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DETERMINATION OF CEFATRIZINE IN SERUM AND URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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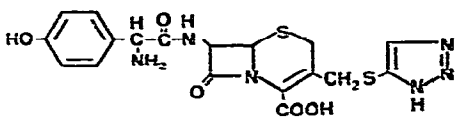
SUMMARY

A fast and simple high-performance liquid chromatographic procedure for the determination of cefatrizine, an orally active cephalosporin, in serum and urine is proposed. Reversed-phase liquid chromatography on an octadecylsilane chemically bonded microparticulate packing, using methanol in 0.03 *M* sodium phosphate buffer (pH 5) as eluent, was used to separate and quantitate the antibiotic. The samples were analysed after deproteinization with trichloroacetic acid and injection of the clear supernatant. The accuracy and reproducibility of the procedure were investigated by determination of the cefatrizine content in spiked serum and urine samples, using cephradine as the internal standard.

INTRODUCTION

Cefatrizine,7-[*R*-α-amino-α-(4-hydroxyphenyl)acetamido]-3-(1,2,3-triazol-5-yl-thiomethyl)-ceph-3-em-4-carboxylic acid, is a new semi-synthetic broad-spectrum and orally active cephalosporin[1,2]. The antibiotic, which is usually available as the water-soluble propylene glycolate solvate, is absorbed well from the gastrointestinal tract. The *in vitro* activity of cefatrizine was shown to be higher than for cephalexin, also an orally active cephalosporin, against gram-negative and gram-positive organisms. It is more active than most of the other cephalosporins against gram-negative bacteria, but less active against the gram-positive bacteria[3–6]. Oral administration of a 500-mg dose of cefatrizine results in a peak serum level of 5–7 μg/ml, which is high enough for the inhibition of most susceptible organisms[5].



Cefatrizine

Quantitative determinations are conventionally performed by microbiological assay by the agar-well diffusion method. Microbiological assay methods are time

consuming and obviously cannot differentiate between cefatrizine and other antibiotics or active decomposition products. Therefore, a chromatographic method is to be preferred for determining the cefatrizine content of biological fluids such as serum and urine. A high-performance liquid chromatographic procedure on an octadecylsilane chemically bonded microparticulate packing material has been developed for determining the cefatrizine content and for differentiating it from other orally active cephalosporins such as cephalexin and cephradine, which can be used as an internal standard in the analytical procedure.

Cefatrizine, as a highly polar and water-soluble compound, cannot be extracted from biological fluids by techniques such as classical two-phase liquid–liquid extraction or by ion-pair extraction with quaternary ammonium salts, which works well with cephalothin[7] and cephapirin[8]. We have also found that deproteinization by ultrafiltration on a membrane filter is inadequate, owing to the high degree of adsorption of cefatrizine on to the membrane. Owing to the stability of cefatrizine in acidic media, the sample preparation could be performed by a simple deproteinization by the addition of an equal volume of a trichloroacetic acid solution to the sample[9,10]. The clear supernatant, obtained after centrifugation, is used directly in the chromatographic procedure.

This paper describes the development of a high-performance liquid chromatographic procedure for the determination of cefatrizine in serum and urine after deproteinization with trichloroacetic acid and an investigation of the accuracy and reproducibility of the proposed procedure.

EXPERIMENTAL

Apparatus and operating conditions for the chromatographic separation

A Varian 4100 liquid chromatograph, equipped with a UV detector (254 nm), was used. The separation was carried out on RSil C18 LL (10 μm) particles. RSil C18 LL is a microparticulate chemically bonded octadecylsilane reversed-phase packing with 9% (low loading) bonded organic material. The 10- and 15-cm stainless-steel columns (4.6 mm I.D.) were cleaned and filled by a slurry technique with glycerol–methanol (20:80) as the suspending medium and water as the pressurizing solvent[11]. The 25-cm columns (4.6 mm I.D.) were used as purchased. The eluent consisted of a mixture of methanol and 0.03 *M* sodium phosphate buffer (pH 5). The composition of the mixture varied from 16% of methanol for the 10- and 15-cm columns to 22% of methanol for the 25-cm columns. The samples were injected by means of a six-way Valco valve, with a loop volume of 20 μl for the urine samples and 50–100 μl for the serum samples. Peak-area measurements were made with a Spectra-Physics Autolab System IV integrator.

