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QUANTITATIVE LIQUID CHROMATOGRAPHIC DETERMINATION OF CEFATRIZINE IN SERUM AND URINE BY FLUORESCENCE DETECTION AFTER POST-COLUMN DERIVATIZATION

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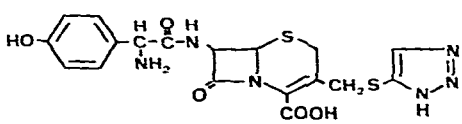
SUMMARY

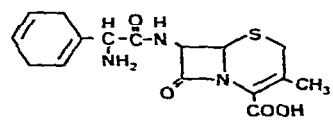
A fast, specific and sensitive high-performance liquid chromatographic procedure for the determination of cefatrizine, an orally active cephalosporin, in serum and urine is proposed. The drug is determined by the internal standard method, using cephradine as the internal standard. The separation is carried out on a reversed-phase column, filled with octadecylsilane chemically bonded microparticles. The eluent is a mixture of acetonitrile with 0.025 *M* sodium phosphate buffer (pH 7). Quantitation is effected by fluorescence detection of the fluorophores formed after post-column derivatization with fluorescamine in a packed-bed reactor. The chromatographic conditions and the conditions for the post-column derivatization are discussed. The method has been applied to serum and urine samples, which were analysed after deproteinization with trichloroacetic acid and injection of the clear supernatant. The accuracy and reproducibility of the procedure were investigated by the determination of the cefatrizine content in spiked serum and urine samples.

INTRODUCTION

Cefatrizine (I), 7-[*R*-α-amino-α-(4-hydroxyphenyl)acetamido]-3-(1,2,3-triazol-5-ylthiomethyl)-ceph-3-em-4-carboxylic acid, an orally active semi-synthetic broad-spectrum cephalosporin[1–4], can be determined in serum and urine by reversed-phase liquid chromatography with UV detection, using cephradine (II) as internal standard[5]. As discussed in a previous paper[5], the sensitivity of this procedure is limited to 1–2 μg/ml of cefatrizine in serum for an injection volume of 100 μl. The serum peak levels of cefatrizine are 5–7 μg/ml after a single oral dose of 500 mg and they



I



II

fall to below 1 μg/ml after a few hours. Hence UV detection is not sensitive enough when the cefatrizine concentration in serum is to be followed as a function of time after oral intake of the drug. In addition, in the determination of these low concentrations in serum at the highest sensitivity of the detector, or in the determination of cefatrizine in urine, the interference of UV-absorbing substances becomes a major problem owing to the non-specificity of the detection system. Therefore, a more sensitive and more specific detection system was necessary for the determination of cefatrizine at concentrations below 1 μg/ml.

Both cefatrizine and cephradine have a primary α-amino function in their 7-acyl side-chain. This amino function reacts readily with fluorescamine[6,7] to form a highly fluorescent reaction product. Reaction with another fluorogenic reagent for primary amines, namely *o*-phthaldialdehyde, was not successful in batch reactions, probably owing to the destruction of the cephalosporins at the high pH values needed for this reaction[8]. The fluorescamine reagent is well suited for use in a post-column reaction system. Many applications of post-column derivatization of various drugs and with various reaction systems have been described recently, and many of them are concerned with the post-column derivatization of primary amines[9–16].

This paper describes the development of a high-performance liquid chromatographic (HPLC) procedure for the determination of cefatrizine in serum and urine with sensitive fluorescence detection after post-column reaction with fluorescamine in a packed-bed reactor. The conditions of the chromatography and the post-column reaction system are discussed. The accuracy and reproducibility of the proposed procedure were investigated on spiked serum and urine samples. The samples were prepared by deproteinization with trichloroacetic acid[17,18] for the reasons given in a previous paper[5].

EXPERIMENTAL

Materials and reagents

Trichloroacetic acid (TCA), acetonitrile and acetone were of analytical-reagent grade (Merck, Darmstadt, G.F.R.). The acetonitrile for the fluorescamine reagent solution was dried over anhydrous calcium chloride and subsequently distilled from phosphorus pentoxide. It was used immediately after distillation. The 0.025 *M* sodium phosphate buffer (pH 7) was prepared by dissolving sodium dihydrogen orthophosphate (analytical-reagent grade) in water and adjusting the pH to 7 with 0.1 *M* sodium hydroxide solution. The fluorescamine used was Fluram (Hoffmann-La Roche, Nutley, N.J., U.S.A.).

