Journal of Chromatography, 229 (1982) 129—139
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1178

SPECIMEN HANDLING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FUROSEMIDE

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(First received July 20th, 1981; revised manuscript received November 20th, 1981)

SUMMARY

A simple high-performance liquid chromatographic method to measure furosemide in plasma and urine is described. Furosemide fluoresces best, but is unstable, at acidic pH and is subject to photochemical degradation. These factors were analysed and the results prompted changes in previously described methods.

All specimens were very carefully protected from light; extraction and acidification were done with acetic acid instead of hydrochloric acid. With these precautions no 4-chloro-5-sulphamoylanthranilic acid was found in biological specimens. The main metabolite was furosemide glucuronide (20% of furosemide excretion). Sensitivity was 0.1 and 0.5 μg/ml for plasma and urine, respectively. The applicability of our method for furosemide studies is demonstrated.

INTRODUCTION

Furosemide (F) is a potent short-acting loop diuretic frequently used in oedematous states. We searched for a reliable method to determine F and possible metabolites in both plasma and urine, for the purpose of pharmacokinetic and pharmacodynamic studies with F in aged patients. A reliable analytical method was deemed necessary, especially for urine samples, because the action of F seems to correlate best with urinary F levels [1—3]. Sensitive methods make use of the fluorescence of F, which is optimal at low pH (4.5), but under these acidic conditions F is rapidly hydrolysed to 4-chloro-5-sulphamoylanthranilic acid (CSA), furfuryl alcohol and other degradation products [6, 7]. This degradation may be even faster if sulphate ions are present [8] as in normal human urine. Human urine is usually weakly acidic, and may be made more acidic by the action of F [9].

Apart from this, mention has been made of photochemical degradation of F

0378-4347/82/0000—0000/$02.75

[6]. Several authors recommend exclusion of light [10—14], mostly without further explanation. Apparently this is necessary during preparation or storage of urine samples only [15].

Some methods of F determination are described only for plasma [16—19], and no degradation problems are mentioned in these papers. Nearly all methods of F determination so far described use hydrochloric-acid extraction (see, for example, refs. 11, 12, 14—16, 20, 21] with the risk of acid hydrolysis of F, and this probably led to the discussion in the literature about the nature of CSA (metabolite in vivo or degradation product; compare refs. 4, 11, 22, 23 with refs. 13, 14, 24). Some authors report the existence of a glucuronide as a metabolite of F [13, 24]. F had a pK_{a1} of 3.8 (pK_{a2} 7.5) and a protein-binding capacity of 95—99%. After testing some of these factors, we developed a method for the high-performance liquid chromatographic (HPLC) determination of F which involves precipitation and acidification of the samples with acetic acid, exclusion of light, extraction with a diethyl ether—*n*-hexane mixture, and the use of desmethylnaproxen as internal standard.

MATERIALS AND METHODS

Reagents

Furosemide and 4-chloro-5-sulphamoylanthranilic acid (CSA) were kindly supplied by Hoechst (Frankfurt, G.F.R.), and desmethylnaproxen by Syntex (Palo Alto, CA, U.S.A.); β-glucuronidase was obtained from Sigma (St. Louis, MO, U.S.A.). Diethyl ether, *n*-hexane, acetic acid, methanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, orthophosphoric acid and sodium hydrogen carbonate were all of analytical grade (E. Merck, Darmstadt, G.F.R.); diethyl ether was distilled shortly before use.

Biological fluids

Human plasma which had been stored at −20°C was obtained from the local blood bank; human urine was collected from a male donor shortly before analysis. From one male volunteer (31 years old, 70 kg) and one patient with congestive heart failure (68 years old, 60 kg) blood and urine were collected after oral administration of a 40-mg F tablet after an overnight fast.

