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SYSTEMATIC APPROACH TO THE DETERMINATION OF CEPHALOSPORINS IN BIOLOGICAL FLUIDS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

M.C. ROUAN*, F. ABADIE, A. LECLERC and F. JUGE

Ciba-Geigy, Centre de Recherche Biopharmaceutique, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

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SUMMARY

The chromatographic behaviour of some cephalosporins as a function of pH and ionic strength of the mobile phase was studied on 10-μm LiChrosorb RP-18. Acidic cephalosporins were retained longest in their neutral form with an acidic eluent. Amphoteric cephalosporins were retained longest in their protonated form with an acidic eluent of low ionic strength. Cefotiam was retained longer with an alkaline mobile phase. LiChrosorb RP-18, Nucleosil C_{18} and μBondapak C_{18} gave rise to different selectivities when an acidic eluent, methanol—water (25:75) containing 0.2% of 1.8 *M* H_2SO_4 was used. This may be related to interactions with residual silanol groups. The studied cephalosporins (with the exception of cefotiam and cefsulodin) were separated from compounds present in biological fluids on 5-μm LiChrosorb RP-18 using the mobile phase 0.2% of 1.8 *M* H_2SO_4 in a mixture of methanol and water with various methanol contents. The determination of cefotiam in biological fluids was performed with an alkaline mobile phase. The preparation of the sample was simple and rapid: precipitation of plasma proteins or dilution of urine. The method was applied to the determination of ceftizoxime in human plasma and urine. Concentrations down to 0.2 μg/ml of plasma and 25 μg/ml of urine could be determined with good reproducibility and accuracy.

INTRODUCTION

In recent years, many liquid chromatographic methods have been reported for the determination of different cephalosporins in plasma and urine. The reversed-phase mode is most commonly used, with precipitation of plasma proteins [1—7] or extraction from plasma with Sep-Pak cartridges [8] prior to injection. The nature and the concentration of the components of the mobile phases used differ arbitrarily from one method to another although cephalosporins have a common 7-aminocephalosporanic acid nucleus. Recently, Brisson and Fourtillan [9] determined seven cephalosporins in plasma in the

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reversed-phase mode by changing only the content of methanol or acetonitrile in the mobile phase. Cephalosporins were extracted and back-extracted in order to obtain clean plasma blanks. However, orally absorbed cephalosporins such as cefroxadin and cephalexin, which possess an α-amino group in their C-7 side-chain, cannot be extracted with the proposed method.

A study of the chromatographic behaviour of cephalosporins on reversed-phase packings may be useful to ascertain whether cephalosporins can be quantitatively determined in biological fluids under similar chromatographic conditions. In a previous paper [10] the influence of the ionization of some cephalosporins was studied with a non-polar octadecylsilyl stationary phase. In the present paper, this influence is examined with mobile phases of lower ionic strengths. On the basis of the results obtained, conditions for the determination of cephalosporins in human plasma, urine, bile and milk, with a rapid sample-preparation step prior to injection, are described.

EXPERIMENTAL

Materials and reagents

The structures of the investigated cephalosporins are given in Table I. Ceftizoxime, cefotiam, cefsulodin and cefroxadin were obtained from Ciba-Geigy (Basle, Switzerland), cefotaxime and its desacetyl metabolite from Roussel Uclaf (Paris, France), cefuroxime and cephalexin from Glaxo (Paris, France), cefamandole and cephalothin from E. Lilly (Saint-Cloud, France), and cefazolin from Allard (Paris, France).

Phosphoric acid (85%), sodium dihydrogen phosphate, disodium hydrogen phosphate, sulphuric acid (95—97%), sodium hydroxide (32%), sodium sulphate and trichloroacetic acid (TCA) were purchased from E. Merck (Darmstadt, G.F.R.). Methanol was purchased from Prolabo (Paris, France). All chemicals were of analytical reagent grade and were used without further purification.

