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QUANTITATIVE ANALYSIS OF EIGHT CEPHALOSPORIN ANTIBIOTICS IN PHARMACEUTICAL PRODUCTS AND URINE BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

A simple and rapid capillary zone electrophoretic (CZE) method has been established for separation and quantification of a mixture of eight cephalosporins – cefadroxil (CFL), cefixime (CIX), cefuroxime sodium (CFR), ceftriaxone sodium (CTR), ceftizoxime (CFT), cefaclor (CFC), cefradine (CFD), and cefotoxime (CTA). Conditions affecting the separation were pH, buffer concentration, and applied potential. Separation was performed in less than 11 min with 50 mM sodium tetraborate buffer, pH 9.0, and an applied potential of 30 kV. Reproducible separation was achieved and calibration plots were linear over two to three orders of magnitude of analyte concentration. Limits of detection were in the range 0.5–5 μg mL^{-1}. Detection was by UV absorbance at 214 nm. When the method was assessed for analysis of cephalosporins in pharmaceutical preparations and in urine, relative standard deviations (*RSD*, $n = 4$) were in the range 0.3–1.9%.

INTRODUCTION

The problem of counterfeiting of pharmaceutical products is growing rapidly. According to the World Health Organization (WHO) as much as 7% of the world's medicine is counterfeit [1]. Counterfeiting of antibiotics, which represents 45% of all cases of fake drugs [2], is a particularly serious problem. Antimicrobial agents that contain less than the stated dose may result in suboptimum levels of circulating drug which may result in both therapeutic failure and the emergence of drug-resistant strains [3].

The cephalosporins are important semi-synthetic antibiotics, fre-

quently used in medicine, with a broad spectrum of activity against Gram-negative bacteria. They are derived from cephalosporin C, which is found among the fermentation products of *Cephalosporium acremonium*. The active nucleus, 7-aminocephalosporanic acid, which is related to the penicillin nucleus, 6-aminopencillinic acid, consists of a β-lactam ring fused to a six-membered dihydrothiazine ring. Side-chains linked to the carbon-7 position may improve the stability and effectiveness of the derivatives and side chains linked to the carbon-3 position may improve the pharmacokinetic characteristics [4–7].

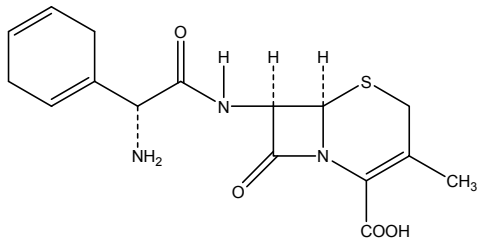
Common methods of analysis of cephalosporin antibiotic include microbiological techniques [8] and high-performance liquid chromatography [9–11]. Capillary electrophoresis (CE) is developing rapidly and has proved to be a powerful technique for analysis of pharmaceutical products and in the field of biomedical analysis. It has also been recognized by the regulatory authorities [12–14]. The main advantages of CE include reduced method development time, low operating cost and solvent consumption, high separation efficiency, and analysis of solutes with limited chromophores. Use of CE has been examined for the analysis of cephalosporins [15], as also have micellar electrokinetic chromatography (MEKC) [16,17] and capillary zone electrophoresis (CZE) [18].

Nishi et al. reported the separation of nine and twelve cephalosporins by MEKC using sodium dodecyl sulphate (SDS) and sodium *N*-lauroyl-*N*-methyltaurate as anionic surfactants with phosphate buffer at pH 9 and pH 6 [19]. Pajchel and Tyski reported determination of six cephalosporins with phosphate–borate buffer at pH 5–8 either alone and with addition of SDS [20]. Cruces et al. reported separation of nine β-lactam antibiotics in 22 min by MEKC [21].

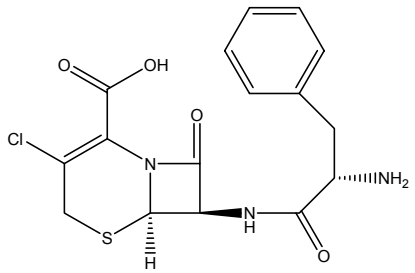
Mrestani et al. separated four and nine cephalosporins by CZE and analysed urine, bile, and plasma samples using either pH 6 citrate buffer or pH 7.2 phosphate buffer, supplemented with pentene-1-sulphonic acid sodium salt [22,23]. Lin et al. [24] observed that the results of Mrestani et al. were not consistent and separated 12 cephalosporins by CZE using three different types of buffer electrolyte including phosphate, citrate, and 2-(*N*-morpholino)ethanesulphonate (MES). To study the stability of cephalosporins in water at different temperatures, Gaspar et al. [25] examined CZE for the separation of fourteen cephalosporins.