Materials and reagents

Trichloroacetic acid (TCA) (UCB, Drogenbos, Belgium) and methanol (Merck, Darmstadt, G.F.R.) were of analytical-reagent grade. The 0.03 *M* sodium phosphate buffer (pH 5) was prepared by dissolving sodium dihydrogen orthophosphate (analytical-reagent grade) in water and adjusting the pH to 5 with 0.1 *M* sodium hydroxide solution. Cefatrizine propylene glycolate, with a potency of 852 μg/mg, was obtained from Bristol Laboratories (Syracuse, N.Y., U.S.A.). Cephradine, which

was used as an internal standard, was purified from the commercial product. The commercial product, containing cephalexin as an impurity[12], was purified by preparative chromatography on a 25 × 1 cm RP-8 Lobar column (Merck) with methanol–water (30:70) as the eluent. The fraction containing the pure cephradine was used after evaporation *in vacuo*. Conical polypropylene test-tubes (690 39/10A; Sarstedt, Herent, Belgium), with a volume of 1.5 ml, were used in the sample preparation. An Eppendorf pipette with a constant volume of 200 μl and a 25-μl Hamilton glass syringe (Series 702) were used to deliver the volumes mentioned in the procedure.

Analytical procedure

Serum and urine samples were deproteinized by the addition of an equal volume of 6% trichloroacetic acid solution containing cephradine as an internal standard. At the same time, a standard graph was prepared using blank serum or urine. The following procedure was used for the sample and standard graph preparation. A 200-μl volume of serum or urine was placed in the conical test-tubes and mixed with 20 μl of water, then 200 μl of 6% TCA solution, containing cephradine as the internal standard, were added. The contents of the tube were mixed for 1 min on a vortex mixer and centrifuged for 30 min at 1100 *g*. The standard graph was prepared by the addition of 20 μl of concentrated cefatrizine standard solution to 200 μl of blank serum or blank urine. After mixing, 200 μl of 6% TCA solution, containing cephradine, were added and the procedure was continued as for the unknown sample. The clear supernatant obtained after centrifugation was used for the liquid chromatographic analysis. An aliquot of 50–100 μl was used for the serum analysis and an aliquot of 20 μl for the urine analysis.

The concentrated cefatrizine standard solutions were prepared in water at ten times the concentration of the unknown by dilution of a stock cefatrizine solution. The cefatrizine concentration in this stock solution was 200 μg/ml for the serum analysis and 2 mg/ml for the urine analysis. The cefatrizine concentration for the preparation of the standard graph after the 20:200 dilution ranged from 1.5 to 20 μg/ml for the serum standard graph and from 40 to 200 μg/ml for the urine standard graph. The internal standard (cephradine) was added to the 6% TCA solution to obtain a concentration of 20 μg/ml of cephradine in the serum analysis and 200 μg/ml of cephradine in the urine analysis. The analytical procedure is shown schematically in Table I.

RESULTS AND DISCUSSION

Chromatographic conditions

The separation of cefatrizine and cephradine in aqueous solution was possible on the reversed-phase packing using a water–methanol mixture as the eluent, but the trichloroacetic acid from the sample solution caused an increase in the capacity factors of both cefatrizine and cephradine. The use of a buffer solution instead of water in the mobile phase was necessary in order to reduce the influence of the trichloroacetic acid.

The pH of the phosphate buffer was important because variations of the pH caused a change in the selectivity between cefatrizine, cephalexin and cephradine. The change in the capacity factors of these compounds as a function of the pH of the

TABLE I

SCHEMATIC REPRESENTATION OF THE PROCEDURE FOR SAMPLE AND STANDARD PREPARATION

Material	*"Unknown"*	*"Standard"*
Unknown sample	200 µl	—
Blank serum or urine	—	200 µl
Water	20 µl	—
Concentrated standard (10-fold concentration)	—	20 µl
	Mix for 30 sec	—
Internal standard in 6% TCA solution	200 µl	200 µl
	Mix for 1 min Centrifugation for 30 min at 1100 *g*	

buffer is shown in Fig. 1, which indicates that the capacity factor of cefatrizine is maximal at pH 5, whereas the selectivity with cephradine is lowest at this pH, but still high enough for a complete separation. The separation of cefatrizine from the natural serum and urine constituents is satisfactory at pH 5 (Figs. 2 and 3). A pH of 5 is very suitable for the stability of the chemically bonded column packing, which is unstable at both high and low pH values, resulting in short lifetimes of the column[13–15]. This pH is also suitable for the good stability of cefatrizine, which is unstable in neutral and alkaline solutions, but stable in acidic solution. However, at pH 5, cefatrizine is no longer baseline separated from cephalexin, which is present as an impurity in cephradine[12], and this makes it necessary to purify the cephradine by preparative chromatography on a reversed-phase RP-8 Lobar column.

