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Rapid and sensitive high-performance liquid chromatographic determination of four cephalosporin antibiotics in pharmaceuticals and body fluids

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Abstract

A rapid, accurate and sensitive method has been developed and validated for the quantitative simultaneous determination of four cephalosporins, cephalexin and cefadroxil (first-generation), cefaclor (second-generation) and cefataxim (third-generation), in pharmaceuticals as well as in human blood serum and urine. A Spherisorb ODS-2 250×4-mm, 5-μm analytical column was used with an eluting system consisting of a mixture of acetate buffer (pH 4.0)–CH_3OH 78–22% (v/v) at a flow-rate 1.2 ml/min. Detection was performed with a variable wavelength UV–Vis detector at 265 nm resulting in limit of detection of 0.2 ng for cefadroxil and cephalexin, but only 0.1 ng for cefotaxime and cefaclor per 20-μl injection. Hydrochlorothiazide (HCT) (6-chloro-3,4-dihydro-7 sulfanyl-2H-1,2,4-benzothiadiazine-1-1-dioxide) was used as internal standard at a concentration of 2 ng/μl. A rectilinear relationship was observed up to 8, 5, 12 and 35 ng/μl for cefadroxil, cefotaxime, cefaclor, cephalexin, respectively. Analysis time was less than 7 min. The statistical evaluation of the method was examined by means of within-day repeatability ($n=8$) and day-to-day precision ($n=9$) and was found to be satisfactory with high accuracy and precision. The method was applied to the determination of the cephalosporins in commercial pharmaceuticals and in biological fluids: human blood serum after solid-phase extraction and urine simply after filtration and dilution. Recovery of analytes in spiked samples was in the range from 76.3 to 112.0%, over the range of 1–8 ng/μl.

Keywords: Cephalosporins; Cefadroxil; Cefaclor; Cefotaxime; Cephalexin

1. Introduction

Cephalosporins are β-lactam antibiotics with the same fundamental structural requirements as penicillin. They are used for the treatment of infections caused by Gram-positive and Gram-negative bacteria. They act by inhibiting the synthesis of essential structural components of bacterial cell wall. They are among the safest and the most effective broad-spectrum bactericidal antimicrobial agents; therefore they are the most prescribed of all antibiotics [1].

Cephalosporin C was first isolated in 1948 from the fungus *Cephalosporium acremonium.* Its chemical modification allowed production of a whole series of semisynthetic cephalosporins used as therapeutics to fight organisms that have become penicillin resistant. As only cephalosporin C is found naturally, the remaining semi-synthetic cephalospor-

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ins are derived from 7-amino-cephalosporanic acid, a product obtained from cephalosporin C hydrolysis [2].

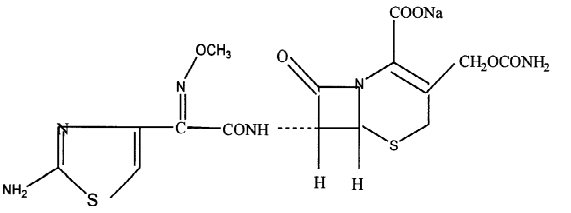
Their composition is accomplished by β-lactam ring fusion with a di-hydrothiazine ring differing in the nature of the substituents attached at the 3- and/or 7-positions of the cephem ring. The substitution at the 3-position affects the pharmacokinetic properties, while the substitution at the 7-position affects the antibacterial spectrum of the cephalosporins.

Cephalosporin antibiotics are divided into four generations: first, second, third and recently fourth-generation compounds. Classification is according to the extent of their effect against Gram-negative activity. Third-generation cephalosporins are characterized by a broader antibacterial spectrum in comparison with the first- and second-generation agents as they are resistant to β-lactamases. The most recent fourth-generation includes molecules with a spectrum similar to the third, but with markedly increased stability to hydrolysis by β-lactamase. Most of these compounds have a short terminal half-life, <1 h. However, some second-, third- and fourth-generation cephalosporins possess longer half-lives. Their excretion occurs mainly through glomerular filtration, but also through tubular secretion [3].

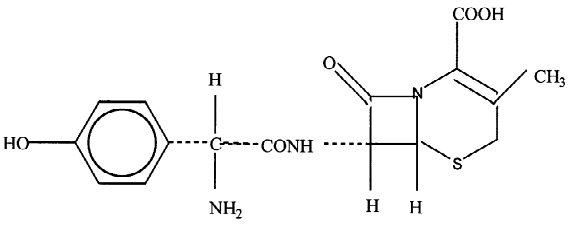
The chemical structures of the examined cephalosporins are shown in Fig. 1.

Cefadroxil and cephalexin are first-generation cephalosporin antibiotics. Cefaclor is a second-generation cephalosporin while cefataxime is a third-generation cephalosporin.