Sample preparation

To find a good precipitation—acidification procedure the following solutions were prepared, before extraction with diethyl ether—*n*-hexane (65:35), in subdued daylight in dark-brown test-tubes (in duplicate; see also Table I, left-hand column):

(1) 5.2 μg of furosemide in 5 ml of diethyl ether—*n*-hexane mixture;

(2) 5.2 μg of furosemide in 5 ml of diethyl ether—*n*-hexane mixture with 20 μl of 1.5 *M* hydrochloric acid;

(3) 5.1 μg of desmethylnaproxen in 5 ml of diethyl ether—*n*-hexane mixture;

(4) 5.1 μg of desmethylnaproxen in 5 ml of diethyl ether—*n*-hexane mixture with 20 μl of 1.5 *M* hydrochloric acid;

(5) 5.2 μg of furosemide and 5.1 μg of desmethylnaproxen in 5 ml of diethyl ether—*n*-hexane;

TABLE I

SAMPLE COMPOSITION AND RECOVERY RATES OF FUROSEMIDE AND DESMETHYLNAPROXEN SOLUTIONS IN DIETHYL ETHER—*n*-HEXANE, USING ACETIC ACID OR HYDROCHLORIC ACID AS ACIDIFICATION AGENT

For details of sample preparation see Materials and Methods section.

	Sample composition				Recovery (%)
	Furosemide	Desmethyl-naproxen	HCl	Acetic acid	
1	+	—	—	—	100
2	+	—	+	—	65, 52
3	—	+	—	—	100
4	—	+	+	—	8, 12
1A	+	—	—	—	100
2A	+	—	—	+	100
3A	—	+	—	—	100
4A	—	+	—	+	100
5	+	+	—	—	100
6	+	+	—	+	100

(6) 5.2 μg of furosemide and 5.1 μg of desmethylnaproxen with 20 μl of 8.5 *M* acetic acid.

The solutions were evaporated to dryness with a gentle stream of nitrogen at 30°C. The residue was dissolved in 1 ml of a methanol—0.01 *M* sodium bicarbonate mixture (3:2). The solutions were measured as described below. The same tests were repeated, using 20 μl of 8.5 *M* acetic acid instead of 20 μl of 1.5 *M* hydrochloric acid (Table I, 1A—4A).

Stability tests

The samples of F in urine obtained during a pharmacokinetic study were divided into three portions and processed immediately and after 20 and 45 days of storage at —20°C in the dark (concentration range was 3—23 μg/ml for urine and 0.1—5.6 μg/ml for plasma). Samples of F in plasma obtained during another pharmacokinetic study were measured after four days and four months of storage.

Solutions of F (130 μg/10 ml) were prepared in phosphate buffer at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.3 in twilight. These solutions were exposed to standard laboratory lighting at room temperature (fluorescent lights Philips TL 40 W/34 de Luxe at 1.5 metres). At time zero, and 0.75, 1, 1.5, 2, 2.25, 3, 3.5, 4 and 5.25 h, 1-ml samples were mixed with 1 ml of methanol and measured directly by injection onto the column as described under Apparatus. The same procedure was carried out with samples of CSA (250 μg per 10 ml) and with solutions of F in human urine.

Solutions of F (130 μg per 10 ml) and CSA were made in phosphate buffer at pH 3.0, 5.5, and 8.0 in twilight. This time the solutions were protected from light; at time zero, and at 1, 2, 3 and 4 h, 1-ml samples of each solution were mixed with 1 ml of methanol and measured directly by injection onto the

column as described under Apparatus. No precautions were taken to prevent contact of the samples with air, and no efforts were made to expel oxygen from the solutions.

Determination of F glucuronide

Every aliquot of urine from a volunteer taking F and from a patient using F chronically was divided between two test-tubes: one for F determination, which was processed as described under Final procedure, and the other for determination of F and F glucuronide together.

The contents of the second test-tube were adjusted to pH 6.8 with KOH (20%) or H_3PO_4 (10%); the added volume of KOH or H_3PO_4 was negligible (maximum 40 µl to 12-ml tube). To an extraction tube containing an amount of internal standard (100 µl of a solution of 6.25 mg of desmethylnaproxen in 100 ml of methanol, evaporated to dryness) were added 0.25 ml of this urine and 0.25 ml of 0.075 *M* phosphate buffer (pH 6.8) containing 500 units of β-glucuronidase. The extraction tube was closed with a screw-cap. The mixture was incubated for 21 h at 37° C in the dark. After chilling to room temperature, 0.5 ml of 8.5 *M* acetic acid and 5 ml of the extraction mixture (diethyl ether—*n*-hexane) were added, and the sample was processed further as described under Final procedure and measured as described under Apparatus.