The purpose of the work reported here was to develop a simple, sensitive, rapid, and reliable analytical procedure which could be used for large-scale screening of commercial cephalosporins in oral dosage forms.

Determination of different generations of cephalosporins could be of value in the detection of counterfeit and substandard drugs. It was considered useful to examine CZE with an uncoated capillary, a simple buffer solution, and wide variations in concentration, suitable for application to the analysis of pharmaceutical preparations and biological samples. The eight most frequently used cephalosporins were examined. Investigation of buffers prepared from six acids and two sodium salts enabled separation and analysis of the compounds in less than 11 min by CZE with borate buffer of pH 9 (Fig. 1). To the best of our knowledge CZE separation of this combination of cephalosporins for possible identification has not been reported.



Cefradine



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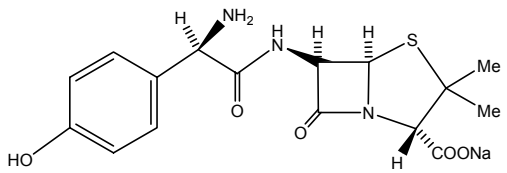
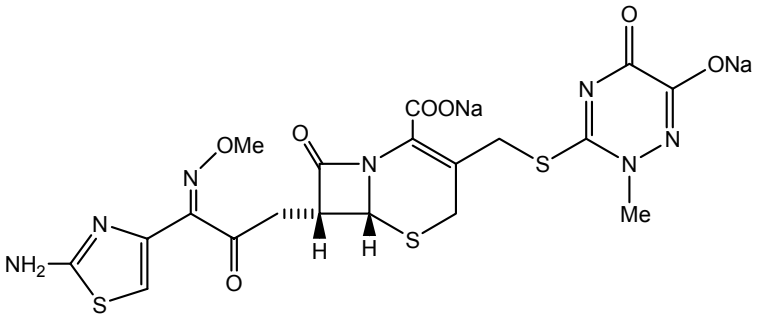
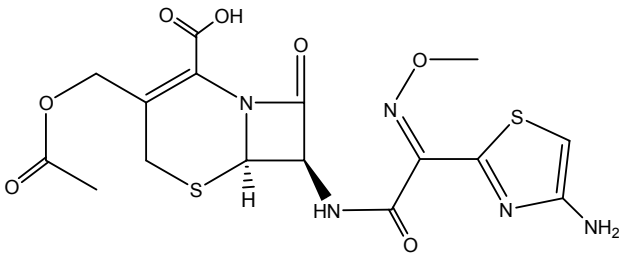