The following reversed-phase materials were used: Co:Pell ODS, 30—38 μm particle size (Whatman, Clifton, NJ, U.S.A.); LiChrosorb RP-18, 5 and 10 μm particle sizes (E. Merck); μBondapak C_{18}, 10 μm particle size (Waters Assoc., Milford, MA, U.S.A.) and Nucleosil C_{18}, 5 μm particle size (Macherey-Nagel, Düren, G.F.R.).

Apparatus

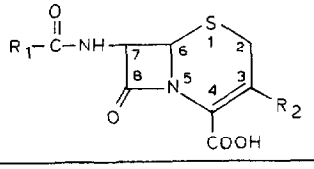
Chromatography was performed on a Hewlett-Packard Model 1081 B instrument equipped with a fixed-wavelength UV detector (254 nm) and an automatic sample injector. The detector was connected to a Hewlett-Packard data system (Model 3353 E).

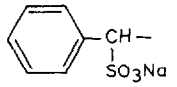
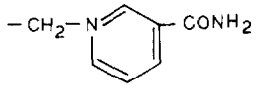
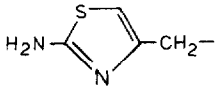
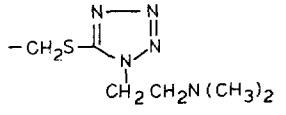
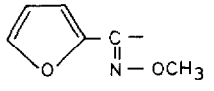
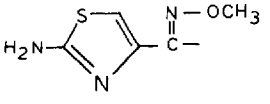
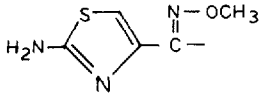
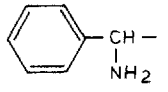
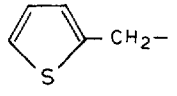
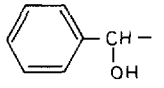
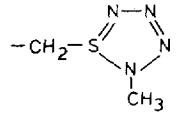
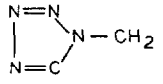
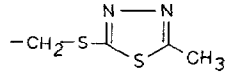
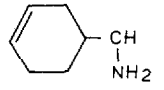
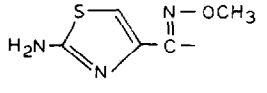
A pre-column (stainless-steel tube, 10 cm × 4.7 mm I.D.), tap-filled with Co:Pell ODS, was used to protect the analytical column. The analytical column (stainless-steel tube, 4.7 mm I.D.) was 15 cm long when filled with LiChrosorb RP-18 and Nucleosil C_{18} and 25 cm long when filled with μBondapak C_{18}. The reversed-phase material was suspended in 10^{-2} mol/l sodium acetate in water—methanol (20:80, v/v) in a ratio of 17:100 (w/v). The filling pressure was set at 420 bars with a pneumatic Haskel pump. Methanol was used as the pressurizing solvent.

TABLE I

CHEMICAL STRUCTURE OF THE INVESTIGATED CEPHALOSPORINS

Cephalosporins



	R_1	R_2
Cefsulodin		
Cefotiam		
Cefuroxime		$-CH_2-O-C-NH_2$ (O)
Cefotaxime		$-CH_2-O-C-CH_3$ (O)
Desacetylcefotaxime		$-CH_2-OH$
Cephalexin		$-CH_3$
Cephalothin		$-CH_2-O-C-CH_3$ (O)
Cefamandole		
Cefazolin		
Cefroxadin		$-O-CH_3$
Ceftizoxime		$-H$

The flow-rate of the mobile phase was 2 ml/min for the 10 μm particle size materials and 1.2 ml/min for the 5 μm particle size materials.

The pH of the aqueous part of the mobile phase was measured with a pH meter (Beckman digital pH meter) and adjusted with NaOH or H_2SO_4.