Final procedure

The experiments were carried out in subdued daylight. In a dark extraction tube with polytetrafluoroethylene (PTFE) screw-cap, 100 µl of internal standard (6.25 mg of desmethylnaproxen per 100 ml of methanol) were pipetted and evaporated to dryness under a gentle stream of nitrogen at room temperature. Aliquots of 0.5 ml of plasma or urine (stored in the dark), 0.5 ml of 8.5 *M* acetic acid and 5 ml of diethyl ether—*n*-hexane (65:35) were successively pipetted into the extraction tube. The extraction tube was closed with the screw-cap and shaken mechanically for 30 min, followed by centrifugation at 1300 *g* for 15 min. The organic layer was pipetted into another dark-brown test-tube and evaporated to dryness with a nitrogen stream at 30° C. The residue was dissolved in 0.6 ml of methanol (1 ml for urine). After addition of 0.4 ml of 0.01 *M* $NaHCO_3$ (1 ml for urine), 10 µl of this mixture were injected onto the column and measured under the conditions described under Apparatus. Before quantitative measurements were undertaken, an internal standard method was calibrated with the aid of a Hewlett-Packard 3353 Lab Auto System (with the option of measuring peak height instead of peak area), using a plasma calibration solution of 5 µg/ml furosemide and 12.5 µg/ml desmethylnaproxen. This was done to enable the computer to discriminate between the internal standard peak and the furosemide peak. After calibration, a standard series with the following concentrations was injected: 0.27, 0.53, 1.06, 2.12, 3.18, 4.24, 5.30 µg/ml of plasma. A basic programme calculated the regression line of the standard curve with the peak height ratio of furosemide to internal standard. The concentrations of the samples were calculated according to the regression line.

The same procedure was carried out for urine. The calibration solution for urine was 50 µg/ml furosemide and 12.5 µg/ml desmethylnaproxen. The stan-

dard series had the following concentrations: 1.04, 2.60, 5.19, 10.38, 20.76, 31.14, 51.3 μg/ml of urine.

Apparatus

A Hewlett-Packard HP1081B high-performance liquid chromatograph equipped with a variable-volume injector was used. The stainless-steel column had a length of 15 cm and an internal diameter of 4.6 mm, and was packed with LiChrosorb RP-8, particle size 5 μm (Merck). The oven temperature was 35°C and the injection volume was 10 μl. Furosemide and the internal standard desmethylnaproxen were measured with a fluorescence spectrometer Model 3000 (Perkin-Elmer); the excitation wavelength was 275 nm (for urine 235 nm), the emission wavelength was 410 nm (for urine 400 nm), and the excitation and emission slits were 10 nm.

The mobile phase, consisting of methanol—0.02 *M* phosphate buffer, pH 3.0 (1:1), was delivered at a rate of 1.0 ml/min, the resulting pressure being 240 bars.

Pharmacokinetic pilot study

A male volunteer (31 years old, 70 kg) took a standard 40-mg furosemide tablet (Lasix®) at 9.00 a.m. after an overnight fast. Blood samples were taken at time zero, and at 0.5, 1, 1.5, 2.25, 3, 5.5 and 6.5 h. A blank urine sample was voided at time zero and further samples at 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 5, 8 and 12 h, directly into dark-brown bottles.

One male patient (68 years old, 60 kg), who during 10 days had been using furosemide for congestive heart failure, took his eleventh standard tablet of 40 mg furosemide (Lasix®) at 9.00 a.m. after an overnight fast. Blood was sampled at time zero, and at 0.5, 1, 1.5, 2, 2.5, 3 and 4 h. Urine samples were collected after spontaneous voiding, which was rather irregular. By mistake two specimens were not protected from light immediately.