RSil C18 LL (RSL, St.-Martens-Latem, Belgium) is a microparticulate chemically bonded octadecylsilane reversed-phase packing with *ca.* 9% (= low loading) of bonded organic material. Permaphase ODS (DuPont, Paris, France) is a pellicular chemically bonded octadecylsilane packing of uniform particle size (31 ± 6 μm).

Cefatrizine propylene glycolate, with a potency of 852 μg/mg, was obtained from Bristol Laboratories (Syracuse, N.Y., U.S.A.). Cephradine was obtained from R.I.T. (Genval, Belgium) as a commercial product and contained cephalexin as an impurity[19].

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 Series 702 glass syringe were used to deliver the volumes mentioned in the procedure.

Apparatus and operating conditions

Fluorescence apparatus. An Aminco-Bowman spectrophotofluorimeter (Catalogue No. 4-8203 DE, American Instrument Co., Silver Spring, Md., U.S.A.) was used to record the spectra and the fluorescence intensity after batch reaction with fluorescamine.

Chromatographic system. A Waters Model 6000A solvent pump was used to deliver the eluent to the column. The separation was carried out on a 100 × 4.6 mm I.D. stainless-steel column, filled with 10-μm RSil C18 LL particles. The column was filled by a slurry technique with glycerol–methanol (1:4) as the suspending medium and water as the pressurizing solvent. The eluent consisted of a mixture of acetonitrile and 0.025 *M* sodium phosphate buffer (pH 7), the acetonitrile content varying from 10 to 7.5% in order to optimize the separation and the retention times of the compounds. The samples were injected by means of a six-way Valco valve with a sample loop of 10 μl for the urine samples and 20–100 μl for the serum samples.

Post-column reaction system and detection. The column outlet was connected to an unmodified 1/16-in. Swagelok union tee by means of a short piece of 1/16-in. stainless-steel capillary tubing of 0.25 mm I.D. The Fluram reagent (15 mg of fluorescamine in 100 ml of dried acetonitrile) was added to the other side of the union by means of a reciprocating piston pump (Milton Roy Model 396 minipump), equipped with a pulse-damping system consisting of two large-volume Bourdon gauges and a 5 m × 0.25 mm I.D. stainless-steel capillary tube as a restriction. The vertical arm of the Swagelok union tee was connected to the reactor by means of a short piece of capillary tubing (5 cm × 0.25 mm I.D.). The reactor was a packed-bed type and consisted of a stainless-steel tube (100 × 4.6 mm I.D.) filled with Permaphase ODS particles. The reactor column was filled by the "tap and fill" method, which is a dry packing procedure[20]. The outlet of the reactor column was connected by 1/16-in. capillary tubing (0.25 mm I.D.) to the fluorescence detector (Waters Model 420 Filter Fluorescence detector), equipped with a 395-nm excitation filter and a 460-nm emission filter. Calculations were made by peak-height measurement. The column and packed-bed reactor were kept at a constant temperature of 25°. A schematic diagram of the complete system is shown in Fig. 1.

Analytical procedure

The serum and urine samples were deproteinized by the addition of an equal volume of 6% trichloroacetic acid solution, which contained cephradine as the internal standard. The analytical procedure was carried out as described in a previous paper[5] and is shown schematically in Table I.

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 100 μg/ml for the serum analysis and 1 mg/ml for the urine analysis. The cefatrizine concentration in the standard graph after the 20:200 dilution ranged from 0.1 to 10 μg/ml of cefatrizine

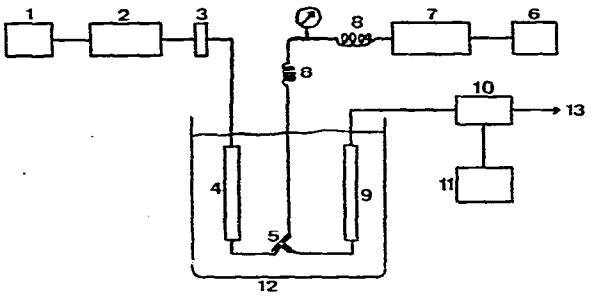


Fig. 1. Schematic diagram of the HPLC system with post-column reaction system. 1 = Eluent reservoir; 2 = high-pressure pump; 3 = injection device, six-way valve with sample loop; 4 = analytical column; 5 = unmodified 1/16-in. Swagelok union tee; 6 = reagent reservoir; 7 = reagent pump; 8 = pulse-damping system (Bourdon gauge + spiral capillary tubing); 9 = reactor column, packed-bed reactor; 10 = fluorescence detector; 11 = recorder; 12 = thermostated water-bath; 13 = waste.

