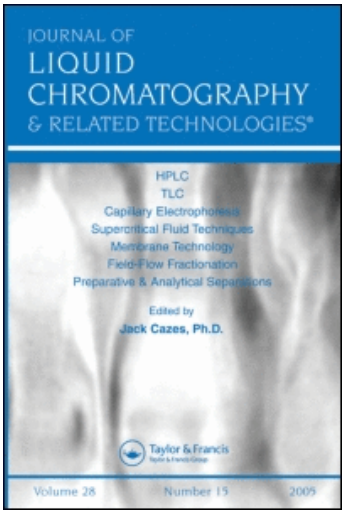

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FLUORESCAMINE POST-COLUMN DERIVATIZATION FOR THE HPLC DETERMINATION OF CEPHALOSPORINS IN PLASMA AND URINE

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ABSTRACT

A post-column fluorescamine derivatization procedure is proposed for the determination of cephalosporins having an α-primary amino group in their side chain (cefaclor, cephalexin, cephradine, cefroxadine, cefaloglycine and cefadroxil). The linearity, repeatability and detection limits of fluorescence emission and UV absorption detection are compared under the same chromatographic conditions. Fluorescence detection is about two times more sensitive than UV absorption detection. Application to the determination of these cephalosporins in plasma and urine shows an improved selectivity by comparison with UV detection.

INTRODUCTION

Cephalosporins are a series of β-lactam antibiotics extensively used for antibacterial treatment of infectious diseases. For the pharmacokinetic studies and the therapeutic monitoring of these drugs in serum and urine, high performance liquid chromatography (HPLC) with ultraviolet (UV) absorption detection is in most cases, the routine method. However, this

detection mode lacks specificity. For the last few years, we have investigated the possibility of using new detection modes for the determination of cephalosporins in biological fluids or pharmaceutical formulations (1-4). We have shown that thin layer chromatography with fluorescence detection (1-2) and HPLC with electrochemical detection (3-4) give lower detection limits than those obtained using UV absorption detection.

In this paper, the feasability of using fluorescence as a selective and/or sensitive detection mode in HPLC for the determination of cephalosporins in plasma and urine is presented. The method is based on the formation of fluorescent derivatives with fluorescamine reagent for cephalosporins having an α-primary amino group in their side-chain. For this purpose, a post-column derivatization procedure in a tubular reactor at ambient temperature is used. The linearity, repeatability and detection limits obtained using fluorescence emission or UV absorption detection are compared for cefaclor, cephalexin, cephradine, cefroxadine, cefaloglycine and cefadroxil. The interest of fluorescence detection for a selective determination of these cephalosporins in plasma and urine is shown.

BACKGROUND

Fluorescence detection after HPLC separation has been used in a few instances for the determination of cephalosporins (5-9). The procedures presented are based either on the formation of fluorescent compounds after off-line degradation or post-column derivatization.

• Off-line degradation of cephalexin and cephradine (5) under drastic conditions (reaction with hydrogen peroxide at a pH of 2.5 and heating for 55 min. at 100°C) yields highly fluorescent products which after extraction can be chromatographed. The detection limits in plasma samples (0.2 ng for cephalexin, 1 ng for cephradine) are low but the technique is too laborious and time-consuming to be applied for routine use.

The two other fluorescence procedures proposed are based on the post-column derivatization of the primary amino group of some cephalosporins with OPA (6-7) or fluorescamine (8-9).

• The reaction with OPA was carried out for cephalexin and cephradine in a tubular reactor, at a pH of 12 and a temperature of 90°C. The detection limits are 280 ng and 160 ng for cephalexin and cephradine, respectively. The selectivity in fermentation broth was reported to be better than that obtained using UV absorption detection although no comparative data or chromatograms were presented. The corrosive nature of the reagent (outlined by the authors) under the conditions used, seems a serious limit for a routine use of the method, in addition to the fair detection limits obtained.