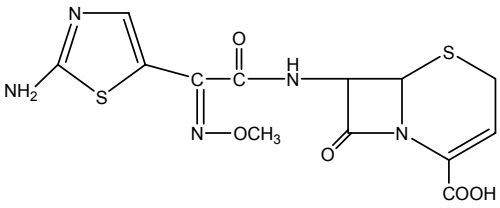
Cefadroxil



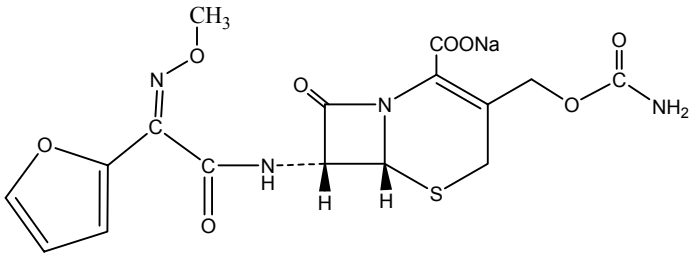
Ceftriaxone



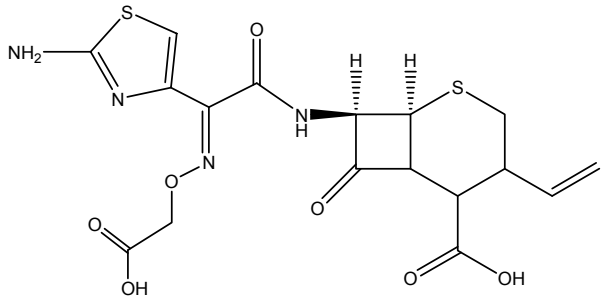
Cefotoxime



Ceftizoxime



Cefuroxime



Cefixime

Fig. 1

The structures of the cephalosporins

EXPERIMENTAL

Instrumentation

Capillary electrophoresis (CE) was performed with a Beckman Instruments (Fullerton, CA, USA) Coultor P/ACE MDQ instrument equipped with autosampler, photo-diode array UV detector, and a data-handling system comprising an IBM personal computer and P/ACE MDQ system (32 Karat) software. Fused silica capillaries of 57 cm total length, 50 cm effective length, 75 μm i.d., 375 μm o.d. were obtained from Beckman. The temperature of the capillary and the sample was maintained at 25°C.

Reagents and Solutions

Pure cephalosporin standards cefadroxil (CFL), cefradine (CFD), and cefixime (CIX) were from Sigma (Germany), cefaclor (CFC) from Fluka (Switzerland), cefotoxime (CTA) from E. Merck (Germany), cefuroxime sodium (CFR) and ceftriaxone (CTR) from Glaxo–Welcome (Pakistan), and ceftizoxime (CFT) from Barrat–Hedgson (Pakistan). Pharmaceutical preparations for analysis of cephalosporin were: Zinacef (Glaxo–Smith Kline, Lahore, Pakistan), Velocef (Bristol Mayers Squib Pharma, Karachi, Pakistan), Ceclor (Eli Lilly, Hyderabad, Pakistan), Cefizox injection (Bosch Pharma, Karachi, Pakistan), Cefomerc injection (Merck Marker, Quetta, Pakistan), Helicef (Helix Pharma, Karachi, Pakistan), Neucef (Sami Pharmaceutical, Karachi, Pakistan), Evacef (Highnoon Laboratories, Lahore, Pakistan), and Fixitil (Tabros Pharma, Karachi, Pakistan).

All reagents were of analytical grade and solvents were of chromatography purity. Methanol, sodium tetraborate, sodium hydroxide, and boric acid of GR grade were obtained from E. Merck. The pH of sodium tetraborate (50 mM) was adjusted to 5, 6, 7, 8, 9, and 10 by addition of boric acid (50 mM) or sodium hydroxide (50 mM) while maintaining the concentration of buffer at a constant value of 50 mM. Buffer electrolyte solutions were prepared fresh daily. pH was measured with an Orion Research (Boston, USA) 420A pH meter connected to a glass electrode and an internal reference electrode. All the solutions were prepared with deionized double-distilled water.

Standard solutions

Separate stock solutions (1 mg mL^{-1}) of cephalosporins CFC, CFD, CFR, CTR, CFT, and CTA were prepared by dissolving 0.1 g in 100 mL water. For CFL and CIX, 1:4 (*v*/*v*) methanol–HCl (0.1 M) was used instead of water. All stock solutions were stored under refrigeration at 4°C. Further dilutions were made with the same solvent as required. A mixture of all eight cephalosporin was prepared by dissolving 10 mg of each of drug in 2:1:1 (*v*/*v*) water–hydrochloric acid (0.1 M)–methanol in a 10-mL volumetric flask.

Analytical Procedure

At the beginning of the day the capillary was regenerated and conditioned by washing, in sequence, with methanol for 1 min, water for 0.5 min, hydrochloric acid (0.1 M) for 2 min, water for 0.5 min, sodium hydroxide (0.1 M) for 2 min, water for 0.5 min, and, finally, running buffer for 2 min.

Solutions containing CFD (3–1000 μg mL^{-1}), CTA and CFC (15–1000 μg mL^{-1}), CFT, CFR, and CFL (5–1000 μg mL^{-1}), CTR (10–1000 μg mL^{-1}), and CIX (15–1000 μg mL^{-1}) were placed in septum vials (1.5 mL). Before sample injection the capillary was washed sequentially with sodium hydroxide (0.1 M) for 2 min and water for 0.5 min and then equilibrated with running buffer for 2 min. Samples were injected by means of the autosampler and the hydrodynamic method (4 s at 0.5 psi). The running buffer was 50 mM sodium tetraborate (pH 9) and the applied potential 30 kV. Detection was performed by UV absorbance at 214 nm.