RESULTS

Studies on the influence of acidification

Using hydrochloric acid (1.5 *M*) as acidification agent, we obtained a recovery of about 50% for F and 10% for the internal standard because of decomposition of F and desmethylnaproxen. Using acetic acid (8.5 *M*) as acidification agent, no degradation of F or desmethylnaproxen was observed. Details are given in the right-hand column of Table I and under sample preparation in Materials and Methods.

Stability tests: storage of urine and plasma

If the pharmacokinetic samples of urine were immediately protected from light and stored at −20°C, we found a recovery of 97 ± 7% (mean ± S.D.) after 20 days and of 94 ± 7% after 45 days. This recovery was the same with biological samples of urine of pH 5—8 and various F concentrations of 3—23 μg/ml. In the plasma samples measured after four months, we found a recovery of 102 ± 3% in the lower as well as in the higher concentration range.

Stability tests: influence of light at various pH values

If F was exposed to light (see Table II), fast hydrolysis took place which resulted in at least three compounds, highly dependent on the pH of the F solution. The degradation products had retention times of 1.93 min (F_1), 2.85 min (F_2) and 5.43 min (F_3). A degradation product of F with a retention time of 2.85 min (F_2) in the chromatographic system was much more fluorescent under the detection conditions than furosemide itself (Fig. 1). No degradation product with a retention time identical to CSA (2.1 min) was found. Decomposition of F appeared to obey first-order kinetics, and consequently a decomposition half-life of F could be calculated as shown in Table II. Apart from this we found further decomposition of pure CSA solutions, which we could not identify because the peak of the decomposition product of CSA (1.94 min) overlapped with the CSA peak (2.10 min) (see Fig. 2). When instead of water we used human urine in view of the possible influence of sulphate ions, the results were not significantly altered (nor more pronounced either). When the samples of F in 0.01 *M* phosphate buffer of pH 3.0—5.5 and 8.0 were protected from light at room temperature, there was 100% recovery after 4 h, whereas after 24 h we found only negligible decomposition peaks.

TABLE II

RECOVERY OF FUROSEMIDE (%) FROM SOLUTIONS OF DIFFERENT pH WHEN EXPOSED TO LIGHT

Time exposed to light (h)	pH									
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.3
0	100	100	100	100	100	100	100	100	100	100
0.75	75	81	89	97						
1					95	95	96	99	100	94
1.5	46	60	81	91						
2					90	90	92	95	98	89
2.25	26	39	59	67						
3					81	84	82	84	88	84
3.5	10	24	55	62						
4					76	78	78	81	84	84
5.25	7	18	51	ND*						
$t_{½}$ decomp. (h)	1.1	1.7	4	5	10	11	11	13	16	16

*ND = not determined.

Final procedure

When we used blood bank plasma and human urine, the recovery of F in plasma was 85 ± 2% at concentration 5 μg/ml and 89 ± 3% at concentration 0.5 μg/ml; in urine the recovery was 95 ± 2% at concentration 50 μg/ml, 97 ± 3% at concentration 25 μg/ml and 95 ± 2% at concentration 2.5 μg/ml.

With plasma furosemide concentrations of 0.27—5.3 μg/ml, there is a linear relationship between the peak height ratio of furosemide to internal standard (Y) and the plasma F concentration (X), as given by the equation $Y = 0.114X$