• In the procedure proposed by Crombez for the determination of cefatrizine in serum and urine, the post-column reaction with fluorescamine was conducted in a reaction bed at a pH of 7 and at ambient temperature. The satisfactory reproducibility and determination limits (10 ng) were reported to be better than those obtained using UV absorption detection, but the two detection modes were not compared under the same chromatographic conditions. The procedure was not extended to other cephalosporins.

The purpose of our work was to find out a sensitive, specific and reliable fluorescence detection mode after HPLC separation, to be used for cephalosporins in routine analysis. For this reason, a post-column reaction was chosen because it does not require a further sample preparation (and consequently yields a better precision than pre-column derivatization) and can be easily automated. Among the selective fluorogenic reagents available for a post-column reaction with a primary amino group, fluorescamine was selected from the literature survey (6-10) because (i) its reaction with primary amines proceeds at a fast rate and usually at ambient temperature, (ii) the reagent and its major hydrolysis products are not fluorescent which avoids the necessity of separating the fluorescent derivatives formed from the excess of reagent (iii) moreover it has proved to be a very sensitive detection reagent in TLC experiment (1).

EXPERIMENTAL

Apparatus

A high performance liquid chromatograph (Merck LMC System) fitted with a Rheodyne injection valve and a 10 µl loop

was used. The column was a 25 x 0.4 cm i.d. stainless steel cartridge packed with 7 µm Lichrosorb RP 18 stationnary phase. A 4 x 10 mm i.d. guard column packed with a Lichrosorb RP 18 phase was fitted prior to the analytical column. The flow rate was 1 ml.min^{-1} and the pressure was about 80 bar. Detection was carried out using a variable wavelength UV detector (LC 313) and in series a post-column reaction system (655-A 13 reaction pump Merck) coupled with a fluorimetric detector (Aminco SPF 500) fitted with a 12 µl quartz flow through cell. The post-column reaction system was a 4.50 m x 0.25 mm i.d. PTFE capillary coil with a hold-up time of 10.6 sec. at the reagent flow rate used (0.25 ml.min.$^{-1}$). All experiments were conducted at ambient temperature.Fig. 1 shows the experimental set-up schematically.UV absorbance measurements were carried out at 262 nm, maximum absorbance wavelength for all the compounds.

Fluorescence measurements were carried out with a xenon lamp at an excitation-emission wavelength of 385 nm and 485 nm, respectively (maxima excitation-emission wavelengths) for all the fluorophores formed. A 10 nm slit was used.

Chemicals and solutions

Cephalosporins were obtained as gifts from different laboratories : cefaclor (I) cephalexin (II), Eli-Lilly France (Saint Cloud, France) ; cephradine (III), Smith Kline French (Pessac, France) ; cefroxadine (IV), Ciba-Geigy (Rueil Malmaison, France) ; cefaloglycine (V), Eli-Lilly (Indianapolis, USA) ; cefadroxil (VI), Bristol (Paris, France) (see structures table 1).

Fluorescamine (Fluram® Roche) was purchased from Sigma (Buchs, Switzerland). Acetonitrile was HPLC grade. All other chemicals were of analytical reagent grade.

Aqueous stock solutions of cephalosporins (500 mg.l^{-1}) were daily prepared and suitably diluted in water before use.

Fluorescamine solution was 200 mg.l^{-1} in acetonitrile.

Different mobile phases were used. Mobile phase A for compounds I-IV was acetonitrile-0.025 M phosphate buffer pH 7 (10 + 90). Mobile phase B for compound V was acetonitrile - 0.025 M phosphate buffer pH 7 (15 + 85). Mobile phase C for compound VI was acetonitrile-0.025 M phosphate buffer pH 7 (5 + 95)