TABLE I

SCHEMATIC REPRESENTATION OF THE PROCEDURE FOR SAMPLE AND STANDARD PREPARATION

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

for the serum standard graph and from 5 to 100 µg/ml of cefatrizine for the urine standard graph. The internal standard cephradine was added to the 6% trichloroacetic acid solution to obtain a concentration of 10 µg/ml of cephradine in the serum analysis and 100 µg/ml of cephradine in the urine analysis.

RESULTS AND DISCUSSION

Chromatographic conditions

Both cefatrizine and cephradine form fluorescent reaction products after reaction with fluorescamine with an excitation maximum at 385–390 mm and an emission maximum at 480 nm. The fluorescence intensity varies with the pH of the reaction medium and reaches its optimum at pH 7 for both compounds (Fig. 2).

In previous work, we found that the separation between cefatrizine and the serum and urine impurities was optimal at pH 5, while at pH 7 the resolution, recorded with UV detection, between cefatrizine and the serum impurities decreased[5]. However, pH 5 is not as suitable for the post-column derivatization with fluorescamine. This pH of the eluate necessitates adjustment of the pH by the addition of another buffer to the eluate before the addition of the fluorescamine reagent, which has to be added separately in a non-hydrolytic solvent. In order to avoid this two-

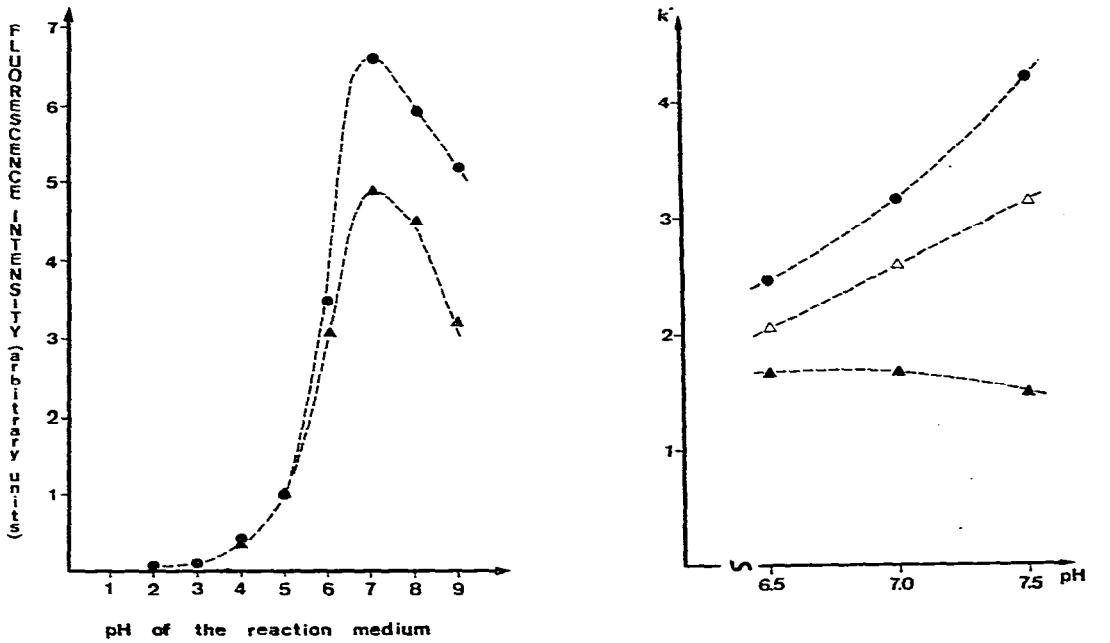


Fig. 2. Fluorescence intensities (arbitrary units) of cefatrizine (▲) and cephradine (●) after reaction with fluorescamine as a function of the pH of the reaction medium. Sample concentration: 1 mg of cephalosporin per 100 ml of buffer solution. Buffer solutions: pH 1–5, 0.2 *M* sodium citrate buffer; pH 6, 0.2 *M* sodium phosphate buffer; pH 7–9, 0.2 *M* sodium borate buffer. Reagent: 20 mg of fluorescamine in 100 ml of acetone. Reaction: 1.5 ml of sample solution + 0.5 ml of reagent. Fluorimeter: $\lambda_{exc.} = 385$ nm; $\lambda_{em.} = 480$ nm.