Sample preparation

Plasma samples were prepared as follows, unless otherwise specified: 150 μl of plasma, 50 μl of TCA (10:100, w/v) aqueous solution, and 15 μl of aqueous solution of the studied cephalosporin, were introduced into a 10-ml conical glass tube, mixed on a Vortex mixer for 15 sec and centrifuged for 3 min. A 20—40 μl volume of the clear solution was injected onto the column.

Human milk and bile samples were prepared as those of plasma, and 20 μl of the supernatant were injected after a two- to ten-fold dilution.

For urine samples, 100 μl of urine, 100 μl of standard solution of the cephalosporin and 800 μl of water were introduced into a 10-ml glass tube and mixed on a Vortex mixer; 10—20 μl of the solution were injected onto the column.

RESULTS AND DISCUSSION

Effect of pH and ionic strength of the mobile phase on the retention of cephalosporins

The effect of pH and ionic strength of the mobile phase was investigated on 10 μm LiChrosorb RP-18.

Cephalosporins contain one or more groups ionizable in the pH range studied (pH 2—8), and the retention of these compounds will depend on the extent of dissociation of these groups. All cephalosporins possess a carboxylic group and some have another acidic function (cefsulodin) or an amino group (cefroxadin, cephalexin and cefotiam). Furthermore, some new cephalosporins (cefotiam, cefotaxime and ceftizoxime) possess an aminothiazolyl moiety with an amino group which exhibits weak basic properties. The pK_a values which relate to the 4-carboxylic group and to the 7-side-chain amino group have been reported to be about 3—4 and 7—7.5, respectively, at 20°C and ionic strength 0.15 [10]. The three pK_a of cefotiam were reported to be $pK_{a_1} = 2.6$, $pK_{a_2} = 4.6$, $pK_{a_3} = 7.0$ in aqueous solution [11].

At pH 8, all cephalosporins are mainly present in anionic form. When the ionic strength of the mobile phase was increased by addition of sodium phosphates at this pH, the retention of all the investigated cephalosporins also increased, as illustrated in Fig. 1A. This is in agreement with the findings of Van de Venne et al. [12], who stated that with an increasing concentration of cations from the buffer, the adsorption of ionized acids on the organic silica is increased owing to compensation of their negative charges by these cations.

The ionic strength was further increased at pH 2.7 by addition of sodium sulphate. The dissociation of carboxylic groups is partly suppressed at this pH. The effect of the ionic strength was low for cefuroxime and cefazolin, whereas the retention decreased with increasing salt concentration for cephalosporins having an amino group. This is illustrated in Fig. 1B. Such a decrease in retention of protonated amino compounds on octadecyl-silicas with increasing salt