Analysis of Pharmaceutical Preparation

Six tablets each Zinacef for analysis of CFR, Velocef for analysis

of CFD, and Ceclor capsule for analysis of CFC were separately ground to a fine powder and an amount equivalent to 5 to 10 mg of each active ingredient was dissolved in water. Well mixed Cefizox injection powder for analysis of CFT and Cefomerc injection for analysis of CTA corresponding to 5–10 mg active ingredient was weighed and dissolved in water. Eight tablets each of Helicef, Neucef, and Evacef for analysis of CFL and Fixitil for analysis of CIX were separately ground to fine powder. A quantity equivalent to 5 to 10 mg CFL or CIX was weighed and dissolved in 1:4 (*v*/*v*) methanol–hydrochloric acid (0.1 M). Each sample was shaken thoroughly to dissolve the solid, the volume was adjusted to 10 mL, and the sample was sonicated for 10 min. The final solution was filtered through filter paper (Whatman no. 42) and an appropriate volume was diluted with deionized water as required. The clear solution was analysed as described above and quantification was achieved by use of an external calibration plot.

Analysis of Pharmaceutical Preparation by the Standard Addition Technique

Solutions of Zinacef tablets for analysis of CFR and Velocef tablets for analysis of CFD were prepared in duplicate and analysed as described above. Another 100 μg CFR or CFD was added to each and the solutions were analysed again. Quantification was achieved by use of an external calibration plot and from the increase in the response when the standard was added.

Analysis of Urine Samples

Two urine samples were collected from two volunteers approximately 8 h after separate administration of Zinacef and Velocef tablets containing CFR and CFD (250 mg/tablet), respectively. The samples were filtered through Whatman no. 42 filter paper and 1 mL was diluted to 10 mL. The clear solution was analysed as described above and quantification was achieved by use of an external calibration plot.

Analysis of Urine Samples by the Standard Addition Technique, and Determination of Recovery

CFR or CFD, 100 or 200 μg respectively, was added to urine samples (1 mL) obtained as described above and the volume was adjusted to 10 mL. Analysis was performed as described above and quantitation was achieved by use of an external calibration plot and from increase in the response when the standard was added. Recovery (%) was then calculated.

Mobility Calculations

The electrophoretic mobility of each cephalosporin was calculated from the observed migration times by use of the equation:

$$\mu = \frac{L_t L_d}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right)$$

where μ is the electrophoretic mobility of the compound, L_t is the total length of the capillary, L_d is the length of the capillary between injection and detection, V is the applied potential, t_m is the migration time measured directly from electropherogram, and t_0 is the migration time for an uncharged solute (acetone). The migration time of each component was recorded separately first then as a mixture.

RESULTS AND DISCUSSION

The cephalosporins are widely used in human and veterinary medicine. The abundant and, occasionally, improper use of the cephalosporins may, however, have lethal effects on human and veterinary health. Separation of the eight cephalosporins was examined by CE to demonstrate the separating power of CE for such compounds. Each of the compounds could be identified for qualitative purposes and analysed quantitatively in complex mixtures such as pharmaceutical preparations and biological fluids.

Development of the Capillary Electrophoretic Separation

Capillary zone electrophoresis (CZE) is the most widely used type of CE because of its simplicity and versatility. If a molecule is charged it can easily be separated by CZE. Separation in CZE is based on differences between electrophoretic mobilities, which result in different velocities of migration of ionic species in the electrophoretic buffer contained in the capillary. The separation mechanism is mainly based on differences between solute size and charge at a given pH. Initial study of electrolyte optimization was therefore based on the plots of migration time against pH for the cephalosporins. It was not possible to achieve separation at pH < 6.0 (Fig. 2). Some separation was observed in the pH range 6–7, but unsymmetrical peaks were observed for all eight cephalosporins. The pH range 8.0–10.0 was therefore selected for preliminary study. Three buffers were examined – tris-HCl (pH 8.0), sodium tetraborate (pH 9.0), and sodium

carbonate (pH 10.0) – with the objective of investigating the CE separation behaviour of a standard mixture of CFL, CFD, CIX, CFC, CTA, CFR, CTR, and CFT. Maximum separation was achieved with sodium tetraborate buffer of pH 9, although separation of CFT, CTA, and CFR was incomplete, with resolution, $R_S < 1.5$. Borate buffer of pH 8.5, 9.0, and 9.5 was therefore investigated. Improvement of the separation was not observed and sodium tetraborate buffer of pH 9 was therefore selected. This buffer was studied at four concentrations in the range 25–100 mM, at intervals of 25 mM, under otherwise constant conditions (potential 30 kV, injection time 4 s, temperature 25°C, wavelength 214 nm). The optimum separation was obtained with 50 mM borate buffer.