Fig. 3. Capacity factors of cefatrizine (▲), cephalexin (△) and cephradine (●) as a function of the pH of the sodium phosphate buffer in the eluent. LC conditions: as in Fig. 4 except pH of the phosphate buffer. Post-column reaction conditions and sample: as in Fig. 4.

step reagent addition, coupled with the need for a third solvent pump, the separation was investigated from pH 6.5 to 7.5, which is a suitable pH range for the post-column reaction.

As shown in Fig. 3, the resolution between cefatrizine, cephalexin and cephradine increased with increasing pH of the buffer, while the capacity factor of cefatrizine decreased at increasing pH. However, at pH 7.5 the cefatrizine component is still almost completely separated from the natural serum and urine constituents, which give only a small signal on the fluorescence detector owing to the high specificity of the post-column derivatization. The intensity of the early eluting peaks, due to natural serum and urine components, increased with increasing pH of the eluent phosphate buffer, probably owing to the reaction of early eluting amino acids present in serum and urine. The amino acids have their pH optimum at pH 9 for the fluorescamine reaction. For these reasons a phosphate buffer of pH 7 was chosen as the eluent. At this pH cefatrizine was completely separated from hephalexin and cephradine and also from the natural serum and urine impurities.

Although a good separation was obtained with a methanol–phosphate buffer

(pH 7) mixture as eluent, the methanol was completely replaced with acetonitrile because the presence of methanol in the column eluate had a negative influence on the intensity of the fluorescence signal. The intensity was twice as high with acetonitrile in the eluent as with methanol.

The influence of temperature on the separation was investigated over the range 20–55°. As shown in Table II, the selectivity between cefatrizine and cephalexin increased with increasing temperature, while the selectivity between cephalexin and cephradine remained constant over the whole temperature range. At high temperature, however, the capacity factor of cefatrizine becomes very small and cefatrizine is no longer separated from the natural serum and urine constituents.

TABLE II

CAPACITY FACTOR (k') AND SELECTIVITY (α) OF CEFATRIZINE, CEPHALEXIN AND CEPHRADINE AS A FUNCTION OF THE TEMPERATURE OF THE COLUMN

t_0 was calculated from the signal of trichloroacetic acid or from an unretained amino acid (aspartic acid) (t_0 = 90 sec).

Temperature (°C)	*Cefatrizine* k'	α	*Cephalexin* k'	α	*Cephradine* k'
20	1.93	1.34	2.60	1.18	3.07
25	1.67	1.51	2.53	1.21	3.07
30	1.41	1.70	2.40	1.25	3.00
35	1.27	1.83	2.33	1.23	2.87
45	0.87	2.30	2.00	1.30	2.60
55	0.62	2.73	1.69	1.38	2.33

The sodium phosphate concentration, which was varied between 0.01 and 0.05 *M*, only influenced the capacity factors, which increased with increasing phosphate concentration. The resolution remained almost constant over the whole range, as did also the fluorescence intensity of the compounds. A concentration of at least 0.02 *M* was necessary in order to reduce the influence on the retention times due to the acidic nature of the trichloroacetic acid-containing sample.

Increasing the acetonitrile content in the mobile phase caused an expected decrease in the capacity factors, while the resolution was only slightly influenced. A concentration of 7.5–10% of acetonitrile in the eluent was suitable for performing an analysis in a reasonable time, and cefatrizine was sufficiently well separated from the early eluting serum or urine constituents.

Post-column reaction conditions

As the fluorescence reaction of primary amines with fluorescamine is very rapid either a helically coiled capillary tube reactor or a packed-bed reactor was suitable. A packed-bed reactor was chosen because less peak broadening was observed. The successful use of the packed-bed reactor has also been described in the literature[9,13,21]. Owing to the lack of small spherical glass beads of uniform particle size (± 15 μm), Permaphase ODS particles were used as the packing material for the packed-bed reactor. This reversed-phase packing had no adverse effect on the separation as the fluorescent compounds were not retained on this low-capacity reversed-phase column.