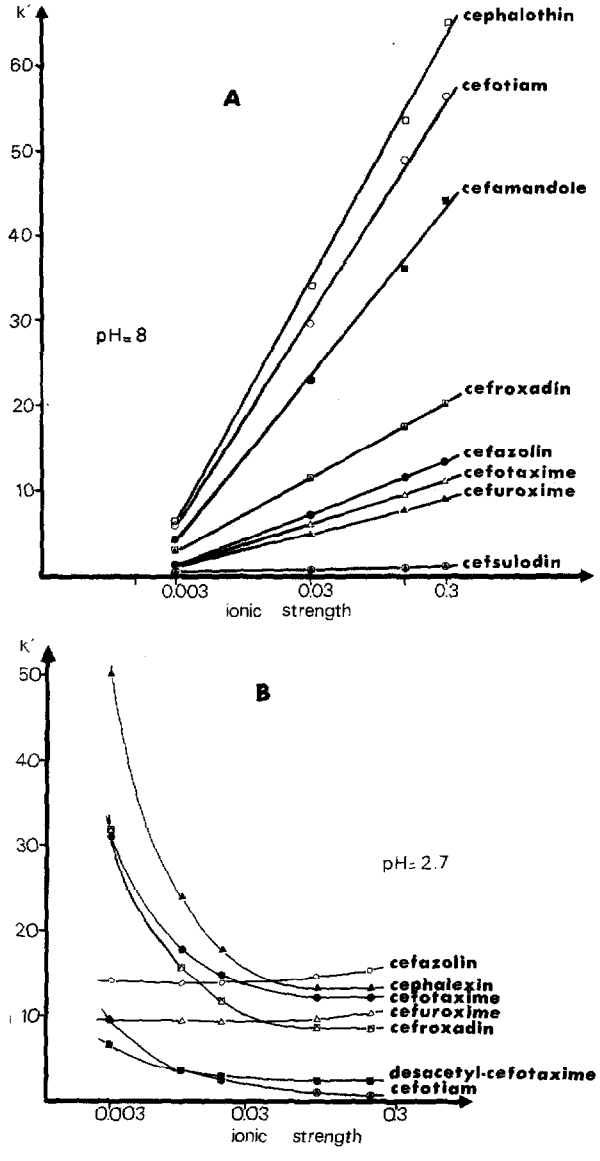


Fig. 1. Plots of capacity factors against ionic strength. Column: LiChrosorb RP-18, 10 μm. Methanol content in the mobile phase: 15% (v/v).

concentration was previously reported [13–15]. This decrease may be interpreted by the dual-retention model [13], which involves both solvophobic interactions with the hydrocarbonaceous layer and silanophilic interactions with unreacted silanol groups that remain on the surface of the stationary phase. With increasing concentration of cations from the buffer, an attenuation of silanophilic interactions may result from a competitive effect of these cations on residual silanols. Cephalexin and cefroxadin are fully protonated at this low pH and their retention is more markedly reduced than that of cefotaxime, which exhibits weak basic properties.

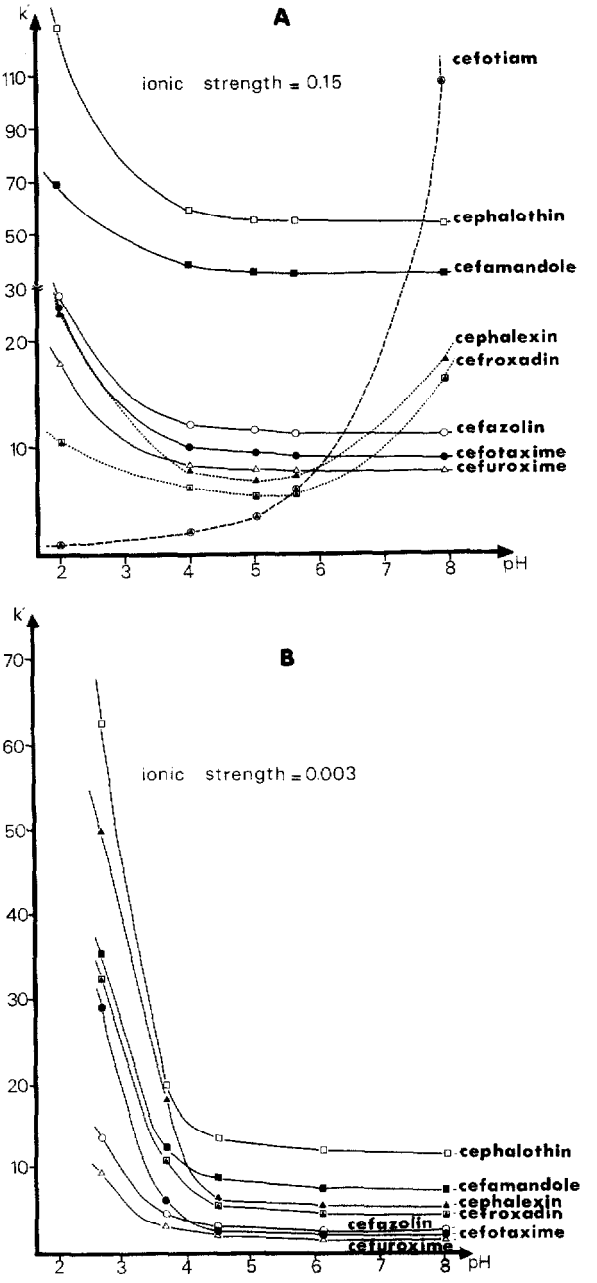


Fig. 2. Plots of capacity factors versus mobile phase pH. Column: LiChrosorb RP-18, 10 μm. Methanol content in the mobile phase: 15% (v/v).