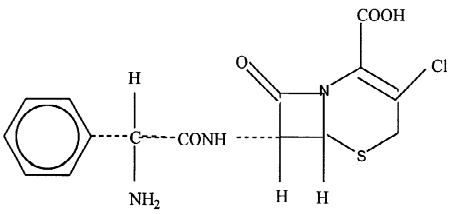
Various methods and reviews have been published covering the analysis of cephalosporins in biological matrices and pharmaceuticals. Among the published methods of determination of cephalosporins TLC, GC, HPLC, CE, and microbiological assays are available. Microbiological techniques lack sensitivity and specificity. HPLC methods have been described for the determination of cephalosporins in biological fluids using different stationary phases, mobile phases with different buffer systems, mostly phosphates or ion pairing agents, detection mode, e.g., UV and electrochemical and sample preparation procedures [2,4–11,13]. Derivatization process for cefotaxime determination has been also reported [12].



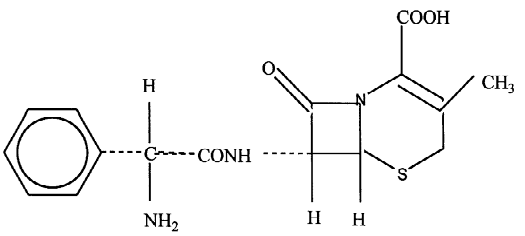
Cefotaxime sodium



Cefadroxil



Cefaclor



Cephalexin

Fig. 1. Structures of cephalosporins investigated.

Only two of the reported methods in literature determine simultaneously the four examined cephalosporins among other antibiotics, though only in pharmaceutical formulations [2,8]. The other published methods are determining one or some of the four examined compounds. Best sensitivity in pharmaceuticals is achieved with electrochemical de-

tection in the range of ppb levels; however, analysis time is more than 20 min [2].

One method reported on biological samples avoiding SPE requires high sample volumes: 5 ml of serum and 2.5–10 ml of urine [7].

Since numerous new molecules belonging to this group of antibiotics are continuously being developed, pharmacokinetic studies are necessary for a better understanding of their activity. Clinical research and therapeutic drug monitoring in routine hospital use clearly indicate the need for a fast and reliable method.

A simple, rapid assay method for the simultaneous determination of cephalexin, cefadroxil, cefaclor and cefataxim, in pharmaceutical formulations and biological fluids such as blood serum and urine is described here, requiring small volumes of biological fluids. The method gives good separation and is sensitive and linear over a wide concentration range.

2. Experimental

2.1. Instrumentation and chromatography

A Shimadzu (Kyoto, Japan) LC-10AD pump was used to deliver the mobile phase to the analytical column, Spherisorb ODS-2 250×4 mm, 5 μm purchased by MZ Analysentechnik (Mainz, Germany). Sample injection was performed via a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 20-μl loop. Detection was achieved by an SSI 500 UV–Vis detector (SSI, State College, PA, USA) at a wavelength of 265 nm and a sensitivity setting of 0.002 AUFS. A Hewlett-Packard (Avondale, PA, USA) HP3396 Series II integrator was used for quantitative determination of eluted peaks. A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using 0.2-μm membrane filters obtained from Schleicher & Schuell (Dassel, Germany). Degassing of solvents was achieved by helium sparging before use. Dissolution of compounds was enhanced by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany). A Glasscol, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pre-treatment.

All evaporations were performed with a nine-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA). UV spectra of cephalosporins for selecting the working wavelength of detection were taken using a Varian DMS 100S UV–Vis double-beam spectrophotometer.

UV spectra comparison was performed by SPD-M6A Shimadzu photodiode array UV–Vis detector complied with Data acquisition software Class-M10A. The mobile phase was prepared by mixing sodium acetate–acetic acid buffer, pH 4.0, solution and methanol in a ratio of 78:22 (v/v). The flow-rate was 1.2 ml/min. The injection volume was 20 μl.

2.2. Chemicals and reagents

Cefotaxime sodium salt and cephalexin hydrate were purchased from Fluka Chemie (Buchs, Switzerland). Cefaclor, cefadroxil and hydrochlorothiazide (internal standard) were supplied from Sigma (St. Louis, MO, USA). Methanol and acetonitrile and acetic acid were supplied from Merck (Darmstadt, Germany), while sodium acetate was from Riedel-de-Haen (Seelze, Germany).

Diol and RP-8 SPE BondElut cartridges were purchased from Analytichem, A division of Varian (Harbor City, USA). Other SPE cartridges examined are Discovery by Supelco (Bellefonte, PA, USA), OASIS HLB by Waters (Waters Corporation, MA, USA) and Adsorbex C-8 by Merck.

Commercial cefotaxime, and cefaclor pharmaceuticals were purchased from a local market. Ceclor a product by Eli Lilly & Co. (Indianapolis, IN, USA) in tablets and oral suspension was manufactured and packed by Vianex (Patras, Greece). Tablets contained 500 mg of cefaclor as monohydrate while suspension after dilution to water provided a solution of 125 mg/5 ml. Claforan a product of Hoechst Marion Roussel (Rommainville, France), is formulated as dry powder to be diluted in 2 ml of water, containing 0.524 g cefotaxime sodium salt corresponding to 0.5 g i.m/i.v.of cefotaxime is manufactured and packed by Vianex SA (Metamorfosi-Attikis, Greece).

Serum samples were kindly provided from the Blood Donation Unity of a State Hospital.

Urine samples for calibration curve construction and recovery assay were taken from healthy volunteers.