The buffer concentration, which was varied from 0.03 to 0.065 *M*, affected the capacity factors but not the selectivity of the separation. A buffer concentration of 0.03 *M* was sufficient to overcome the influence of trichloroacetic acid and to

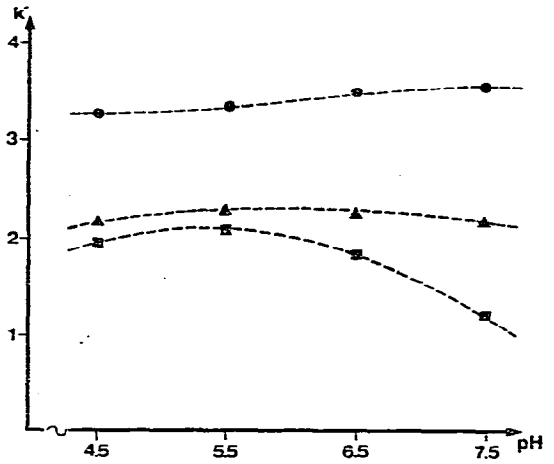


Fig. 1. Influence of the pH of the buffer component in the mobile phase on the capacity factors of cephradine (●), cephalexin (▲) and cefatrizine (■). Column: RSil C18 LL (250 × 4.6 mm I.D.). Eluent: methanol–0.05 *M* sodium phosphate buffer (20:80). Flow-rate: 1.3 ml/min. Temperature: 25°.

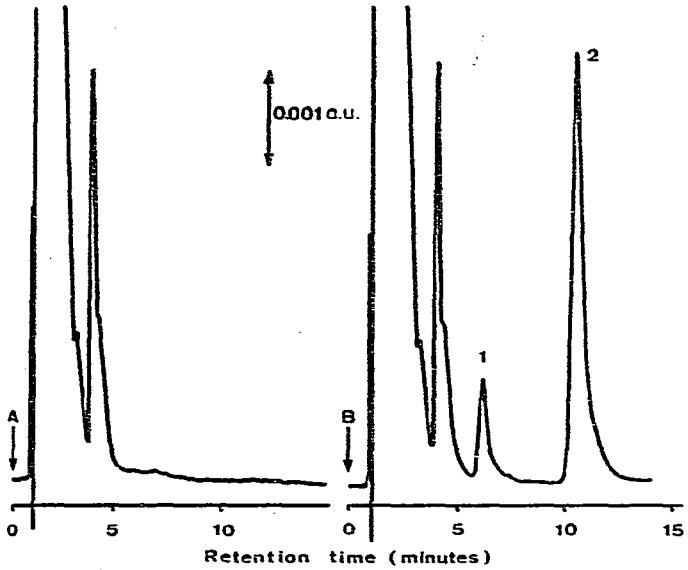


Fig. 2. Chromatography of (A) blank human serum and (B) serum containing 4.8 μg/ml of cefatrizine and cefradine (20 μg/ml) as internal standard (50-μl injection volume). Column: RSil C18 LL (10 μm) (100 × 4.6 mm I.D.). Mobile phase: methanol–0.03 *M* sodium phosphate buffer (pH 5) (16:84). Flow-rate: 1.0 ml/min. Retention times: cefatrizine (1) 369 sec and cephradine (2) 627 sec. Temperature: 25°. Detection: UV (254 nm), 0.01 a.u.f.s.