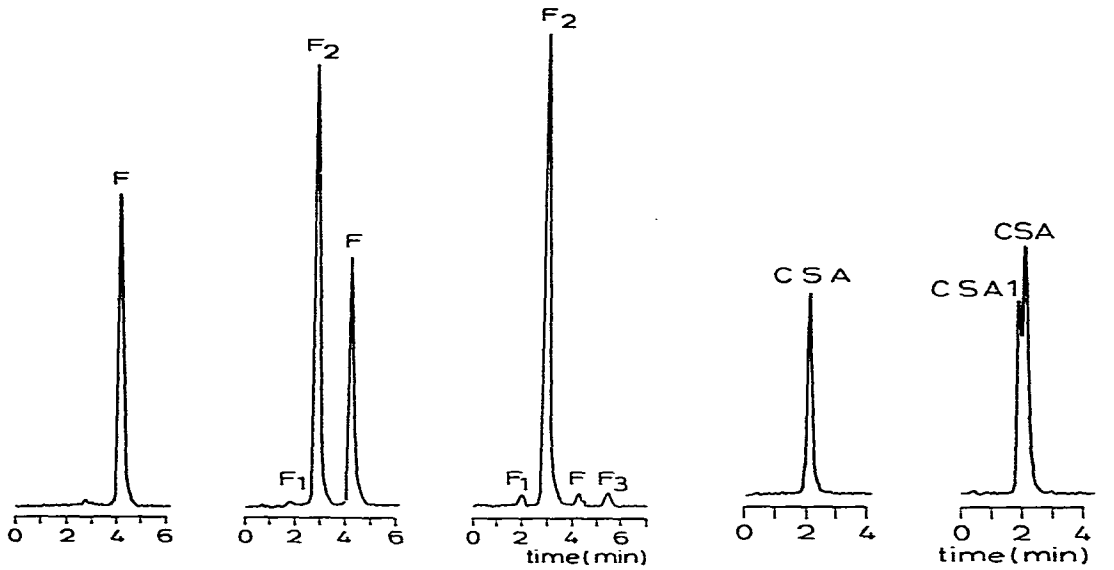

Fig. 1. Effect of light on a furosemide solution consisting of 130 μg of F dissolved in 10 ml of 0.01 *M* phosphate buffer (pH 4.0); 1-ml samples were processed and measured. Left: chromatogram of sample protected from light. Centre: chromatogram of sample processed after 0.75 h under fluorescent lights; degradation products F_1 and F_2 are visible. Right: chromatogram of sample processed after 5.25 h under fluorescent lights; degradation products F_1, F_2 and F_3 are present.

Fig. 2. Effect of light on a CSA solution formed by dissolving 250 μg of CSA in 10 ml of 0.01 *M* phosphate buffer (pH 4.5); 1-ml samples were processed and measured. Left: chromatogram of a sample protected from light. Right: chromatogram of a sample processed after 0.75 h under fluorescent lights; the overlap of degradation peak CSA_1 and original CSA peak results in an apparent rise in CSA peak height.

+ 0.0013 (r = 0.9998, n = 7). With urine concentrations of 1.04—51.9 μg/ml, there is a linear relationship between urinary F concentration (X) and peak height ratio (Y), described by the equation $Y = 0.020X - 0.0038$ (r = 0.9999, n = 7).

At a given plasma F concentration of 0.54 μg/ml, the coefficient of variation is 2.67% (n = 10): 0.5401 ± 0.0144 (range 0.514—0.560). At a given plasma F concentration of 2.04 μg/ml the coefficient of variation is 3.50% (n = 10): 2.038 ± 0.0713 (range 1.98—2.18). At a given urinary F concentration of 3.61 μg/ml, the coefficient of variation is 2.35% (n = 9): 3.614 ± 0.085 (range 3.47—3.70). At a given urinary F concentration of 21.81 μg/ml, the coefficient of variation is 1.60% (n = 10): 21.814 ± 0.3482 (range 21.36—22.26). Under the conditions described under Apparatus, the retention time of CSA was 2.1 min, that of F was 4.2 min and that of the internal standard desmethylnaproxen was 6.0 min (Figs. 3 and 4). In the case of accidental exposure to light during the procedure, peaks of degradation products appear. The lower limit of detection in plasma with spiked samples using an internal standard of 2.5 μg of desmethylnaproxen instead of 12.5 μg/ml is 0.1 μg/ml (0.1 ± 0.08, n = 10, range 0.09—0.12). The lower limit of detection in urine is 0.5 μg/ml (0.47 ± 0.02, n = 10, range 0.45—0.52). This covers very satisfactorily the drug levels at which the diuretic effects of F occur.