2.3. Standard solutions

Separate stock standard solutions of each cephalosporin were prepared by dissolving an accurate weight of 10 mg for each compound in 100 ml water. Working standard solutions were freshly prepared in deionized water at concentrations 0.01, 0.05, 0.1, 0.5, 1, 3 and 5 ng/μl for cefotaxime, up to 8 ng/μl for cefadroxil, up to 12 ng/μl for cefaclor and up to 35 ng/μl for cephalexine.

A 20-μl aliquot was injected onto the column. Quantitative analysis was based on peak area measurements as ratios towards the peak area of internal standard.

Pooled human drug-free serum obtained from healthy volunteers was spiked from the stock cephalosporin solutions to attain concentrations of 1, 2, 3, 5 and 8 ng/μl.

A pooled human drug-free urine sample was 100-fold diluted with water and filtered using 0.2-μm filters. Aliquots from this sample were spiked from the stock cephalosporin solutions to attain concentrations of 1, 2, 3, 5 and 8 ng/μl.

2.4. Method validation

Method validation was performed in terms of sensitivity and specificity, precision and accuracy, linearity and stability.

2.4.1. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank matrices.

2.4.2. Precision and accuracy

Method validation regarding reproducibility was achieved by replicate injections of standard solutions at low, medium and high concentration levels, where peak areas were measured in comparison to the peak area of the internal standard.

Intermediate precision study (day-to-day reproducibility) was conducted during routine operation of the system over a period of nine consecutive days. Statistical evaluation revealed relative standard deviations at different values for six injections.

Within-day repeatability was studied by eight replicate measurements at three concentration levels.

2.4.3. Stability

Problems of stability are usually encountered with these compounds, mainly affecting plasma concentrations at room temperature. From blood sampling to analysis, storage in the freezer eliminates decomposition.

The stability of cephalosporins was verified by storing sample solutions refrigerated for 6 months. Concentrations were measured once a week.

2.5. Pharmaceuticals sample preparation

2.5.1. Capsules

A number of commercial capsules were finely powdered in a porcelain mortar and an accurately weighed portion of the pooled sample equivalent to the antibiotic content of one capsule 0.5680 g, was quantitatively transferred to a volumetric flask and dissolved and diluted to volume with water. Further dilution provided solutions containing the following concentrations 1.0, 2.0 and 4.0 ng/μl.

2.5.2. Oral suspension

Granules are diluted to 60 ml providing a concentration of 125 mg/5 ml. Aliquots of the prepared suspension were suitably diluted with water to provide three solutions at 1.25, 2.0 and 5.0 ng/μl.

2.5.3. Sterile powder for injection

The total content of one package of the injection in dosage form of sterile powder was transferred into a 1000-ml volumetric flask, dissolved and diluted to volume with water. Aliquots of the prepared solution were suitably diluted to provide three solutions at 1.0, 2.0 and 3.0 ng/μl.

All working solutions originated from pharmaceutical formulations contained the internal standard HCT at a concentration of 2.0 ng/μl. Aliquots of 20 μl were injected onto the HPLC analytical column.

2.6. Sample extraction procedure

Five different sorbents were assayed for the extraction of the investigated cephalosporins: Oasis HLB (60 mg) by Waters, C_{18} (500 mg) Discovery by Supelco, Adsorbex RP-8 by Merck, and RP-8 and 2-OH Bondelut by Varian. Up to 20 different extraction protocols were assayed using different wash-

ing and eluting solvents. Optimum extraction protocol in terms of highest extraction efficiency is described in Fig. 2. Washing step was avoided as a significant analytes loss was noticed during this stage. Recovery was enhanced when a serial elution with 1 ml of methanol followed by 1 ml of acetonitrile was performed. Optimal extraction protocol was then applied to standards solutions of cephalosporins.

2.7. Serum sample preparation

Aliquots of 40 μl human blood serum were spiked with 100 μl of cephalosporin solutions at different concentration levels and treated with 200 μl of CH_3CN in order to precipitate proteins. After vortex mixing for 2 min, the sample was centrifuged at 800 *g* for 15 min and the supernatant was evaporated, at 45 °C, under nitrogen stream, to remove organic solvents. Subsequently the sample was quantitatively transferred and slowly applied to the solid-

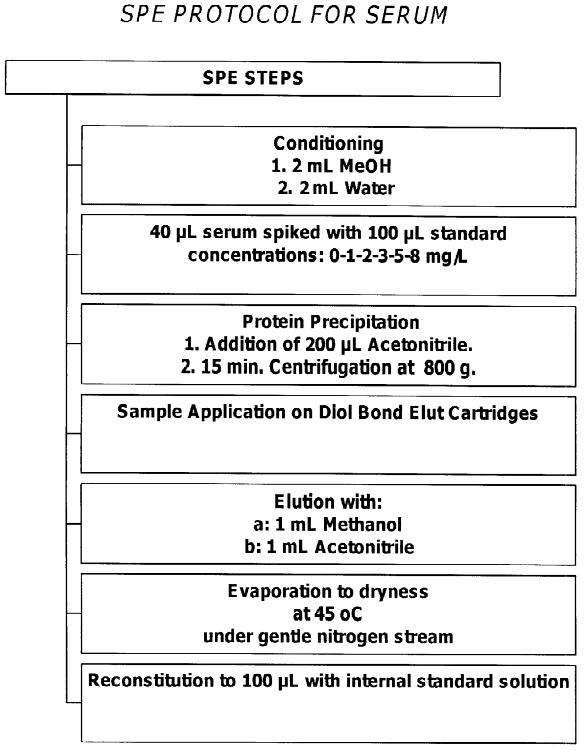


Fig. 2. SPE protocol for cephalosporins recovery from serum samples.

