of Transfusion (Debrecen, Hungary); wound drainage and cerebrospinal fluid samples were from the Department of Neurosurgery, University of Debrecen (Hungary). For serum

Table I. Analytical data from determination of the cephalosporins.

Cephalosporin	Migration time (min)	Mobility, μ ($cm^2kV^{-1}min^{-1}$)	*LOD** ($\mu g\,mL^{-1}$)	*RSD* (%)** peak area	*RSD* (%)** migration time	Theoretical plates, *N*
Ceftazidime	2.913	– 7.06	0.478	2.42	0.76	218 713
Cefotaxime	3.105	– 8.70	0.206	2.72	0.77	131 489
Cefuroxime	3.228	– 9.66	0.361	2.77	0.79	298 957
Ceftriaxone	4.054	–14.55	0.230	3.18	0.84	226 433

* $3 \times s$; ** $c = 100\,\mu g\,mL^{-1}$, $n = 10$.

and urine the simplest sample pretreatment process was chosen – fivefold dilution of the samples with distilled water before analysis.

Before CE analysis all samples and buffers were filtered through 0.45 μm syringe filters. Electrophoretic runs were performed as quickly as possible, and no later than 4 h after solution preparation.

Results and Discussion

Optimization of Separation Conditions

The cephalosporins investigated have slightly different molecular sizes (weight) and are anionic compounds over a wide pH range, suggesting the possibility of their complete separation by capillary zone electrophoresis. Initially it was intended to perform the separation with a buffer solution containing no surfactant. In CZE mode buffer pH can be regarded as having the greatest effect on resolving power, and so the effect of pH in the range 2.5 – 10 was examined. The effect of pH on the migration time of the cephalosporins is shown in Figure 2. Although very high resolution can be achieved by use of an acidic buffer electrolyte, the separation requires a very long time (almost 30 min at pH 4.5) and baseline separation can be achieved with an alkaline electrolyte within 10 min. Borate solution of pH 9.2 was chosen as buffer electrolyte because of its simplicity and because no pH adjustment was necessary after preparation of the borate solution. The concentration of the borate solution was 25 mM; this generally resulted in an electrophoretic current of approximately 39 μA in our system (25 kV). When this concentration of borate was used, as much as 100 mM SDS additive could be added with use of the same voltage without excessive current and Joule-heat generation. It was found that the buffer concentration did not have a substantial effect on the separation. To achieve rapid separation, but with undistorted peak shapes, a 25 kV voltage was used. Under these conditions the four cephalosporins were eluted after the EOF at approximately 2.9 (ceftazidime), 3.1 (cefuroxime), 3.2 (cefotaxime), and 4.0 min (ceftriaxone) (Figure 3). This order of migration can be explained in terms of the electric charge and size of the analytes.

In addition to the CZE mode, the effect on analyte migration times of surfactant concentration (0 – 300 mM SDS in 25 mM borate) was also investigated. The migration times of the analytes gradually increased with increasing SDS concentration. Because the four cephalosporins have similar, low, lipophilic character, only a slight micellar solubilization effect can occur. These analytes are, on the other hand, anionic compounds with carboxyl groups, and the electrostatic repulsion between the analytes and the SDS micelles might suppress the micellar solubilization of the cephalosporins. The other reason for the increasing migration times with increasing concentrations of the SDS might be that the higher salt (SDS) concentration results in greater ionic strength and consequently a smaller electroosmotic flow. Although addition of SDS to the buffer electrolyte did not result in higher resolution, study of the SDS content of the electrolyte was important, because the direct analysis of biofluids with a high protein content requires anionic surfactant in the electrolyte solution.

Table II. Linearity regression data for the cephalosporins (Conditions as for Figure 3).

Cephalosporin	Regression equation	Correlation coefficient	Range ($\mu g\,mL^{-1}$)
Ceftazidime	$y = 0.136x - 0.034$	0.9990	2 – 150
Cefotaxime	$y = 0.140x + 0.024$	0.9994	2 – 150
Cefuroxime	$y = 0.292x + 0.158$	0.9991	2 – 150
Ceftriaxone	$y = 0.267x + 0.057$	0.9992	2 – 150

Analytical Performance

Tables I and II summarize the analytical performance of the CZE separation of a mixture of four cephalosporins in aqueous solution. The detection limits (signal-to-noise ratio, $S/N = 3$) were between 0.206 and 0.478 $\mu g\,mL^{-1}$ and the quantification limits ($S/N = 10$) were between 0.686 and 1.593 $\mu g\,mL^{-1}$. The precision of migration times and peak areas was measured for ten replicate injections. The precision of migration times was < 0.84 *RSD*%. Such precision was achieved after at least 10 min preconditioning of the capillary with the buffer electrolyte; replenishing the buffer vial before each electrophoretic run did not improve the precision further. The precision of the response was calculated on the basis of peak areas, correction of peak areas for migration time did not result in substantially better precision. The main reason we could not achieve better precision than 2.4 *RSD*% might be that the cephalosporins slowly decompose in aqueous medium, resulting in slowly decreasing peak areas for replicate measurements [12].