The pH was increased at constant ionic strength (I = 0.15). The k'—pH profiles obtained (Fig. 2A) reflect the ionization of the compounds, as previously reported for some cephalosporins [10]. This indicates that the retention occurs essentially by way of solvophobic mechanisms at the studied ionic strength. As the pH increases, the decrease in retention is due to gradual formation of

anionic or zwitterion compounds, which are less retained on the hydrocarbonaceous surface than neutral or cationic compounds, respectively. A minimum retention is observed at the isoelectric point for the two amphoteric compounds cephalexin and cefroxadin where they have two opposite charges. This does not appear for cefotiam and cefotaxime, probably because their pK_a are too close. Cefotiam, which has two basic functions, behaved as a basic compound, in spite of its carboxylic group: its retention gradually increased with pH.

When pH was increased at a lower ionic strength (I = 0.003), the retention decreased and then tended to become constant for all investigated cephalosporins (Fig. 2B). The k'—pH profiles obtained for cephalexin and cefroxadin did not exhibit a minimum at a pH corresponding to their isoelectric point. This may be related to the fact that retention occurs by way of both solvophobic and silanophilic interactions at this low ionic strength.

Comparison of reversed-phase packing materials

The retention of cefuroxime, cefazolin, cephalexin, and cefotaxime was examined on 5 μm LiChrosorb RP-18 and 5 μm Nucleosil C_{18} with the mobile phase methanol—water—H_2SO_4 (1.8 M) (25:75:0.2, v/v). The results are shown in Table II. The retention of cephalexin, which is present in its protonated form, is the most modified by the nature of the solid phase. The retentions of cefuroxime and cefazolin, which are predominantly present in their neutral form, are similar on both packing materials. The elution order on μBondapak C_{18} was similar to that obtained on Nucleosil C_{18}. The different behaviour of the cephalosporins cannot be explained on the basis of a single retention mechanism. Such selectivity differences between reversed-phase materials were reported by Melander et al. [16]. They were attributed to silanophilic interactions. This result thus supports the hypothesis that silanophilic interactions may be partly responsible for the retention of cephalosporins having an amino group when acidic eluents of low ionic strength are used. LiChrosorb RP-18, which may have more residual silanol groups than the two other solid phases, was shown to give more peak tailing than μBondapak C_{18} [17] for protonated amines. This was not observed for cephalosporins.

TABLE II

RETENTION (k' VALUES) OF SOME CEPHALOSPORINS ON TWO PACKING MATERIALS

Eluent: methanol—water—H_2SO_4 (1.8 M) (25:75:0.2). Flow-rate: 1.2 ml/min. Ambient temperature.

Cephalosporin	Packing material	
	5-μm Nucleosil C_{18}	5-μm Lichrosorb RP-18
Cefuroxime	6.9	6.4
Cefazolin	7.9	7.6
Cephalexin	3.9	12.7
Cefotaxime	4.1	9.0