The influence of temperature on the fluorescence intensity was investigated by changing the temperature of the reactor column from 20° to 60°, while the separation column was kept at 25°. Fig. 4 shows the decrease in the intensity as a function of increasing temperature above 30°. For this reason 25° was chosen as the operating temperature because the fluorescence intensity was optimal and a good separation was obtained (Table II).

The influence of the fluorescamine concentration in the reagent on the fluorescence intensity was investigated from 5 to 30 mg of fluorescamine in 100 ml of dried acetonitrile. The fluorescence intensity increased with increasing fluorescamine concentration, but the relationship was not linear. A reagent with 15 mg-% of fluorescamine yielded 80% of the fluorescence intensity obtained with a 30 mg-% reagent solution. We decided to use a 15 mg-% solution, because this reagent concentration was sufficient for determining concentrations of 0.1–0.2 μg/ml of cefatrizine in the serum samples. The acetonitrile for the reagent was dried in order to avoid a degradation of the fluorescamine by trace amounts of water.

The influence of the reagent flow-rate on the fluorescence intensity was checked from 7 to 21 ml/h, while the fluorescamine concentration and the eluent flow-rate were kept constant. The intensity varied only slightly and a maximal fluorescence signal was obtained with a reagent flow-rate of 10–11 ml/h at an eluent flow-rate of 1 ml/min.

The intensity of the fluorescence signal increased with increasing reactor column length but remained constant for a column length of 10 or 15 cm. A reactor column length of 10 cm proved to be sufficient for an almost complete reaction. The residence time of the compounds for this column was approximately 33 sec.

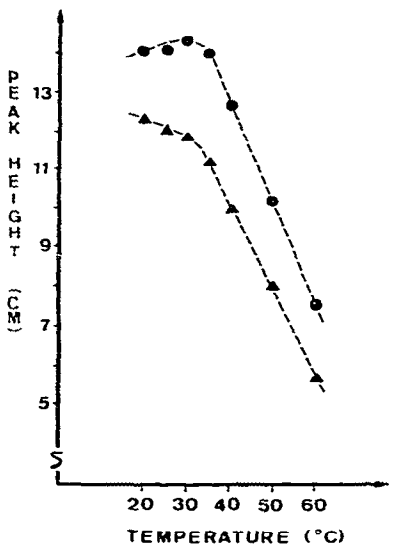


Fig. 4. Fluorescence intensity of cefatrizine (▲) and cephradine (●) as a function of the temperature of the packed-bed reactor in the post-column reaction system. LC conditions: column, RSil C18 LL, 00 × 4.6 mm I.D.; temperature, 25°; eluent, acetonitrile–0.025 *M* sodium phosphate buffer (pH 7) 00:90); flow-rate, 1.0 ml/min; fluorescence detection, $\lambda_{exc.} = 395$ nm, $\lambda_{em.} = 460$ nm. Post-column reaction conditions: reagent, 10 mg of fluorescamine in 100 ml of acetonitrile at 8 ml/h; reactor column, 100 × 4.6 mm I.D. Permaphase ODS. Sample: 20 μl of a cefatrizine–cephradine solution each 5 μg/ml).