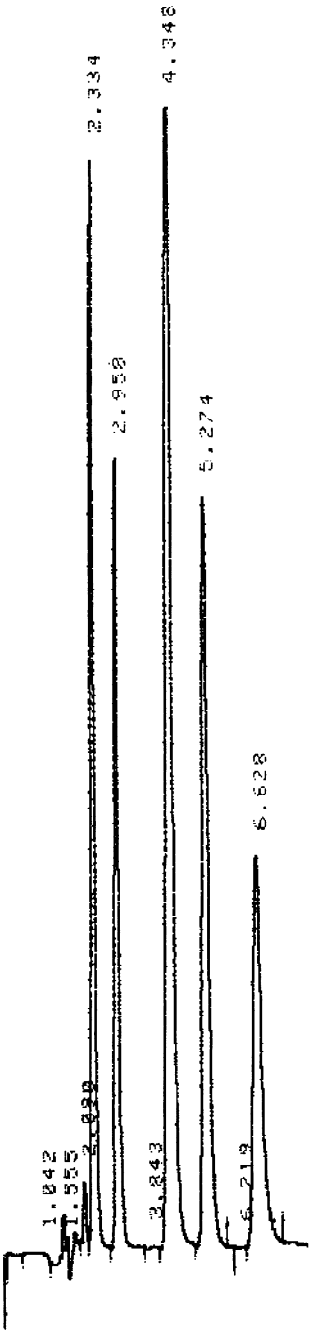


Fig. 3. High-performance liquid chromatogram of cephalosporins: cefadroxil (2.334 min), cefotaxime (4.348 min), cefaclor (5.274 min), cephalexin (6.628 min) with hydrochlorothiazide 2 ng/μl, 2.958 min, as internal standard. Chromatographic conditions are described in text.

Table 1
Retention times, calibration data and sensitivity of cephalosporins determination using HCT as internal standard

Compound	Retention times mean value±SD (min)	RSD (%)	LOD (ng/μl)	LOQ (ng/μl)	Upper limit (ng/μl)	Regression equation	*R*
Cefadroxil	2.326±0.050	2.2	0.01	0.03	8	$Y = (0.12626 \pm 0.00893) + (0.01060 \pm 0.00010)X$	0.9999
Cefotaxime	4.370±0.121	2.8	0.005	0.015	5	$Y = (0.02202 \pm 0.04381) + (0.02398 \pm 0.00074)X$	0.9995
Cefaclor	5.264±0.139	2.6	0.005	0.015	12	$Y = (0.00883 \pm 0.01689) + (0.01225 \pm 0.00016)X$	0.9998
Cephalexin	6.669±0.190	2.8	0.01	0.03	35	$Y = (-0.01310 \pm 0.02567) + (0.01127 \pm 0.00027)X$	0.9994

Y, peak area ratio; *X*, ng.

phase cartridge, which was preconditioned with methanol and water.

The sample was subsequently treated according to the procedure described under solid-phase extraction paragraph.

The same procedure can be followed when samples are analyzed after cephalosporins administration. In this case the reconstitution should be made to 40 μl in order to avoid analyte dilution.

2.8. Urine sample preparation

Urine samples were initially at a volume ratio 1:50 and filtered using 0.2-μm filters. A subsequent 1:1 dilution was performed by adding equal volume of internal standard solution, at a concentration of 4 ng/μl in order to provide the desired final concentration of 2 ng/μl. Spiked urine drug-free samples were also prepared in the same manner by adding the appropriate concentrations of standard solutions. Aliquots from these samples were injected into the HPLC, analyzed and chromatographed under the conditions described above.

2.8.1. Ceclor urine samples

Urine samples were collected after 30 min, 45 min and 2 and 3 h of Ceclor administration to a patient. The volume of the urine was measured and recorded after each collection. A portion of the sample was filtered and suitably diluted with internal standard

Table 2
Day-to-day (over a period of nine consecutive days) and within-day ($n=8$) precision and accuracy study for determination of cephalosporins