Determination of the investigated cephalosporins in biological fluids

Interactions with residual silanol groups have previously been shown to be useful to obtain an adequate selectivity for the separation of peptides [13]. A good retention is obtained with mobile phases of low pH and low ionic strength, not only for amphoteric cephalosporins (with the exception of cefotiam) but also for acidic ones. Silanophilic interactions may consequently also be useful for the determination of cephalosporins in biological fluids with closely related mobile phases, assuming that the specific separation of the cephalosporin from endogenous compounds will be facilitated if one selects a mobile phase that allows a good retention of the cephalosporin. An acidic eluent containing only a low concentration of H_2SO_4 was used to chromatograph the cephalosporins. When the mobile phase methanol—water—H_2SO_4 (1.8 *M*) (18:82:0.2, v/v) was used with 5 μm LiChrosorb RP-18, cefroxadin was the least retained of the investigated cephalosporins, and it was well separated from endogenous plasma and urine components, as illustrated in Fig. 3. LiChrosorb RP-18, 5 μm, was chosen as the packing material instead of the 10-μm one, in order to obtain better column efficiency. The methanol content in the above-mentioned mobile phase was increased, in order to decrease the retention of the other cephalosporins. A convenient separation could be obtained for each of the studied cephalosporins. The different methanol contents, which afford a compromise between adequate separation and rapid analysis, are indicated in Table III. Cefsulodin, the most polar compound, was not sufficiently retained with such conditions to obtain a separation allowing good sensitivities for the determination in plasma and urine.

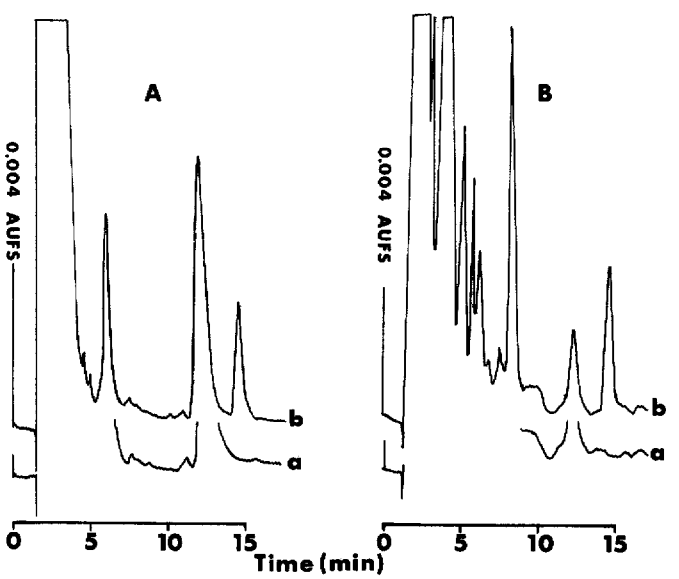


Fig. 3. (A) Chromatograms of blank plasma (a) and plasma containing 2 μg/ml cefroxadin (b); injection volume = 20 μl. (B) Chromatograms of blank urine (a) and urine containing 20 μg/ml cefroxadin (b); injection volume = 20 μl. Column 15 cm × 4.7 mm LiChrosorb RP-18, 5 μm; mobile phase, methanol—water—H_2SO_4 (1.8 *M*) (18:82:0.2, v/v); ambient temperature; flow-rate, 1.2 ml/min; UV detection at 254 nm.

TABLE III

METHANOL CONTENT USED FOR THE DETERMINATION OF CEPHALOSPORINS IN PLASMA AND URINE WITH A MOBILE PHASE OF METHANOL—WATER CONTAINING 0.2% (v/v) OF H_2SO_4 (1.8 *M*)

Column: 5 μm LiChrosorb RP-18. Flow-rate: 1.2 ml/min. Ambient temperature.

	Methanol content (%)
Desacetylcefotaxime	15
Cefroxadin	18
Cefuroxime	20*
Cefazolin	21.5
Cefotaxime	25
Cephalexin	28
Cefamandole	30*
Cephalothin	36

*The methanol contents are 19% for cefuroxime and 28% for cefamandole for the determination of these compounds in urine.

When the eluents described in Table III were used, cephalosporins could also be determined in human milk and bile, after appropriate dilution of the sample, as described above.