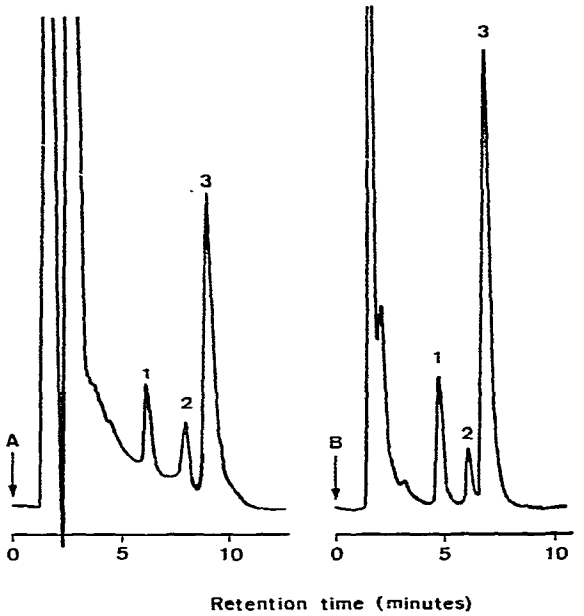


Fig. 5. Chromatography of (A) serum containing 0.2 μg/ml of cefatrizine and cephradine as the internal standard (injection volume 100 μl); (B) urine containing 20 μg/ml of cefatrizine and cephradine as the internal standard (injection volume 10 μl). LC conditions: column, RSil C18 LL, 10 μm, 100 × 4.6 mm I.D.; temperature, 25°; eluent, acetonitrile–0.025 *M* sodium phosphate buffer (pH 7) (8:92); flow-rate, 1 ml/min; fluorescence detection, $\lambda_{exc.}$ = 395 nm, $\lambda_{em.}$ = 460 nm; attenuation 128 (A) and 16 (B). Post-column reaction conditions: reagent, 15 mg of fluorescamine in 100 ml of acetonitrile at 11 ml/h; reactor column, 100 × 4.6 mm I.D. Permaphase ODS; temperature, 25°. Peaks: 1 = cefatrizine; 2 = cephalexin; 3 = cephradine.

TABLE III

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 1 to 10 μg/ml of cefatrizine for determinations 1–3, from 0.5 to 5 μg/ml for determinations 4–7 and from 0.1 to 1 μg/ml for determinations 8–10. The correlation coefficient of the standard graph was in all instances between 0.9980 and 0.9999.

Serum No.	*Cefatrizine content (μg/ml)*		*Recovery (%)*	*Coefficient of variation (%)*
	Theoretical	*Experimental*		
1	4.280	4.180	97.6	2.2
2	4.200	4.220	100.4	2.5
3	1.984	1.989	100.2	2.0
4	2.016	2.035	100.9	3.6
5	1.008	0.992	98.5	4.6
6	0.992	0.966	97.6	4.2
7	0.416	0.411	98.7	1.7
8	0.426	0.422	99.1	2.9
9	0.212	0.2117	99.8	4.1
10	0.197	0.196	99.7	2.8

The reproducibility of the chromatographic system with fluorescence detection after post-column derivatization was investigated by repeated analyses of the same cefatrizine–cephradine sample (each 4 μg/ml). The ratio of the peak heights of cephradine and cefatrizine showed good reproducibility with a coefficient of variation of 1.4% ($n = 30$). The linearity of the fluorescence response as a function of the cefatrizine concentration was investigated over the range 10–100 ng of cefatrizine on the column (100-μl loop of 0.1–1 μg/ml) and 200 ng–2 μg on the column (20-μl loop of 10–100 μg/ml). In both instances there was good linearity, with correlation coefficients > 0.9990.

TABLE IV

DETERMINATION OF THE CEFATRIZINE CONTENT IN URINE

The cefatrizine was added to blank urine. Calculation was carried out by peak-height measurement. Standard graph from 10 to 100 μg/ml of cefatrizine. The correlation coefficient was >0.9990 in all instances.

Urine No.	*Cefatrizine content (μg/ml)*		*Recovery (%)*	*Coefficient of variation (%)*
	Theoretical	*Experimental*		
1	80.64	80.55	99.9	2.1
2	68.32	70.10	102.6	1.25
3	58.56	60.17	102.7	1.4
4	50.80	51.45	101.3	1.0
5	40.16	40.19	100.1	2.5
6	30.12	29.79	98.9	0.95
7	20.08	19.90	99.1	1.2
8	20.96	20.77	99.1	1.4
9	10.40	10.52	101.1	1.4
10	5.20	5.27	101.5	1.3

Accuracy and reproducibility of the proposed procedure

The accuracy and reproducibility of the sample preparation and the chromatographic system with post-column derivatization were checked by the analysis of spiked serum and urine samples, where known amounts of cefatrizine were added to blank serum and urine (Fig. 5). The results of the serum analysis are given in Table III. Each serum sample was analysed ten to fifteen times with coefficients of variation ranging from 2 to 4%. The results were obtained by measurement of the peak heights of cefatrizine and the internal standard.

The results of the urine analysis are given in Table IV. Each urine sample was analysed ten to fifteen times. The analysis showed good reproducibility with coefficients of variation ranging from 1 to 2%.

CONCLUSIONS

The proposed liquid chromatographic procedure with post-column derivatization with fluorescamine and fluorescence detection allows the fast and selective quantitative determination of cefatrizine in serum and urine down to concentrations of 0.1–0.2 μg/ml with good reproducibility. Compared with UV detection at 254 nm

there were less interfering peaks, especially near to the solvent front. Owing to its selectivity and sensitivity, the method is suitable for monitoring cefatrizine levels in serum and urine after administration of the drug.

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