Added (ng)	Within-day			Day-to-day		
	Found±SD (ng)	RSD	Relative error %	Found±SD (ng)	RSD	Relative error %
Cefadroxil						
10	10.1±0.5	4.9	1.1	9.8±0.7	6.9	−1.9
20	19.1±0.2	1.2	−4.5	19.5±1.3	6.6	−2.6
100	99.9±0.8	0.8	−0.1	101.2±2.4	1.9	1.2
Cefotaxime						
10	10.0±0.3	3.1	0.1	9.9±0.5	4.6	−1.0
20	18.3±0.1	0.6	−8.5	18.7±0.04	0.2	−6.6
100	99.6±2.4	2.4	−0.4	101.3±2.0	1.9	1.3
Cefaclor						
12	12.1±0.6	4.5	0.7	12.4±0.3	2.6	3.4
24	24.0±0.3	1.3	−0.2	23.8±0.7	2.9	−0.6
120	122.2±1.0	0.8	1.8	123.5±3.2	2.6	2.9
Cephalexin						
9.6	9.9±0.5	5.1	3.0	10.0±0.2	2.4	4.2
19.2	18.4±0.2	1.4	−4.3	19.1±0.7	3.6	−0.3
96	96.0±1.4	1.5	0.0	98.2±3.0	3.1	2.3

solution. A 20-μl volume was injected into the HPLC in triplicate for each solution and chromatographed under the conditions described above. Cefaclor levels were calculated using the calibration curve for urine samples.

3. Results and discussion

3.1. Chromatography

A chromatogram obtained using the developed methods conditions is illustrated in Fig. 3. Resolution factors ranged from 1.54 to 2.78 indicating satisfactory separation of resolved analytes.

Retention time precision for a 10-day period is summarized in Table 1.

3.2. Method validation

3.2.1. Linearity and sensitivity

Calibration curves were obtained by least-squares linear regression analysis of the peak area ratio of analyte/internal standard versus analyte concentration. The method was linear up to 8, 5, 12 and 35 ng/μl for cefadroxil, cefotaxime, cefaclor and cephalexin, respectively. Correlation coefficients ranged from 0.9994 to 0.9999.

Table 2 summarizes the results of the method validation regarding accuracy, within-day and day-to-day precision assays. The measured concentra-

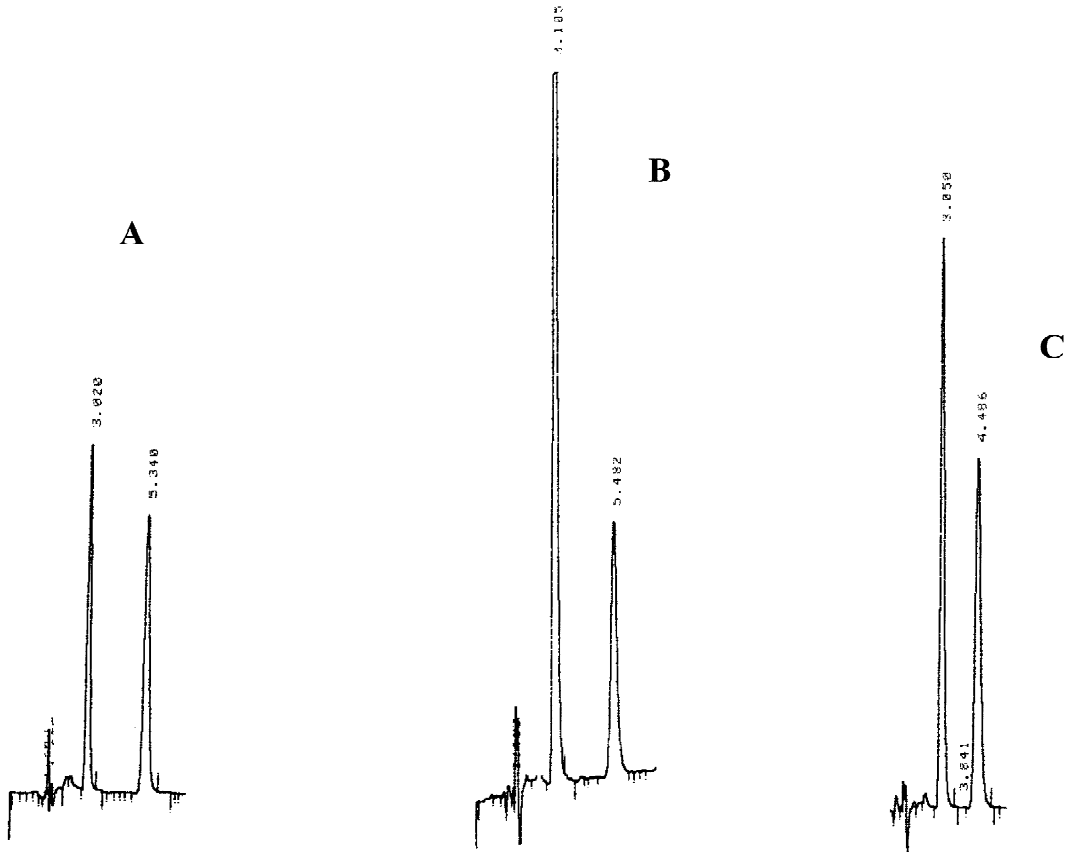


Fig. 4. High-performance liquid chromatogram of cephalosporins in commercial pharmaceuticals with I.S. 2 ng/μl. (A) Cefaclor capsules: HCT (3.020 min), cefaclor (5.340 min). (B) Cefaclor syrup: HCT (3.105 min), cefaclor (5.482 min). (C) Cefotaxime injection powder: HCT (3.050 min), cefotaxime (4.480 min). Chromatographic conditions are described in text.

tions had RSD values <7%, with relative error (inaccuracy) in the range of −6.6 to 4.2%.