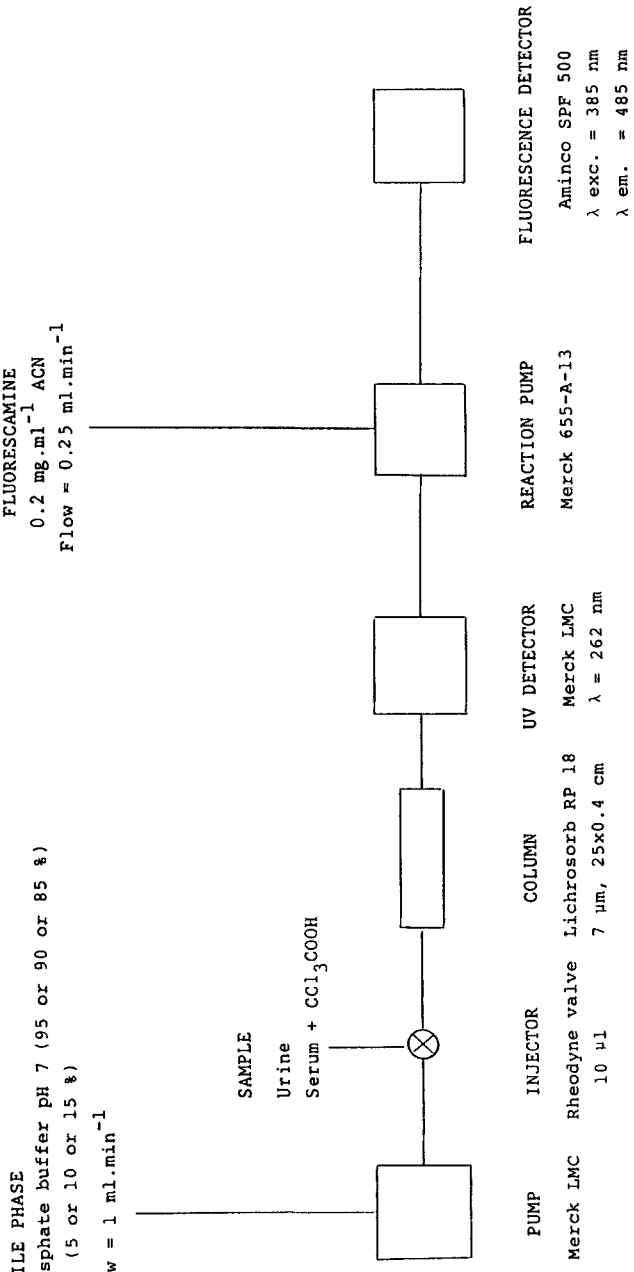
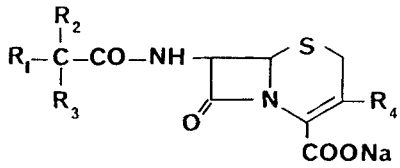
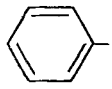
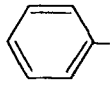
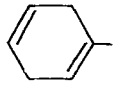
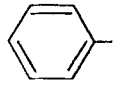
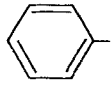
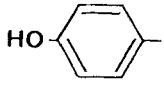


FIGURE 1. Experimental set-up.

TABLE 1 - Structure of the Cephalosporins Studied.



	R_1	R_2	R_3	R_4
Cefaclor		NH_2	H	Cl
Cephalexin		NH_2	H	CH_3
Cephradine		NH_2	H	CH_3
Cefroxadine		NH_2	H	OCH_3
Cefaloglycine		NH_2	H	CH_2OCOCH_3
Cefadroxil		NH_2	H	CH_3

The mobile phases were filtered through a 0.45 µm Millipore filter, degassed and kept under helium throughout the procedure.

Sample preparation

For recovery studies from serum, 50.0 µl or 100.0 µl of standard aqueous solutions (400 $mg.l^{-1}$, 100 $mg.l^{-1}$ or 40 $mg.l^{-1}$) were added to 1.0 ml of plasma samples. Deproteination was carried out by addition of 1.0 ml of 6 % (v/v) trichloracetic acid followed by a centrifugation for 10 min. at 4000 rpm. The deproteinated samples are stable for at least 6 hours at ambient temperature.

For recovery studies in urine, 5.0 ml or 10.0 ml of the same standard aqueous solutions were added to 100.0 ml of urine.