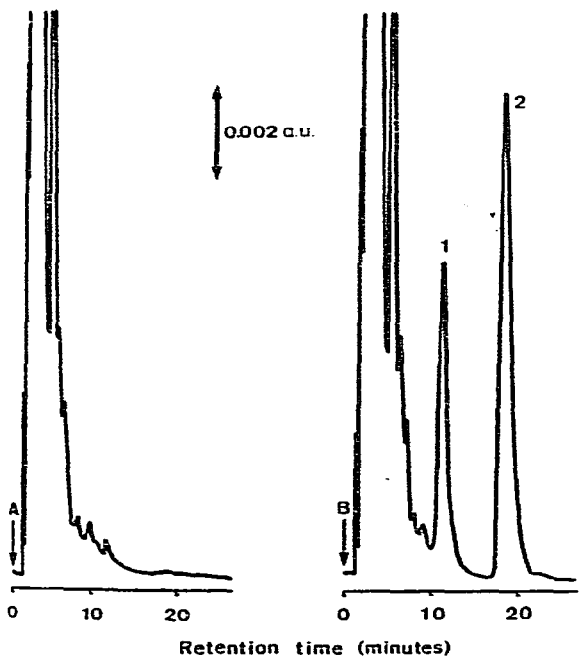


Fig. 3. Chromatography of (A) blank human urine and (B) urine containing 90 μg /ml of cefatrizine and cephradine as internal standard (20-μl injection volume). Column: RSil C18 LL (10 μm) (150 × 4.6 mm I.D.). Eluent: methanol–0.03 *M* sodium phosphate buffer (pH 5) (15:85). Flow-rate: 1.0 ml/min. Retention times: cefatrizine (1) 670 sec and cephradine (2) 1080 sec. Temperature: 25° Detection: UV (254 nm), 0.02 a.u.f.s.

achieve reproducible retention times, with a coefficient of variation of 0.5% for analyses carried out on the same day. A higher buffer concentration was not used, in order to increase the lifetime of the column[16,17] and to avoid crystallization of the buffer salts on the column or in the chromatographic system.

The methanol content in the mobile phase was adjusted daily to obtain an optimal separation between cefatrizine and the natural impurities in serum and urine. A reduction in the content of the modifier resulted in increased capacity factors for cefatrizine and cephradine and in a better separation between cefatrizine and the natural impurities, but also in an increased and impractical analysis time. The separation of cefatrizine from the serum and urine impurities was also better at lower temperature. Therefore, the column temperature was maintained at 25° by means of a water-jacket.

The sensitivity of the procedure for the 10-cm column is limited to 1 μg/ml of cefatrizine in serum for an injection volume of 100 μl. The column efficiency did not decrease with increased injection volumes up to 100 μl. The plate number increased from 2400 to 2700 theoretical plates for cefatrizine on the 10-cm column on doubling the injection volume from 50 to 100 μl. This result is probably due to a pre-concentration effect of the compounds on top of the analytical column due to the high polarity of the sample solution[18].

The column efficiency decreased rapidly after a few days of use and could be restored to nearly the initial efficiency by replacing the upper part of the column (1–2 mm) with new packing material. Probably owing to the large amounts of trichloroacetic acid solution injected on to the columns, their lifetime was only 8–10 working days. Then the column efficiency decreased permanently and the column back-pressure increased slightly.

Analytical procedure

The method sample preparation, consisting of a simple deproteinization by the addition of a trichloroacetic acid solution, was possible owing to the stability of both cefatrizine and cephradine towards acids. The stability of cefatrizine and cephradine in the trichloroacetic acid solution at ambient temperature was investigated and both compounds showed no decrease in concentration for at least 3 h.

The use of blank serum and urine for the preparation of the standard graph was necessary because the concentration of cefatrizine and cephradine changed before and after deproteinization, as did also slightly the concentration ratio. However, the change was reproducible when cefatrizine and cephradine were added in a certain ratio to sixteen different serum samples and deproteinized. The change in the concentration ratio was also reproducible when the cefatrizine concentration was varied and the cephradine concentration kept constant, as with the standard graph. The use of blank serum and urine is also useful for obtaining the same chromatographic results for standards and unknown, especially with interfering impurities and problems with baseline separations. The cefatrizine standard solutions were not prepared by dissolution of cefatrizine in serum, but a 10-fold concentrated standard solution in water was added to the blank serum or urine in the analytical procedure. In this way, the problems of the standard preparation in serum and the use of large amounts of blank serum were avoided. Also, cefatrizine is not stable in serum at pH 7.4, but is relatively stable in water.

The reproducibility of the procedure was investigated by preparing the same solution fourteen times, whereby 20 μl of a concentrated standard solution were added to 200 μl serum and deproteinized by the addition of 200 μl trichloroacetic acid solution containing the internal standard. Analysis of the supernatant gave results with a reproducibility of ± 3.8% (coefficient of variation). The accuracy and reproducibility of the procedure were checked by the analysis of spiked serum and urine samples, known amounts of cefatrizine being added to blank serum and urine. The results for the serum analysis are given in Table II. Each serum sample was analysed 10–15 times with reproducibilities ranging from 2 to 4% (coefficient of variation). The standard graph was always linear in the concentration range used, with a correlation coefficient of >0.9995. The results obtained by measurement of the peak-height ratio were more reproducible than those obtained by integration of the peak areas, especially with the low cefatrizine concentrations that occur in serum samples.