The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three. Respective LOD values for each compound based upon this criterion are shown in Table 1. These values were observed for 10 samples. The limit of quantitation of the assay was evaluated as the concentration equal to 10 times the value of the signal-to-noise ratio and found to be 0.3 ng for cefotaxime and cefaclor and 0.6 ng for cefadroxil and cephalexin.

3.2.2. Precision and accuracy

The precision of the method based on within-day repeatability was performed, by replicate injections ($n=8$) of three standard solutions covering different concentration levels: low, medium and high, where peak areas were measured, in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations, at different values. Results are shown in Table 2.

The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of nine consecutive days. Reproducibility results are illustrated in Table 2.

Accuracy was determined by replicate analysis of three different levels (1.0, 2.0 and 5.0 ng/μl). Expressed as relative error can be calculated by the equation:

$$\text{Relative Error (\%)} = \frac{[\text{Mean determined value} - \text{theoretical (added amount)}]}{\text{theoretical}} \times 100$$

Table 3
Results of cephalosporins determination in commercial pharmaceuticals

Added cefaclor (ng)	Measured±SD (ng)	RSD %	Found[a] (mg) ±SD
Ceclor: capsules (declared 500 mg)			
20	20.9±0.3	1.6	523.8±8.2
40	38.1±0.7	1.8	476.1±8.5
80	77.9±1.5	2.0	487.0±4.2
Found[b] (mg) 495.6±6.9			
Added cefaclor (ng)	Measured±SD (ng)	RSD %	Found[a] (mg/5 ml) ±SD
Ceclor: oral suspension declared 125 mg/5 ml			
25	26.5±0.4	1.6	132.6±2.4
40	38.6±0.8	2.1	120.7±2.6
100	105.8±0.3	0.3	132.3±0.4
Found[b] (mg/5 ml) 128.5±1.8			
Added cefotaxime (ng)	Measured±SD (ng)	RSD %	Found[a](g)±SD
Sterile powder for injection Claforan (declared[a] (g)±SD			
20	15.2±0.3	1.6	0.38±0.01
40	34.6±0.9	2.1	0.43±0.01
60	52.9±0.9	1.6	0.43±0.02
Found[b] (g) 0.42±0.01			

[a] Mean of six measurements.
[b] Mean of six measurements×three different concentrations.

Table 4
SPE recovery results using different protocols for cephalosporins isolation

Analyte	Recovery (%)										
	Varian RP-8[a]	Varian RP-8	Varian RP-8[b]	Discovery C_{18}	OASIS	Merck-RP-8[c]	Merck-RP-8[d]	2-OH[e]	2-OH[f]	2-OH[g]	2-OH[h]
Cefadroxil	53.4	83.3	45.6	47.6	97.7	72.5	48.4	79.0	87.5	89.6	108.0
Cefotaxime	48.6	51.9	66.2	75.2	81.3	45.4	43.3	48.9	70.0	80.4	88.3
Cefaclor	36.2	75.1	243.4	141.9	40.2	200	60.6	≫ 200	81.4	73.6	81.0
Cephalexin	33.5	44.9	34.5	54.2	65.7	77.2	55.8	65.6	63.2	63.1	69.0

[a] Conditioning: CH_3OH–acetate buffer.
[b] No washing.
[c] Washing: CH_3OH–H_2O (10:90, v/v).
[d] Washing: CH_3OH–H_2O (2:98, v/v).
[e] Elution with CH_3CN.
[f] Washing: CH_3OH–H_2O (5:95, v/v).
[g] Elution by a mixture of CH_3OH–CH_3CN (50:50, v/v).
[h] Serial elution by CH_3OH and CH_3CN. No washing.

3.2.3. Stability

Standard solutions stored at 4 °C were stable for periods up to 6 months for all examined compounds except for cephalexin that was found to be stable for 5 months. Also eluted samples from SPE columns dried on the same day were found to be stable when stored at 4 °C for 2 weeks. Thus extracted serum and urine samples can be stored at 4 °C for subsequent HPLC analysis. In deep-frozen biological fluids all compounds were found to be stable for 5–6 months.

Table 5
Recovery of cephalosporins from human blood serum and urine

Serum			Urine		
Added (ng)	Found (ng) ±SD	Recovery (%)	Added (ng)	Found (ng) ±SD	Recovery (%)
Cefadroxil					
40	37.4±0.01	93.4			
60	61.8±4.3	103.0			
100	99.2±3.1	99.2			
Cefotaxime					
40	35.1±0.5	87.7	40	38.3±0.6	95.7
60	62.8±0.2	104.7	60	59.1±0.5	98.4
100	100.2±0.7	100.1	88	87.9±0.5	99.9
Cefaclor					
44.8	45.9±1.8	102.5	44.8	43.2±0.6	96.6
67.2	68.8±1.4	102.4	67.2	65.3±0.5	97.1
112	85.5±0.8	76.3	89.6	85.2±0.5	95.1
Cephalexin					
40	39.9±2.4	99.8	40	34.3±0.7	86.0
60	67.2±1.1	112.0	60	61.4±0.6	102.2
100	107.5±1.0	107.5	100	79.2±0.7	99.0

3.3. Analysis of pharmaceuticals

High-performance liquid chromatograms of cephalosporins in pharmaceutical formulations are shown in Fig. 4. The experimental results from these analyses are given in Table 3.