RESULTS AND DISCUSSION

1) Optimization of the operating conditions

Reactor. Fluorescamine reaction is not achieved using a hold-up time of 10.6 sec. in the capillary. Doubling the capillary length resulted in an increase of the fluorescence intensity (calculated by peak area). This increase varied between 5 % and 30 % according to the cephalosporins injected. However, as doubling the length of the capillary resulted in a non negligible band broadening (σ^2 = 20 $sec.^2$) and a subsequent loss of resolution, a 10.6 sec. hold-up time was chosen in the further stages of development of the method. For a higher sensitivity a longer residence time has to be preferred.

Solvent. Fluorescamine gives fluorescent products only with primary amines, but is reactive with nucleophiles, such as alcohols which slow down the reaction with primary amines (11). Therefore acetonitrile was preferred to methanol as organic solvent in the mobile phase. Fluorescamine solutions were also prepared in acetonitrile to avoid a precipitation and a clogging of the capillaries due to the buffer salt. Fluorescamine solutions in acetonitrile are stable for at least 12 weeks (12).

Reaction pH. The influence of the pH on the fluorescence intensity was investigated from batch study within a pH range of 3-9, using a spectrofluorimeter and within a pH range of 4-8 under dynamic conditions using the post-column reaction system. Fig. 2 shows the dependency of the fluorescence intensity (calculated from peak area) on the pH of the mobile phase for six cephalosporins having an α-amino group. It has been established that the pH interferes both on the completion of the reaction and on the fluorescence of the fluorophores formed. The reaction yield depends on the pH of the reaction since the $R-NH_2$ is the reacting species and a loss of fluorescence is generally noted at pHs lower than 6 due to the cyclation of the lactone ring of fluorescamine (11) and at pHs higher than 9 due to the hydrolysis of fluorescamine yielding non reactive products (13). A maximum in the fluorescence intensity is observed at a pH of around 8. The decrease we observe in the fluorescence intensity at a pH < 8, for the cephalosporins studied which have a pk_a of around 7 (14) and at a pH > 9 in batch experiments is in accordance with these observations.

From fig. 2 it can be seen that a pH of 8 has to be preferred if a maximum sensitivity is required. However, a pH of 7 was chosen for subsequent experiments in order to prolonge the life-time of the column.

The fluorescamine reaction was also investigated for cephalosporins with an aminothiazole substituent on their side-chain (cefotaxime, ceftizoxime, cefmenoxime, ceftazidime, ceftriaxone). No fluorescence was obtained either in batch experiments or under dynamic conditions at a pH of 7.0 for these cephalosporins which have a pk_a of the amino group of about 3 (15).

These results are in agreement with previous observations made using fluorescamine as spray reagent in TLC (1). In contrast to cephalosporins with an α-amino group, cefotaxime was found to react slowly. The difference in the reactivity observed between the two groups of cephalosporins cannot be easily explained.

Fluorescamine concentration. The influence of the fluorescamine concentration on the fluorescence intensity was studied