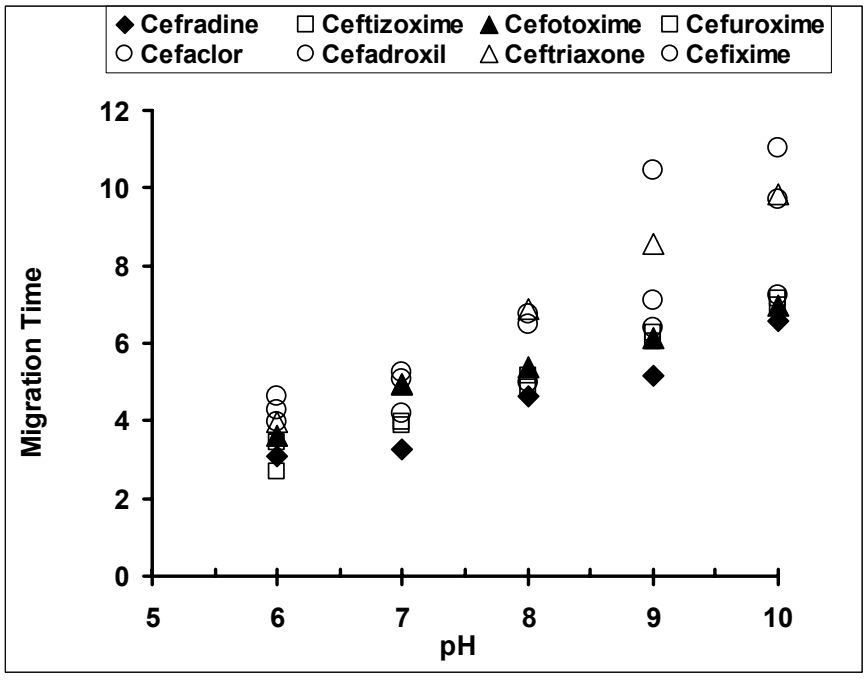


Fig. 2

Effect of pH on the migration times of the cephalosporins

The effect of applied voltage on the separation was examined in the range 20–30 kV at intervals of 5 kV. The migration time increased with decreasing applied voltage, without any improvement in the resolution of the cephalosporins (Fig. 3). An applied voltage of 30 kV was therefore selected, to achieve the shortest analysis time and the highest separation

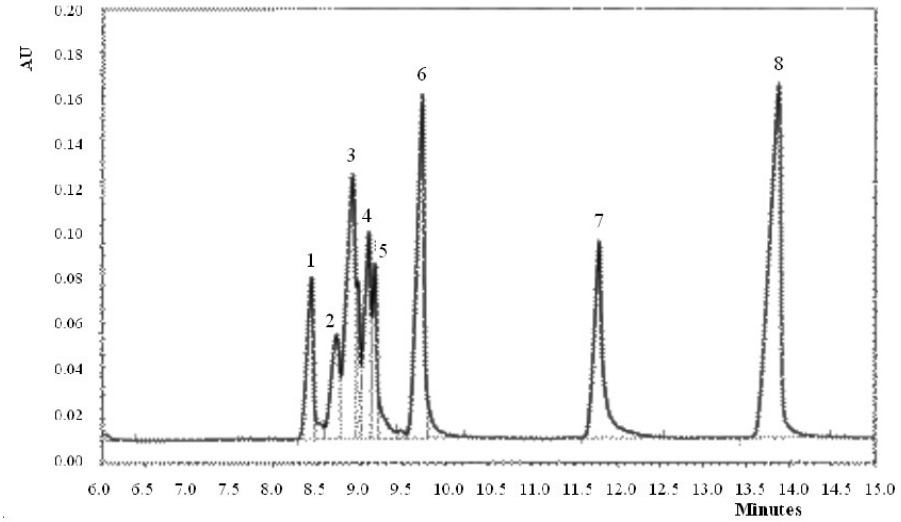


Fig. 3

Typical electropherogram obtained from a mixture of the cephalosporins. CE conditions: applied potential 20 kV; buffer 50 mM sodium tetraborate, pH 9; detection wavelength 214 nm. 1, cefradine; 2, ceftizoxime; 3, cefotoxime; 4, cefuroxime; 5, cefaclor; 6, cefadroxil; 7, ceftriaxone, and 8, cefixime