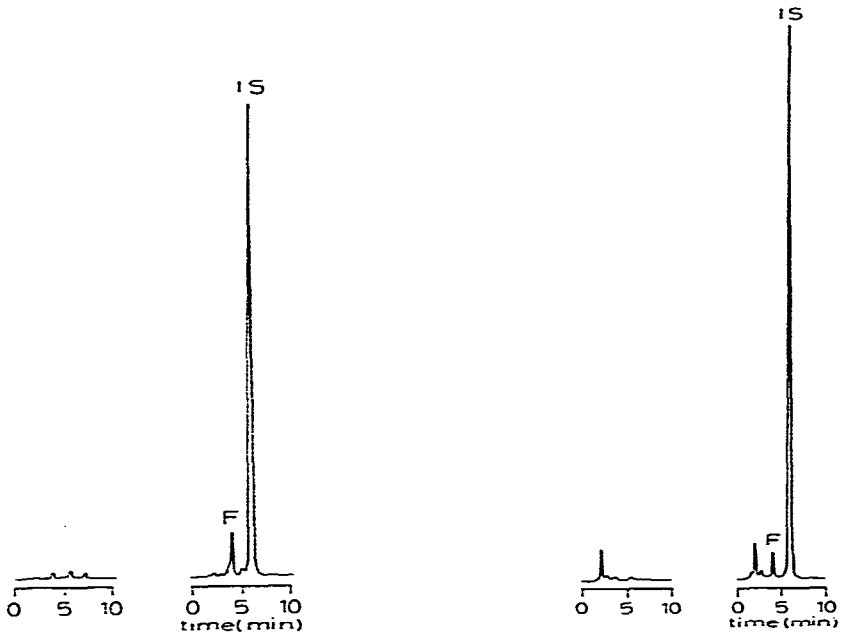

Fig. 3. Left: chromatogram of blank plasma with small peaks of endogenous fluorescent substances at 3.8 and 5.3 min. Right: chromatogram of plasma containing 1 μg/ml furosemide at 4.2 min and 25 μg/ml desmethylnaproxen (internal standard, I.S.) at 6.0 min.

Fig. 4. Chromatograms of (left) blank urine and of (right) urine containing 2.5 μg/ml furosemide and 25 μg/ml desmethylnaproxen (internal standard, I.S.).

Pilot pharmacokinetic studies

In the adult volunteer the peak plasma level was 1.8 μg/ml at 1 h. The elimination half-life was determined as 1.06 h and the area under the curve (AUC) for plasma as 3.6 mg h per l. The renal furosemide clearance was 70 ± 20 ml/min. The recovery of F in urine was 14.54 mg after 12 h; recovery of F glucuronide in urine was 3.55 mg; total recovery in urine during 12 h was 18.1 mg (45%). No trace of CSA or other metabolites was found. There was a good correlation ($r = 0.84$, $p < 0.01$) between urinary F excretion per min and urinary water excretion (as a global parameter of diuretic effect) during the first 3 h; this correlation extended also over the period 0—8 h ($p < 0.001$, $r = 0.88$) (see Fig. 5).

In the older patient, resorption was somewhat delayed: a peak plasma F level of 1.7 μg/ml was attained at 1.5 h, elimination half-life was 0.8 h and AUC for plasma was 3.1 mg h per l (renal furosemide clearance was approximately 73 ± 27 ml/min, but only four specimens were available for this estimation). The recovery of F in urine was 13.7 mg after 24 h, and recovery of F glucuronide in urine was 3.9 mg; in the two specimens exposed to light by the patient we found no CSA, but after pretreatment of the specimen with β-glucuronidase (in the dark) we found a major peak in the chromatogram with a retention time of 2.85 min (F_2, Fig. 1). No trace of this substance was found