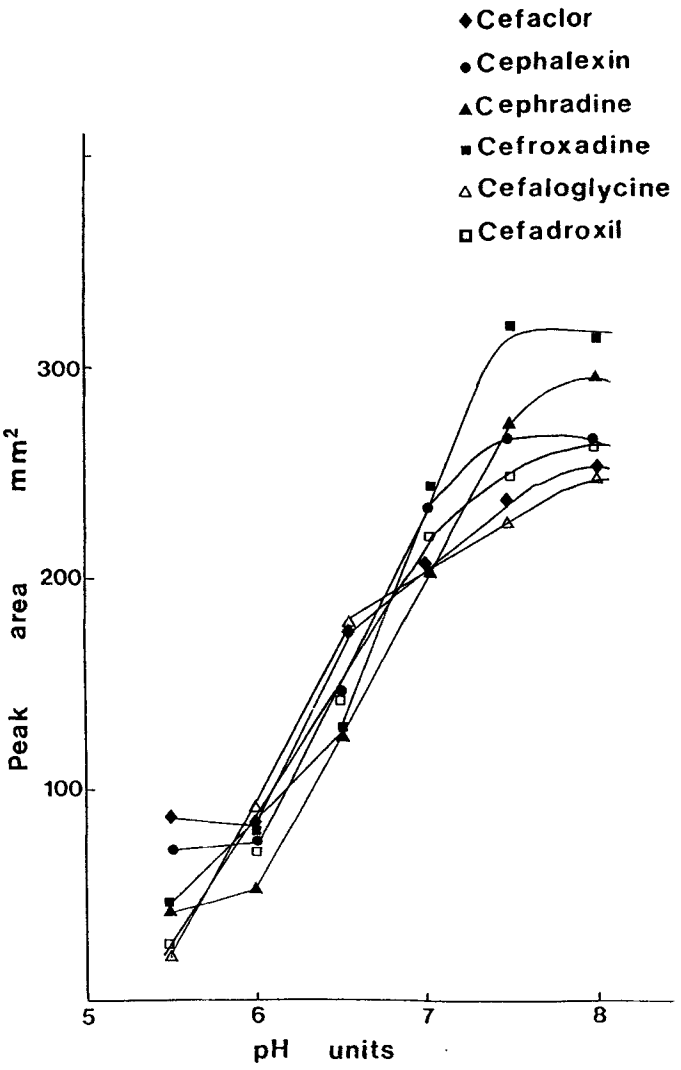


FIGURE 2. Influence of pH on the fluorescence intensity.

at a pH of 7 under hydrodynamic conditions. The fluorescamine concentration was varied from 50 $mg.l^{-1}$ to 500 $mg.l^{-1}$ using a constant flow rate of 1 $ml.min.^{-1}$ and 0.25 $ml.min.^{-1}$ for the mobile phase and the reagent, respectively. Fig. 3 shows the influence of the fluorescamine concentration on the fluorescence response evaluated from peak height measurements. The optimum fluorescamine concentration is about 250 $mg.l^{-1}$.

Reagent flow. A reagent flow of 0.25 $ml.min.^{-1}$ was found to give a reasonable band broadening.

2) Quantitative determination

The response of standard solutions of I-IV was compared using UV absorbance and fluorescence emission detection, under the conditions given in the experimental section. Capacity ratio ranged from k' = 3 (VI) to k' = 5.25 (I).

The linearity was checked for I-VI within the range 10 - 500 ng and the response evaluated by peak height measurements. The parameters of the linear regression equation were calculated. The correlation was highly significant in all cases ($r > 0.999$). The confidence limits on the intercept (P = 95 %) showed that all the calibration graphs passed through the origin.

The precision was assessed by injecting 100 ng of each cephalosporin, 12 times successively. The peak height was calculated and the repeatability expressed by the coefficient of variation (CV, %). The CV obtained are of the same order of magnitude using UV or fluorescence detection (from 1.6 to 3.3 % according to the cephalosporins). These data show that a complete reaction is not necessary if the post-column system give a reproducible reaction.

The detection limits defined for a signal-to-noise ratio of two, are lower using fluorescence detection (Table 2). These detection limits can be still lowered of about 20 % using a mobile phase with a pH of 8 or doubling the length of the capillary.

3) Application to the determination of cephalosporins in plasma and urine.

The method was applied to the determination of the six cephalosporins in plasma and urine and validated under the