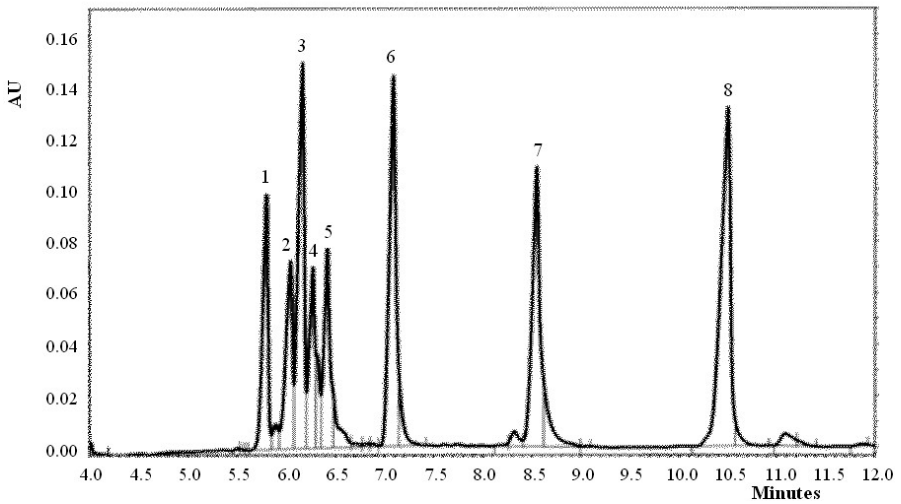


Fig. 4

Typical electropherogram obtained from a mixture of the cephalosporins. CE conditions: applied potential 30 kV; buffer 50 mM sodium tetraborate, pH 9; detection wavelength 214 nm. 1, cefradine; 2, ceftizoxime; 3, cefotoxime; 4, cefuroxime; 5, cefaclor; 6, cefadroxil; 7, ceftriaxone and 8, cefixime

efficiency. Under these optimized conditions the order of appearance of the cephalosporin peaks in electropherograms was as indicated in Fig. 4 and Table I.

The resolution (R_S) between pairs of adjacent peaks was calculated by use of an IBM personal computer and P/ACE MDQ system (32 Karat) software. Resolution between the peak pairs 1 and 2, 4 and 5, 5 and 6, 6 and 7, and 7 and 8 was >1.5; for peak pairs 2 and 3, and 3 and 4, however, resolution was 1.42 and 1.45, respectively (Table II). This resolution was sufficient for identification of individual peaks from migration times and by spiking with standard drug solution. The electrophoretic mobility of each of the cephalosporins was calculated, first separately and then in a mixture, and found to be in the range −7.486 to −14.867 $cm^2\ kV^{-1}\ min^{-1}$ with *RSD* in the range 0.5–1.5% (Table I); acetone was used as uncharged species. The repeatability of migration times, peak areas, and peak heights was examined for all eight cephalosporins ($n = 6$); *RSD* were in the ranges 0.5–1.1% for migration times and 0.3–1.4% for both peak areas and peak heights. Intra-day variation was also examined ($n = 5$) and *RSD* was in the ranges 0.6–1.6% and 0.5–1.8%, respectively. The effect of cephalosporin concentration on average peak height ($n = 6$) was examined and calibration plots were linear over two to three orders of magnitude (CFD 3–1000 μg mL^{-1}, CFT, CFR, and CFL 5–1000 μg mL^{-1}, CTR 10–1000 μg mL^{-1}, and CTA, CFC, and CIX 15–1000 μg mL^{-1}) with coefficients of determination (R^2) in the range 0.9926–0.9993 (Table III). Detection limits measured for a signal-to-noise ratio of 3:1 were in the range 0.5 to 5 μg mL^{-1} (Table I).

Table I

Analytical data for determination of cephalosporins

No.	Name	Migration time (min)	Mobility, μ_{ep} ($cm^2\ kV^{-1}\ min^{-1}$) (95% confidence limit)	*LOD* (μg mL^{-1})	% *RSD*	
					Peak area	Migration time
1	Cefradine	5.779	−7.486 ± 0.03	0.5	0.4	1.0
2	Ceftizoxime	6.025	−8.160 ± 0.008	1	0.8	0.9
3	Cefotoxime	6.146	−8.464 ± 0.015	1	1.4	0.7
4	Cefuroxime	6.250	−8.721 ± 0.021	1	1.0	0.7
5	Cefaclor	6.396	−9.072 ± 0.035	5	0.3	1.1
6	Cefadroxil	7.096	−10.535 ± 0.019	1	1.0	0.5
7	Ceftriaxone	8.537	−12.806 ± 0.003	5	0.6	0.6
8	Cefixime	10.488	−14.867 ± 0.012	2	0.8	0.7