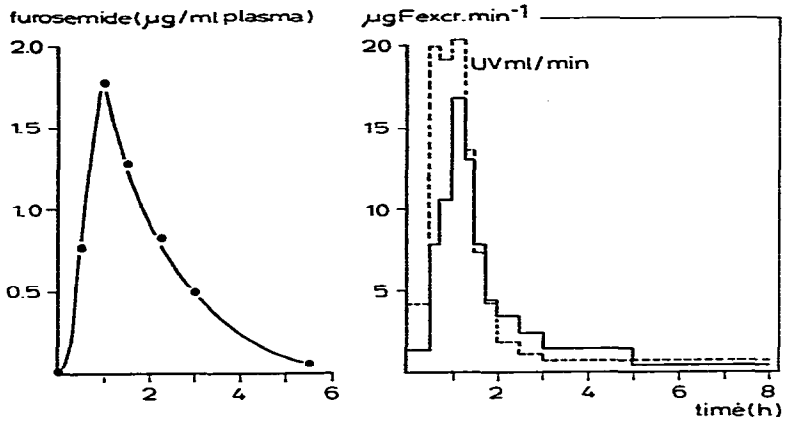

Fig. 5. Left: plasma level versus time curve after ingestion of a 40-mg furosemide tablet by a healthy volunteer. Right: urinary furosemide excretion (µg F excr.) and urinary volume (UV) versus time.

in the properly handed specimens. The patient was unable to void frequently enough to enable pharmacodynamic studies.

DISCUSSION

Various problems with other methods of F determination are described in the Introduction. Acidification of plasma samples is necessary to separate F from its protein binding and to transfer it to an organic layer. Acidification of urine samples is advisable to minimise the presence of endogenous fluorescing substances in the organic layer.

We did not use N-benzyl CSA [20] as internal standard because it has a longer retention time and is less stable under our experimental conditions than desmethylnaproxen. We did not use dichloromethane or chloroform extraction because these chemicals extract too much endogenous fluorescing substance. Our diethyl ether—*n*-hexane (65:35) extraction mixture was unsuitable if used after HCl precipitation and acidification of the F samples, probably because the organic layer contained traces of water with hydrochloric acid, and furosemide is very unstable in HCl solution, as demonstrated (see Results and Table I).

When acetic acid was used, no degradation of F could be observed. The problem seems to have been encountered before, since Steiness et al. [14] used a different acidification for urine (phosphate buffer) than for plasma (HCl).

Urine samples containing furosemide could not be kept in the deep-freezer for an indefinite time because this is likely to cause a slow decrease in recovery rate. We brought to light the degradation problems in handling furosemide. Especially acidic specimens of F should be kept in the dark from the time of taking the sample until injection onto the HPLC column; decomposition was astonishingly fast, and degradation into various products occurred. The degradation product F_1 of furosemide, with a retention time of 1.93 min in our HPLC procedure, may well be identical to degradation product CSA_1 with a retention time of 1.94 in our system. We were unable to identify CSA_1

in relation to F_1 because chromatographic separation of CSA_1 from CSA was far from complete. With proper handling of samples our method is hardly laborious. In sensitivity and reliability, our method does not differ from other published methods. In plasma samples with concentrations of 0.1—1.0 μg/ml, we now use an internal standard solution of 1.25 mg of desmethylnaproxen per 100 ml of methanol (calibrating a standard curve with 0.1, 0.2, 0.5, 1.0 and 1.5 μg/ml solutions of F). In urine, the concentration range related to the diuretic effect of F is far above our lower limit of detection; it is therefore unnecessary to reduce the amount of interal standard, although this would be possible in the same way. The preliminary studies in man produced results similar to those mentioned in other reports in the literature. Discussion about the existence of metabolites of F in man could hardly be touched in this preliminary study: we did find a glucuronide in considerable amounts; we did not find CSA itself in the specimens from the adult volunteer; from the semi-chronic user of F too few good urine specimens could be obtained to permit detailed analysis. The F_2 glucuronide we found was probably an in vitro breakdown product of F glucuronide, and the F glucuronide excretion in this patient must therefore have been even more than the 3.9 mg mentioned. Further studies are in progress.

ACKNOWLEDGEMENTS

Valuable advice about the HPLC procedure was given by the late Huub Ketelaars. The excellent secretarial assistance of Miss L.W.M. Tempelman is gratefully acknowledged.

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