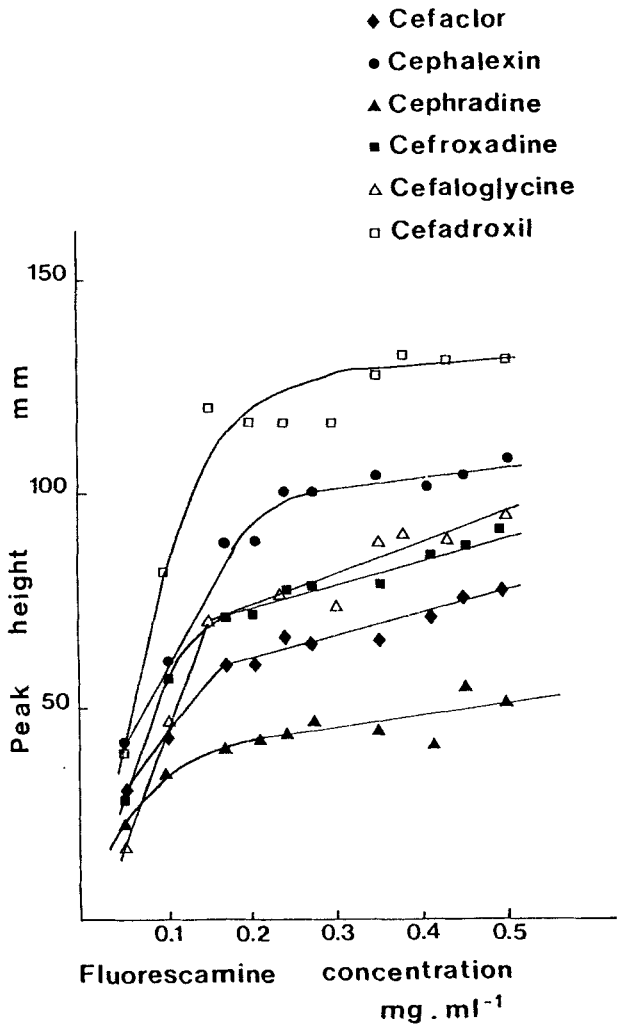


FIGURE 3. Influence of the fluorescamine concentration on the fluorescence intensity.
Cephalosporins concentration : 10 $mg.l^{-1}$.

TABLE 2 - Comparison of Detection Limits (ng) for Cephalosporins using UV Absorption and Fluorescence Detection.

	Fluorescence detection	UV absorption detection
Cefaclor	1.3	1.6
Cephalexin	1.4	1.9
Cephradine	1.8	2.7
Cefroxadine	1.7	2.5
Cefaloglycine	1.0	1.3
Cefadroxil	0.3	0.6

experimental conditions stated in the experimental section.

Plasma. Fig. 4 compares the chromatograms of a blank plasma and a spiked plasma (5.4 $\mu g.ml^{-1}$) recorded using UV absorbance and fluorescence detection mode. No interfering peaks from the matrix are shown, whatever the detection mode used.

In one instance, an interfering peak was noted using UV absorption detection, which indicates that the fluorescence detection can have a higher specificity.

Recovery data from plasma samples (evaluated from peak height measurements) are given in table 3.

A F-test was carried out to compare the recovery data analyzed by the two detection modes, for each compound. There is no significant difference between the variances of the two detection modes at a 95 % confidence level.

The precision was assessed by 12 successive injections of plasma samples spiked with 10 $mg.l^{-1}$ of cephalosporins. The CV were similar using the two detection modes and similar to those found in aqueous solutions (ranging from 1.4 to 3.4 %).

The detection limits (S/N $\sim$ 2) in plasma were similar to these found in aqueous solutions (Table 1).

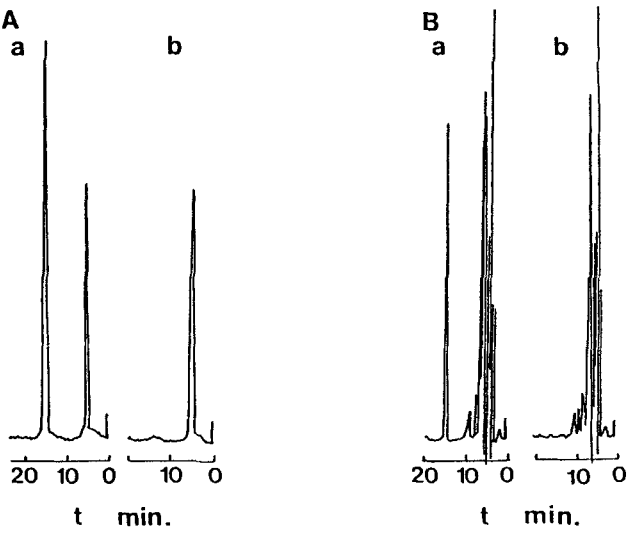


FIGURE 4. Determination of cefaclor in serum
A. Fluorescence detection
B. UV absorption detection
a. Serum spiked with 5.4 $mg.l^{-1}$ cefaclor
b. Blank serum.