Table II

Resolution (R_S) of the cephalosporins

Peaks	Cephalosporin	R_S
1 and 2	Cefradine–ceftizoxime	2.63
2 and 3	Ceftizoxime–cefotoxime	1.42
3 and 4	Cefotoxime–cefuroxime	1.45
4 and 5	Cefuroxime–cefaclor	2.73
5 and 6	Cefaclor–cefadroxil	13.33
6 and 7	Cefadroxil–ceftriaxone	18.51
7 and 8	Ceftriaxone–cefixime	13.63

Table III

Linear regression data for the cephalosporins

No.	Cephalosporin	Regression equation	Determination coefficient, R^2	Range (μg mL^{-1})
1	Cefradine	$y = 612.78x + 4690.5$	0.9928	3–1000
2	Ceftizoxime	$y = 608.9x + 3931.5$	0.9957	5–1000
3	Cefotoxime	$y = 491.25x - 2537.2$	0.9966	15–1000
4	Cefuroxime	$y = 434.16x - 4843$	0.9993	5–1000
5	Cefaclor	$y = 618.01x + 19502$	0.9926	15–1000
6	Cefadroxil	$y = 1014.4x - 51582$	0.9965	5–1000
7	Cefixime	$y = 1606.4x + 45356$	0.9973	15–1000
8	Ceftriaxone	$y = 1000.4x + 1882.4$	0.9969	10–1000

Effect of Excipients

The effect of excipients on analysis of the cephalosporins was examined. The excipients, glucose, lactose, sorbitol, gum arabic, starch, magnesium stearate, and methyl and propyl parabens were added at concentrations at least twice that of the drug. The excipients did not interfere with the determination, and relative error was within ±1.5%. When test mixtures at four different concentrations ($n = 4$) were analysed to examine the validity of the calibration plot the relative error was within ±4%.

Analysis of Pharmaceutical Formulations

A new calibration plot was prepared followed by analysis of the drug from the pharmaceutical preparation after dissolution in water or methanol–hydrochloric acid. The results from analysis of the seven drugs in nine pharmaceutical preparations agreed with the label values with *RSD* ($n = 4$)

within 0.2–1.5% (Table IV). No pharmaceutical preparations of the eighth drug, CTR, were available commercially so this drug was not analysed. Two pharmaceutical preparations, Zinacef and Velocef, were also analysed for CFR and CFD content by standard addition. The amounts found were 240 and 255 mg per tablet with *RSD* 1.0% and 1.1%, respectively. Recovery of the drugs was calculated to be within 99 and 100% (Table V).

Table IV

Results from analysis of pharmaceutical preparations

No.	Compound	Name of preparation	Label declaration (mg per tablet)	Amount found (mg per tablet)	*RSD* (%)
1	Cefuroxime	Zinacef (tablet)	250	240	1.0
2	Cefaclor	Ceclor (tablet)	250	240	1.4
3	Cefadroxil	Evacef (tablet)	500	533	0.3
		Helicef (tablet)	500	530	0.3
		Neucef (tablet)	500	520	0.25
4	Cefixime	Fixitil (tablet)	400	415	1.5
5	Cefradine	Velocef (tablet)	250	255	1.1
6	Ceftizoxime sodium	Cefizox (injection)	1000	1000	0.2
7	Cefotoxime sodium	Cefomerc (injection)	500	525	0.2

Table V

Results from analysis of pharmaceutical preparations by standard addition

No.	Compound	Name of tablet	Label amount (mg per tablet)	Amount added (mg)	Amount found (mg per tablet)	Recovery (%)	*RSD* (%)
1	Cefuroxime	Zinacef	250	0	240	–	1.0
				10	251	100.4	1.5
				20	272	100.7	1.5
2	Cefradine	Velocef	250	0	255	–	1.1
				10	264	99.6	1.7
				20	274	99.6	1.7

Analysis of Human Urine

Urine samples from two volunteers after taking a Zinacef or Velocef tablet containing 250 mg active ingredient CFR and CFD, respectively, were analysed after tenfold dilution with water (Figs 5 and 6). The con-