3.4. Solid-phase extraction

Recovery rates determined by comparing observed analyte concentration in extracted sample to those of non-processed standard solutions, using different sorbents and elution protocols are summarised in Table 4. As shown in this table hydrophilic and lipophilic balanced sorbents octadecyl silica and octyl silica sorbents provide low recoveries, while diol sorbent presents higher efficiency in cephalosporin extraction.

Percentage recovery values higher than 100% are due to carryover effect as SPE cartridges were sometimes reused.

Washing the sorbent with water or mixtures of water with a low percentage of methanol leaded to low recoveries indicating the loss of analytes. This was verified with the analysis of washing step eluant after evaporation to dryness. High concentrations of cephalosporins were measured. When no washing step was involved higher recovery rates were obtained.

As shown in Table 4 serial elution with 1.5 ml of methanol and 1.5 ml of acetonitrile instead of using 3 ml of a 50:50 (v/v) mixture was noticed to provide enhanced recoveries.

3.4.1. Biological fluids: human blood serum and urine

The precision and accuracy studies of SPE of cephalosporins from biological samples were conducted by spiking blood serum samples, with three known concentrations of the compounds and then by comparing obtained results, with those as calculated from regression equations of SPE processed samples. Regression equations revealed correlation coefficients ranging between 0.9991 and 0.9998. Results of recovery studies for serum samples are given in Table 5. Each value represents the mean of six measurements carried out. A blank chromatogram of human blood serum is illustrated in Fig. 5a. The

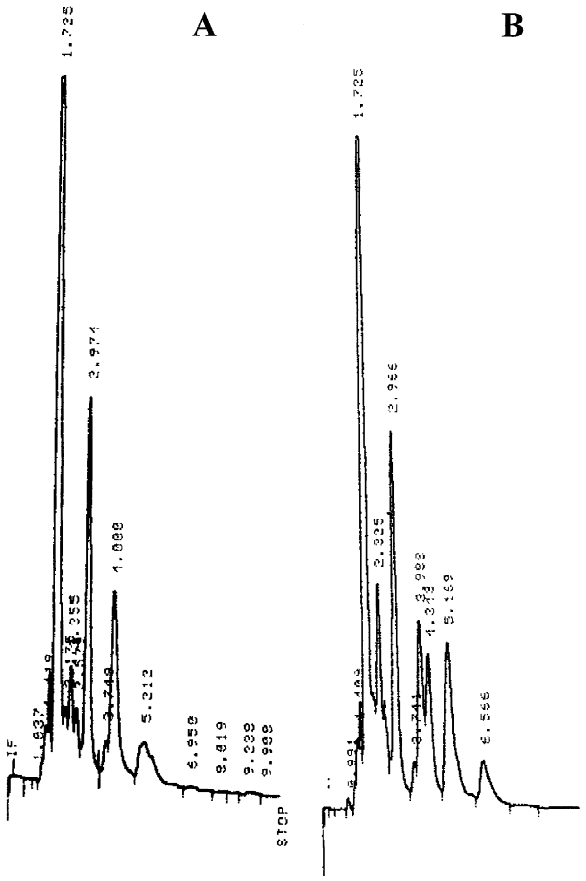


Fig. 5. (a) High-performance liquid chromatogram of blank human blood serum after SPE using the conditions described in text. Peak at 2.974 min is the internal standard hydrochlorothiazide 2 ng/μl. Peak at 4.000 min is an endogenous non-interfering compound, while peak at 5.212 min is paracetamol. (b) High-performance liquid chromatogram of cephalosporins: cefadroxil (2.325 min), cefotaxime (4.343 min), cefaclor (5.169 min), cephalexin (6.566 min) with hydrochlorothiazide 2 ng/μl, 2.966 min, as internal standard, in spiked human blood serum after SPE using the conditions described in text.

endogenous peak appearing at 5.212 min, close to the retention time of cefachlor has been identified to be paracetamol. Identification was performed by UV spectra comparison using a photodiode array detector. High-performance liquid chromatogram of cephalosporins extracted from human blood serum is shown in Fig. 5b. Relative recovery for serum was determined at three different concentrations by comparing the peak area ratios for extracted cephalospor-

ins from serum and the respective values derived from the serum calibration curve.

Urine samples were directly analyzed without SPE. The samples were simply diluted and filtered before analysis. Calibration curve was constructed by spiking pooled drug-free urine sample.

A blank chromatogram of urine sample is shown in Fig. 6a. High-performance liquid chromatogram of cephalosporins in spiked human urine is shown in Fig. 6b. The quantitation of cefadroxil in urine was impaired by interferences caused by an unidentified metabolite. Fig. 6c illustrates the high-performance liquid chromatogram of cefaclor in urine after Ceclor administration.