Urine. Fig. 5 compares the chromatograms of a blank urine and a spiked urine (5 $mg.l^{-1}$) recorded using the two detection modes. The higher specificity of fluorescence detection is clearly shown.

Interfering peaks of the matrix prevented in all cases an accurate determination of the cephalosporins in UV, in contrast to fluorescence detection. The data of recovery studies in urine (Table 4) show the reliability of the fluorescamine procedure.

Potential interferences of some other drugs which could be administrated were also investigated : theophylline, caffeine, acetylsalicylic acid, amidopyrin, diazepam, barbital, lidocaine, tetracaine, aminoglucosides such as kanamycin, gentamycin, netilmicin, dibekacin and tobramycin. Aminoglucosides which do not absorb in UV at 262 nm are susceptible to react with fluorescamine (16). Under the conditions used,

TABLE 3 - Recovery (%) of I-IV from spiked Plasma using Fluorimetric Detection (Fluo) and UV Absorption Detection (UV). A and B refer to two different extractions of a same spiked plasma sample.

Concentration added ($mg.l^{-1}$) / Compound		2		5		10		20		40	
		Fluo.	UV	Fluo.	UV	Fluo.	UV	Fluo.	UV	Fluo.	UV
Cefaclor	A	96.2	97.0	95.2	97.5	89.7	90.3	102.6	100.7	92.2	100.0
	B	101.3	103.0	96.2	95.0	92.7	93.5	100.0	100.7	105.6	100.0
Cephalexin	A	94.9	90.9	97.0	94.9	96.3	92.1	98.0	97.7	94.8	93.5
	B	100.0	103.0	98.0	98.1	92.2	92.2	98.0	97.7	90.9	91.4
Cephradine	A	101.1	100.0	97.1	98.5	103.6	101.5	100.5	102.4	101.1	103.4
	B	101.1	100.0	87.6	98.5	100.0	97.0	98.5	100.0	101.1	101.1
Cefroxadine	A	95.0	94.6	104.9	104.8	103.1	100.0	103.2	102.2	98.4	98.1
	B	90.0	86.0	98.4	104.8	96.9	100.0	102.1	97.4	101.6	100.9
Cefaloglycine	A	99.3	97.6	96.2	101.0	102.4	100.6	99.0	100.0	97.9	99.3
	B	97.9	95.5	94.7	95.0	96.1	95.0	95.8	97.9	97.9	95.7
Cefadroxil	A	103.4	101.5	94.4	97.2	98.3	99.8	94.7	91.3	95.8	98.4
	B	103.4	99.5	99.6	97.2	95.1	99.5	98.8	97.1	88.7	94.9