A convenient separation for cefotiam, which is retained longer on reversed-phase material in alkaline medium, was obtained with the alkaline mobile phase NaH_2PO_4 (1.4×10^{-3} *M*) and Na_2HPO_4 (2.5×10^{-2} *M*)—methanol (76:24, v/v), as previously reported [18]. This method was used for pharmacokinetic studies with sensitivity limits of 0.2 μg/ml of plasma and 5 μg/ml of urine. The method was also found adequate for the determination of the amphoteric compounds cephalexin and cefroxadin in plasma and urine with slightly lower methanol contents than for cefotiam.

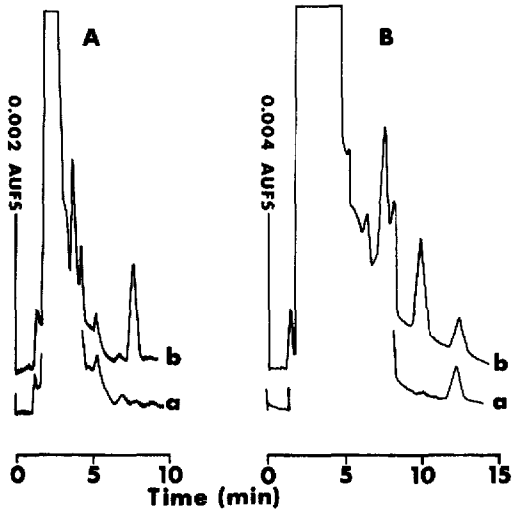


Fig. 4. (A) Chromatograms of blank plasma (a) and plasma containing 1 μg/ml cephalothin (b); injection volume = 30 μl. (B) Chromatograms of blank urine (a) and urine containing 20 μg/ml cephalothin (b); injection volume = 10 μl. Column 25 cm × 4.7 mm, μBondapak C_{18}, 10 μm; mobile phase, methanol—water—H_2SO_4 (1.8 *M*) (28:72:0.2, v/v, for plasma and 26:74:0.2, v/v, for urine); temperature, 35°C; flow-rate, 2 ml/min; UV detection at 254 nm.

Such separations were also found convenient on 10-μm μBondapak C_{18}, the retention times being generally shorter than on 5-μm LiChrosorb RP-18. The methanol contents in the mobile phase were also lower. An example of separation is given for cephalothin in Fig. 4. Cephalothin is bound to serum proteins to the extent of 65—79% [19] and the recovery of the drug was small (about 35%) after precipitation of plasma proteins with TCA. Therefore, proteins were precipitated with methanol for this drug: 150 μl of plasma, 150 μl of methanol, 15 μl of standard solution and 30 μl of H_2SO_4 (6 *M*) were mixed and centrifuged; 30 μl of the supernatant were injected. The recovery of cephalothin was then 65 ± 4%, as compared with that obtained upon direct injection of an aqueous solution.

Application: determination of ceftizoxime in human plasma and urine

Ceftizoxime is a new parenteral cephalosporin that presents structural similarities to cefotaxime (Table I). Consequently, an acidic mobile phase as mentioned above should be adequate to obtain a good retention of this compound.