To determine the linearity of the calibration plot mixed solutions containing 1 – 500 $\mu g\,mL^{-1}$ of the analytes were prepared. For all the cephalosporins investigated the response was found to be linearly dependent on concentration in the range 2 – 150 $\mu g\,mL^{-1}$ ($r > 0.999$). This concentration range covers the therapic concentrations of these antibiotics most commonly used. Although the method seems to be sufficiently precise for reliable quantitative analysis without use of an internal standard, use of an internal standard to compensate for small variations in the injection system of the instrument might be desirable. For these cephalosporins cinnamic acid is recommended as internal standard (it can be easily separated from the analytes, and it has good absorption at 270 nm).

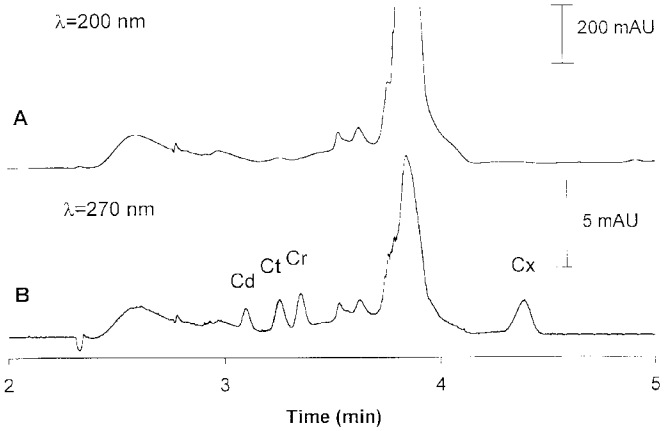

Figure 4. Electropherograms obtained from undiluted human serum spiked with cephalosporins at 50 μg mL^{-1} detected at (**A**) 200 and (**B**) 270 nm. Other conditions were as for Figure 3.

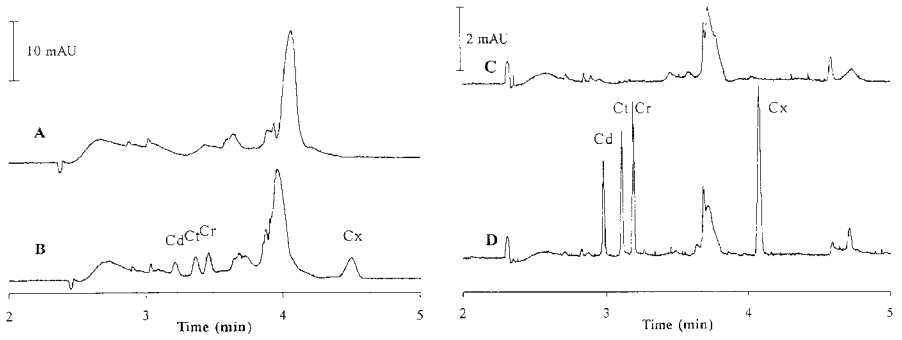

Figure 5. Electropherograms obtained from human serum: **A**. undiluted blank serum; **B**. undiluted serum spiked with cephalosporins at 50 μg mL^{-1}; **C**. fivefold-diluted blank serum; **D**. fivefold-diluted serum spiked with cephalosporins at 50 μg mL^{-1}. Other conditions were as for Figure 3.

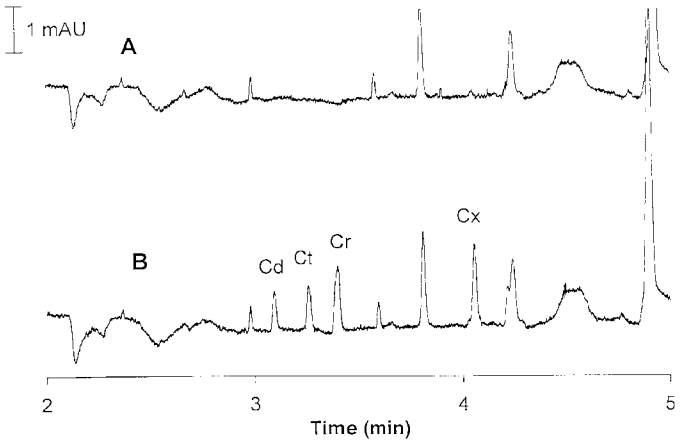

Figure 6. Electropherograms obtained from human serum by use of MECC: **A**. undiluted blank serum; **B**. undiluted serum spiked with cephalosporins at 10 μg mL^{-1}. The borate buffer contained 100 mM SDS. Other conditions were as for Figure 3.