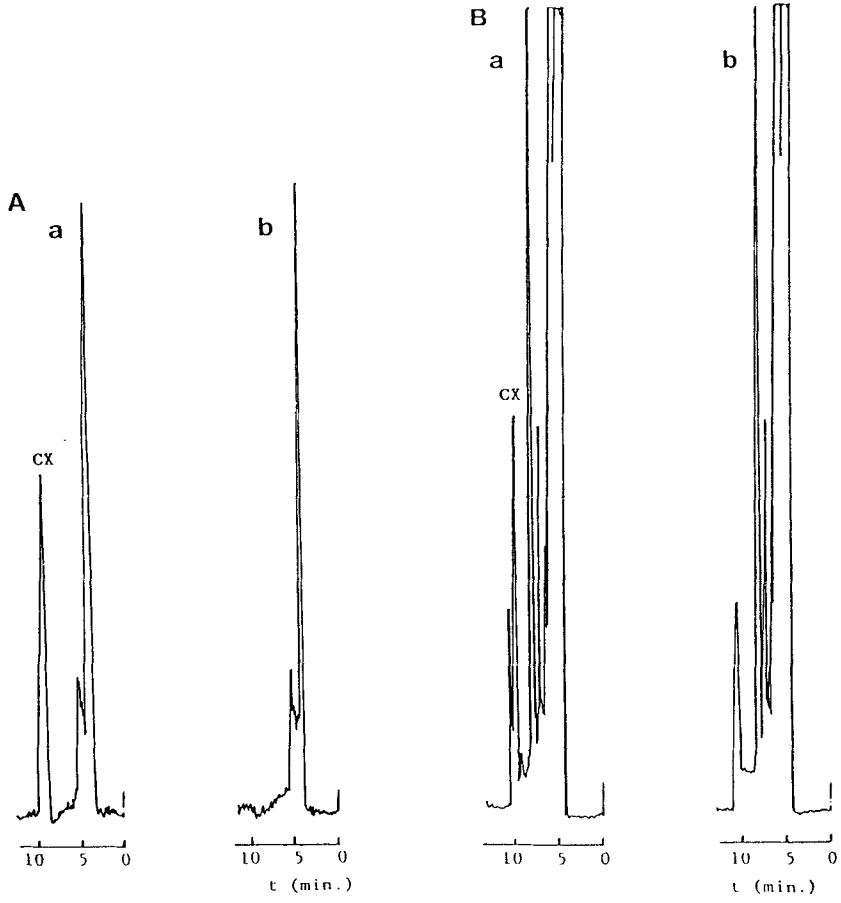


FIGURE 5. Determination of cefroxadine in urine
A. Fluorescence detection
B. UV absorption detection
a. Urine spiked with 5.5 $mg.l^{-1}$ cefroxadine
b. Blank urine.

TABLE 4 - Recovery (%) of I-IV from spiked Urine using Fluorimetric Detection. A and B refer to two different dilutions of a same spiked urine sample.

Concentration added ($mg.l^{-1}$) / Compound	1		2.5		5		10		20	
	A	B	A	B	A	B	A	B	A	B
Cefaclor	100.0	100.0	101.3	95.3	100.0	100.7	101.0	101.5	102.5	100.5
	100.0	91.3	93.3	105.2	102.9	98.5	99.0	99.5	102.5	100.5
Cephalexin	104.7	101.4	101.7	97.9	103.0	100.3	96.7	95.8	101.4	103.1
	104.7	96.8	104.8	95.7	103.0	94.7	105.4	101.5	99.8	103.1
Cephradine	97.7	96.0	102.3	113.6	97.6	96.8	100.1	95.2	95.6	99.0
	94.3	99.5	97.7	97.7	96.8	100.0	103.5	98.8	99.5	101.5
Cefroxadine	98.4	98.4	103.9	102.1	96.8	98.0	104.7	104.7	96.3	93.9
	101.1	98.4	101.0	102.1	99.1	103.6	102.1	102.1	95.1	96.9
Cefaloglycine	101.9	94.8	86.2	96.6	103.2	103.2	101.8	100.0	101.9	100.9
	101.9	90.9	100.0	95.7	103.2	97.6	99.1	98.2	101.9	97.0
Cefadroxil	100.0	96.0	100.4	100.4	102.2	100.0	98.1	98.1	103.0	100.7
	92.0	99.0	100.4	93.9	100.0	104.4	100.9	100.9	101.8	103.4

they were not eluted. All the other drugs investigated strongly absorb in UV but do not give fluorescent compounds with fluorescamine. The advantage of the fluorescence detection is of particular interest for theophylline, caffeine and lidocaine which have a capacity factor close to the cephalosporins studied.

The selectivity of the fluorescamine detection may be also of interest for other coeluting drugs that absorb in UV but lack a free primary amino group.

CONCLUSION

From the data presented, it can be concluded that fluorescence detection after post-column derivatization with fluorescamine can be reliably used for the determination of cephalosporins with an α-primary amino group in their side chain. The method is simple and can be easily automated. The comparison of UV absorption and fluorescence detection shows that this latter gives lower detection limits in serum and urine because of a higher sensitivity and/or specificity of the fluorescamine reaction.

The fact that fluorescamine gives less interfering peaks than UV absorption detection with the highly polar endogeneous compounds of urine may be of interest for the determination of other polar drugs or metabolites susceptible to give fluorescent compounds with fluorescamine. In addition, its specificity towards primary amines allows to substract the interference of many other drugs which could be concomitantly administered.

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