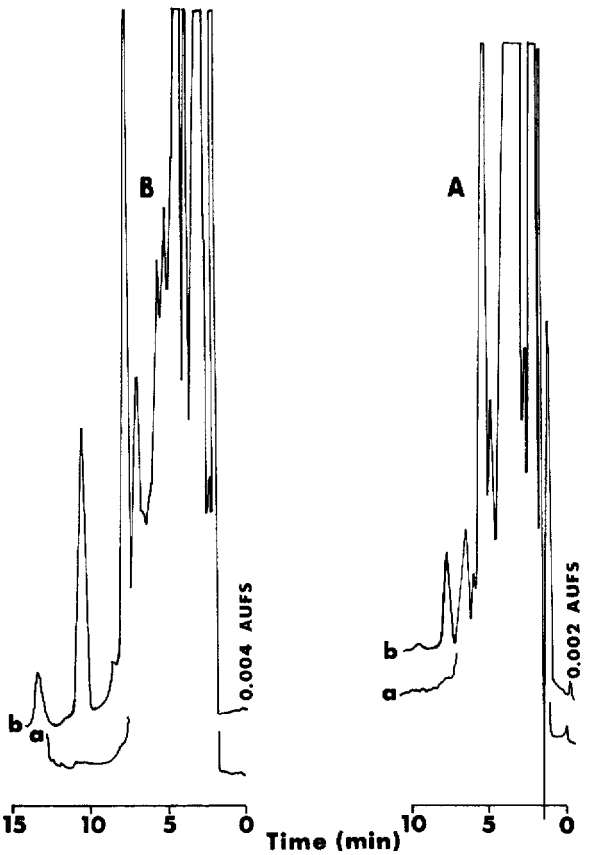


Fig. 5. (A) Chromatograms of blank plasma (a) and plasma containing 0.5 μg/ml ceftizoxime (b); injection volume = 20 μl. (B) Chromatograms of blank urine (a) and urine containing 38 μg/ml ceftizoxime (b); injection volume = 10 μl. Column 25 cm × 4.7 mm, μBondapak C_{18}, 10 μm; mobile phase, methanol—water—H_2SO_4 (1.8 *M*) (15:85:0.2, v/v); temperature, 35°C for plasma, ambient temperature for urine; flow-rate, 2 ml/min; UV detection at 254 nm.

TABLE IV

EVALUATION OF ASSAY REPRODUCIBILITY AND ACCURACY FOR CEFTIZOXIME

Fluid	Concentration added (μg/ml)	Mean* concentration found (μg/ml)	Coefficient of variation (%)
Plasma	0.20	0.20	4.8
	0.50	0.51	4.8
	5.0	4.9	2.0
	10.0	10.0	0.7
	100	98.5	1.8
Urine	25.0	25.0	6.0
	2000	1997	4.9

*Mean of six replicates.

A convenient separation from endogenous compounds of plasma and urine was obtained with the eluent methanol—water—H_2SO_4 (1.8 *M*) (15:85:0.2, v/v), as exhibited in Fig. 5. Plasma proteins were precipitated with TCA, as ceftizoxime is only 31% bound to proteins [20]. The mean recovery of ceftizoxime from plasma was 89 ± 2% in the 0.5—25 μg/ml range. Calibration curves were obtained by plotting peak height (in the range 0.2—2 μg/ml of plasma) or peak area (in the range 0.5—100 μg/ml of plasma and 25—2000 μg/ml of urine) against concentration of ceftizoxime on log—log graphs. The correlation coefficients were better than 0.9990 in all cases. Results of replicate analyses of plasma and urine samples are presented in Table IV. Concentrations as low as 0.2 μg/ml of plasma and 25 μg/ml of urine can be determined with good reproducibility and accuracy.

In routine analysis, more than 500 injections could be performed with the same column. As the column ages, the methanol content in the mobile phase has to be slightly decreased. With such chromatographic conditions, cefroxadin exhibits the same retention time as ceftizoxime, and it can be determined in plasma and urine with the method described for ceftizoxime without modification of the methanol content in the mobile phase. The retention time of cefroxadin is 8 min instead of 15 min on LiChrosorb RP-18.

CONCLUSION

Most cephalosporins are strongly retained on octadecylsilyl stationary phase with an acidic eluent of low ionic strength. It was shown that, with an eluent containing 0.2% (v/v) of H_2SO_4 (1.8 *M*) and various methanol contents, they can be determined in biological fluids after a simple and rapid sample preparation: precipitation of plasma proteins or urine dilution. Short retention times can be obtained when using μBondapak C_{18} material.

The method was checked for ceftizoxime. It allows adequate sensitivity for pharmacokinetic studies.

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