Direct Determination of Cephalosporins in Clinical Samples

The CE analysis of biofluids suffers from special problems compared with determination of standards prepared in water or pure solvents – as is true for other analytical methods. The two most important complications are the possibly high salt and high protein content of the biological samples. Among the biofluids we investigated serum contained the highest concentration of protein; the salt content was highest for urine. Serum and urine samples were therefore expected to be most unsuitable for analysis by direct injection. The other two samples (wound drainage and cerebrospinal fluid) contained relatively small amounts of protein and salts. In this work our intention was to develop direct CE analysis with the simplest and quickest sample pretreatment and measurement procedure.

CZE electropherograms obtained from serum spiked with the four antibiotics are shown in Figure 4. (Although in therapeutics the antibiotics are not administered together, in our studies all four cephalosporins were analysed in the same run to prove the suitability of the method for separation of all four compounds.) Although the UV spectra of the cephalosporins contained two absorption maxima at 200 and 270 nm, other components (proteins, salts) of the biofluids investigated usually absorbed light at 200 nm only. The advantage of detection of cephalosporins in biofluids at 270 nm is therefore obvious. It is apparent from the electropherogram that ceftriaxone was eluted after the largest peak from a serum component (albumin), and the other three cephalosporins appear among the broad zones of the different serum proteins. Although all the four analytes are adequately resolved and are readily identified, the peaks are very broadened and the absorbance values (peak heights) obtained for the cephalosporins are much smaller than those obtained for the same concentrations of the analytes in distilled water. Because of the high protein content of serum, samples for CZE should be diluted at least 1:5 with water; this results in much better resolution and improved peak shape and sensitivity for the analytes (Figure 5). Greater than 1:5 dilution resulted in a further slight improvement in peak shape and sensitivity. In Figure 5 it is also evident that the migration times of analytes shifted toward lower values when the diluted sample was analysed.

Direct quantitative analysis of even undiluted serum samples could, however, be achieved without problems if MECC was used, because the proteinaceous compounds are strongly bound to SDS and are well retained (Figure 6). Although the SDS content of the buffer substantially increases the migration times of proteinaceous components of the serum, the migration times of the cephalosporins barely change.

Another type of biofluid investigated was urine. The determination of urinary cephalosporins is important because some cephalosporins are known to be absorbed from the intestine, transported into the circulatory system, and excreted into urine almost without being metabolized [13]. The high salt content of urine results

in deformation of peak shapes and shifts in migration times. Because of this the urine had to be diluted fivefold before analysis (another solution might be to increase the ionic strength of the buffer electrolyte). Figure 7 shows that quantitative determination of the cephalosporins could be achieved after simple fivefold dilution of the urine.

The cephalosporin content of the wound drainage (Figure 8) and cerebrospinal fluid (Figure 9), important samples in neurosurgery after operations and therapeutics, could be determined by CZE without pretreatment – neither dilution of the sample nor addition of different agents to the buffer electrolyte was necessary.

As has already been mentioned in the Experimental section, when direct injection analysis is performed inter-analysis rinsing is of particular importance for removal of all the proteinaceous components, which have a high tendency to stick to the capillary. After a rinsing with 0.5 M NaOH the capillary was, therefore, also washed with 0.3 M SDS. This rinsing procedure also prevented clogging of the capillary.

Conclusion

Capillary electrophoresis enables qualitative and quantitative determination of cephalosporins in biofluids. UV detection at 270 nm was suitable for measurement of these antibiotics in the therapeutic concentration range ($1-50\,\mu g\,mL^{-1}$) in clinical samples such as wound drainage, cerebrospinal fluid, serum, and urine. In our work a very simple CE system (simple buffer, relatively short capillary, no additives) could be used for complete separation of four cephalosporins in 5 min only. Although the selectivity and separation efficiency of CZE were suitable for the analysis, for samples (serum) with a high protein content the use of MECC was necessary. Although the clinical samples investigated could be analysed without pretreatment, fivefold dilution of the samples was desirable (particularly for urine and serum).