4. Conclusion

Monitoring of the antibiotics concentration in biological fluids such as serum and urine is important in pharmacokinetic studies.

The aim of this study was to develop a simple, fast and sensitive method for the simultaneous determination of four cephalosporin antibiotic agents in

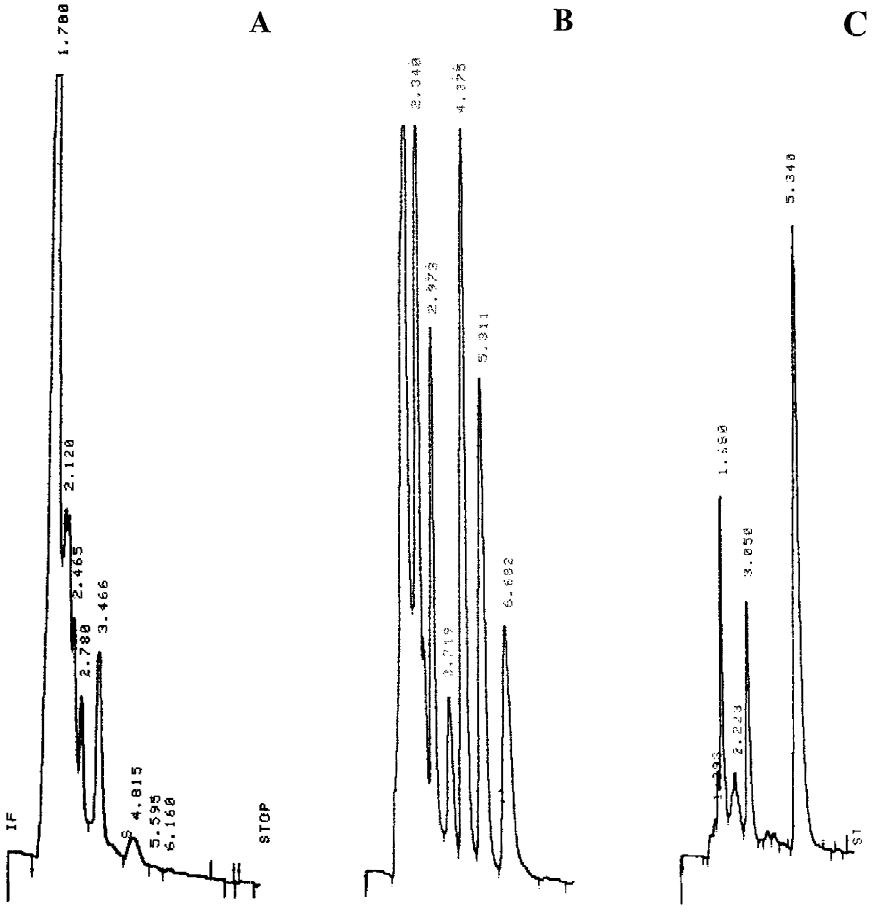


Fig. 6. (a) High-performance liquid chromatogram of blank urine after dilution using the conditions described in text. Peaks at 3.466 and 4.815 min are from endogenous non-interfering compounds. (b) High-performance liquid chromatogram of cephalosporins: cefadroxil (2.340 min), cefataxime (4.375 min), cefaclor (5.311 min), cephalexin (6.682 min) with hydrochlorothiazide 2 ng/μl, 2.973 min, as internal standard in spiked urine sample after dilution using the conditions described in text. (c) High-performance liquid chromatogram of cefaclor (5.340 min), with hydrochlorothiazide 2 ng/μl, 3.050 min, as internal standard in urine sample of a patient after Ceclor administration. Conditions are described in text.

pharmaceuticals and biological fluids. Though simultaneous dosing of the four compounds is unlikely and formulations usually contain only one as active ingredient, the simultaneous determination is useful for routine application in the clinical laboratory as with a single sample preparation protocol the analyst can monitor any of the studied analytes.

As cephalosporins are rather unstable compounds and their stability in solutions depends on several factors as temperature or pH of the solution, time-consuming procedures for their determination must be avoided to minimize drug decomposition.

None of the reported methods is referred to the analysis of the four cephalosporins described in this paper. There are two papers in literature determining the four members of this antibiotic group among others, however these papers apply the developed method only to pharmaceuticals [2,8]. Besides LOQ values reported are in the μg levels much higher than those reported in this method.

The method described herein is a simple validated assay that can readily be used in any laboratory for the quantitative determination of cephalosporins: cefadroxil, cefaclor, cefotaxime and cephalexin in less than 7 min. Phosphate buffers that are usually used in literature are avoided. No ion pairing agent is needed such as it is often used for the analysis of these compounds. Sample preparation time for the urine is minimal since no SPE is required. For serum samples sample pre-treatment step involving SPE can be accelerated by using a SPE manifold. The assay procedures are simple with satisfactory precision and accuracy with RSD$<$7. Reduced sample volumes of biological samples compared to those reported in literature render the method applicable in subjects of any age.

Paracetamol when present was noticed to interfere in serum analysis, while the quantitation of cefadroxil in urine was impaired by interferences caused by an unidentified metabolite.

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