TABLE II

DETERMINATION OF THE CEFATRIZINE CONTENT IN SERUM

The cefatrizine was added to blank serum. Calculation was carried out by peak-height measurement. Standard graph from 4 to 20 μg/ml of cefatrizine for determinations 1–7 and from 1.5 to 7.5 μg/ml for 8–10. Correlation coefficient of the standard graph was >0.9995 in all instances.

Serum No.	*Cefatrizine content (μg/ml)*		*Recovery (%)*	*C.V.*
	Theoretical	*Experimental*		
1	16.50	16.98	102.9	2.53 ($n = 13$)
2	10.00	10.20	102.0	2.48 ($n = 15$)
3	4.92	5.07	103.1	3.94 ($n = 10$)
4	4.88	5.00	102.5	3.25 ($n = 16$)
5	4.80	4.88	101.7	2.73 ($n = 12$)
6	4.20	4.22	100.4	4.02 ($n = 7$)
7	4.20	4.23	100.7	2.15 ($n = 12$)
8	2.03	1.99	98.0	4.90 ($n = 10$)
9	2.16	2.16	100.0	2.87 ($n = 12$)
10	2.03	2.08	102.5	2.40 ($n = 12$)

The results for the urine analysis are given in Table III. Each urine sample was analyzed 10–15 times, with reproducibilities ranging from 2 to 4% (coefficient of variation). Owing to the higher concentrations that occur in urine samples, the results obtained by measurement of the peak-height ratio and by integration of the peak areas showed similar reproducibilities.

The sensitivity of the procedure is limited to 1–2 μg/ml of cefatrizine in serum when a 100-μl sample is injected. In this instance 200 μl of serum are needed for each analysis. The sensitivity of the procedure can probably be enhanced by the post-column formation of a fluorescent reaction product, obtained by reaction of, *e.g.*, fluorescamine with the primary amino function of cefatrizine and cephradine.

Especially with urine samples, the cefatrizine was not always baseline separated from the natural urine constituents. The use of acetonitrile instead of methanol in the mobile phase resulted in a completely altered selectivity, with a better separation between cefatrizine and the urine and serum impurities. However, the separation between cefatrizine and cephradine was no longer complete. A solvent system with

TABLE III

DETERMINATION OF THE CEFATRIZINE CONTENT IN URINE

The cefatrizine was added to blank urine. Calculation was carried out by peak-height measurement. Determinations 1–4 were carried out on a 25-cm column and 5–10 on a 15-cm column. Standard graphs from 40 to 200 μg/ml. Correlation coefficient of the standard graph was >0.9995 in all instances.

Urine No.	*Cefatrizine content (μg/ml)*		*Recovery (%)*	*C.V.*
	Theoretical	*Experimental*		
1	60.3	62.1	102.9	2.63 ($n = 13$)
2	101.5	102.9	101.4	4.34 ($n = 11$)
3	106.4	107.6	101.1	3.48 ($n = 11$)
4	154.5	156.7	101.4	3.34 ($n = 12$)
5	60.0	60.4	100.7	2.29 ($n = 10$)
6	88.0	88.3	100.4	0.77 ($n = 8$)
7	88.0	86.8	98.6	1.94 ($n = 11$)
8	98.5	97.6	99.1	2.52 ($n = 10$)
9	123.3	128.2	103.9	2.42 ($n = 10$)
10	149.7	148.4	99.1	1.85 ($n = 10$)

methanol–acetonitrile (1:1) as the organic modifier gave a baseline separation between cefatrizine and cephradine and also a very good separation between cefatrizine and the impurities. This solvent system also offers a useful alternative for the determination of cefatrizine in serum and urine when interfering impurities are present with the proposed system.

The proposed liquid chromatographic procedure allows the rapid determination of cefatrizine in serum or urine down to concentrations of 1–2 μg/ml with good reproducibility. The method offers the possibility of following the stability of cefatrizine in aqueous solutions and biological fluids, and of monitoring serum and urine concentrations after oral intake of the drug.

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