The results obtained in this and other studies show CZE and MECC to be attractive alternatives to HPLC for the analysis of drugs in clinical samples. In further investigations we plan to use CE to monitor the transport, efficiency, and metabolism of the cephalosporins in the four types of clinical neurosurgery sample studied in this work.

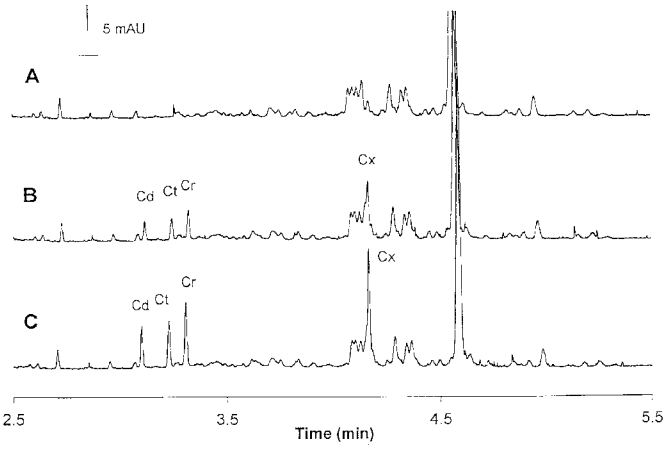

Figure 7. Electropherograms obtained from human urine: **A**. blank urine; **B**. urine spiked with cephalosporins at $10\,\mu g\,mL^{-1}$; **C**. urine spiked with cephalosporins at $25\,\mu g\,mL^{-1}$. The urine was diluted fivefold. Other conditions were as for Figure 3.

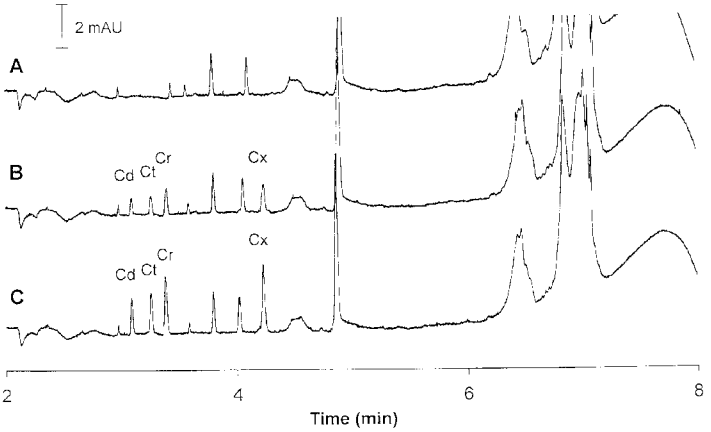

Figure 8. Electropherograms obtained from human wound drainage: **A**. blank content of wound drainage; **B**. wound drainage spiked with cephalosporins at $10\,\mu g\,mL^{-1}$; **C**. wound drainage spiked with cephalosporins at $25\,\mu g\,mL^{-1}$. Other conditions were as for Figure 3.

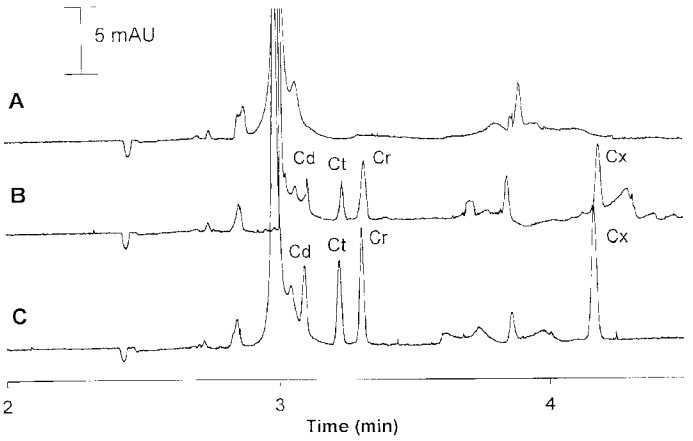

Figure 9. Electropherograms obtained from human cerebrospinal fluid: **A**. blank cerebrospinal fluid; **B**. cerebrospinal fluid spiked with cephalosporins at $25\,\mu g\,mL^{-1}$; **C**. cerebrospinal fluid spiked with cephalosporins at $50\,\mu g\,mL^{-1}$. Other conditions were as for Figure 3.

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