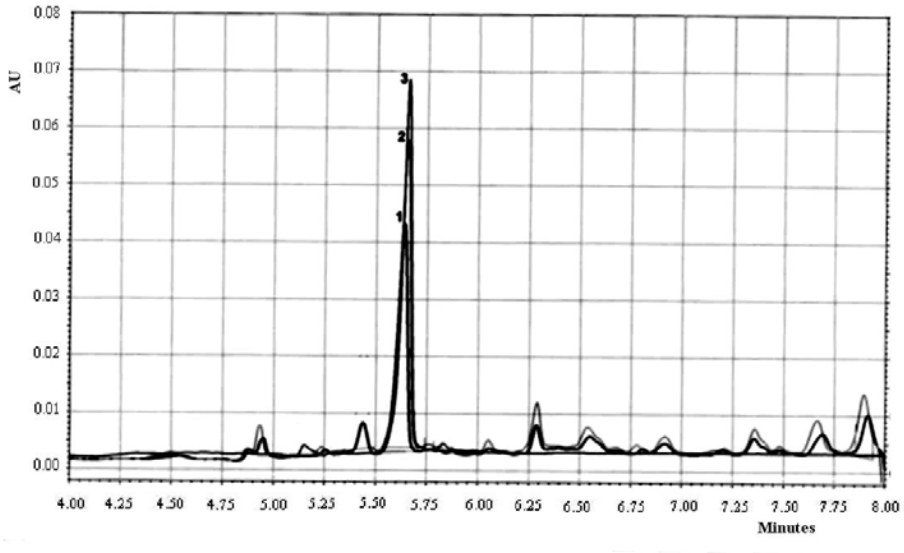


Fig. 5

Typical electropherogram obtained from a urine sample after administration of 250 mg cefradine. CE conditions as for Fig. 3. 1. Urine sample. 2. Urine sample with 100 µg CFD added. 3. Urine sample with 200 µg CFD added

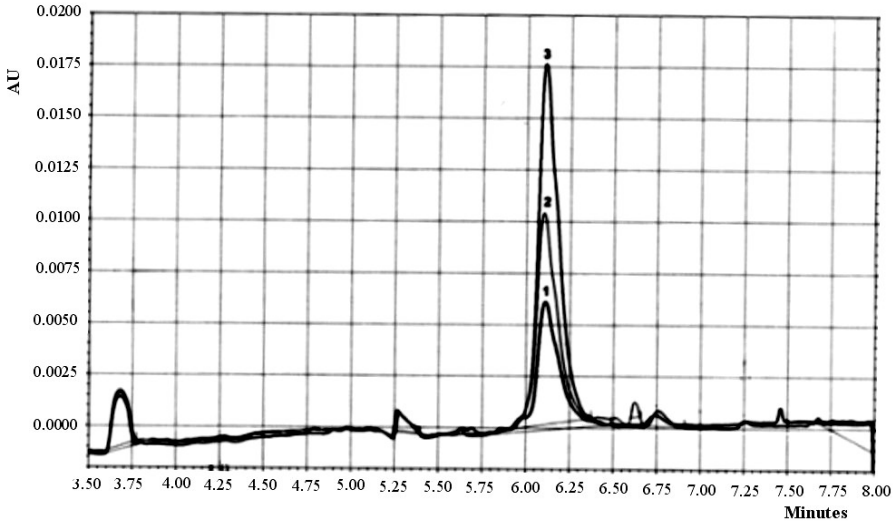


Fig. 6

Typical electropherogram obtained from a urine sample after administration of 250 mg cefuroxime. CE conditions as for Fig. 3. 1. Urine sample. 2. Urine sample with 100 µg CFR added. 3. Urine sample with 200 µg CFR added

centrations of the drugs in the urine were 29.8 and 30.2 μg mL^{-1}, respectively, *RSD* 1.5% and 1.7%, respectively. Recovery of CFR and CFD from urine was calculated by analysis of the same urine samples spiked with 100 and 200 mg of the drug standards and was observed to be between 99 and 100%, *RSD* 1.3–1.9%.

CONCLUSIONS

Simultaneous separation of CFL, CFD, CIX, CFC, CTA, CFR, CTR, and CFT by capillary zone electrophoresis has been achieved within 10.5 min by use of borate buffer of pH 9. Analysis of seven of the cephalosporins in nine pharmaceutical preparations and two in urine samples was conducted. Calibration plots were linear over two to three orders of magnitude of analyte concentration, and detection limits were at sub-μg mL^{-1} levels. Several excipients were investigated and none interfered with the analyses. The analytical method was highly reproducible with inter and intra-day variation, as *RSD*, within the ranges 0.6–1.6% and 0.5–1.8%, respectively. Identification of the drugs is also possible on the basis of migration time.

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