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QUANTITATIVE DETERMINATION OF FUROSEMIDE IN PLASMA, PLASMA WATER, URINE AND ASCITES FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method using spectrofluorometric detection is described for the determination of furosemide in plasma, plasma water, urine and ascites fluid. The extraction procedure decreases interference from endogenous substances. The detection limit of furosemide is 10 ng in 0.5 ml of biological sample. The method is sufficiently sensitive for pharmacokinetic study of furosemide with normal subjects and patients with liver cirrhosis and/or renal disease after oral administration of furosemide in a retard capsule, and for study of protein binding of furosemide in patients with various diseases.

INTRODUCTION

Furosemide (FD) is a potent diuretic agent which is widely used for the treatment of oedema and ascites. Measurements of FD concentration in both

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plasma and urine are required for detailed studies on the pharmacokinetics and pharmacodynamics of FD in man.

Several analytical methods for the determination of FD in plasma and urine have been developed. Although an earlier colorimetric method [1] and a fluorometric method [2] are simple and rapid, they suffer from a lack of sensitivity and selectivity. Mikkelsen and Andreasen [3] reported an analytical method using thin-layer chromatographic (TLC) separation followed by fluorescence analysis. This method allows the determination of FD in plasma and urine in concentrations as low as 100 ng/ml, but the required sample volume is 2 ml. Although another TLC method [4] has a high sensitivity, it extends the complexity of FD assay. Two gas chromatographic (GC) methods [5, 6] based on derivatization of FD to its methyl ester are time-consuming since the incubation time for the derivation is about 1 h.

Recently high-performance liquid chromatographic (HPLC) methods [7–13] for the determination of FD in plasma and urine have been developed. In these methods, however, the complete separation of FD from endogenous substances in urine is not accomplished with a minimum detectable FD concentration of less than 100 ng/ml. Therefore, these HPLC methods can not be applied to the measurement of low FD concentrations (approximately 20 ng/ml) in plasma after its administration in retard capsules, or to the study of binding of FD to plasma protein at therapeutic plasma levels.

The present paper describes an HPLC method which has a high sensitivity, specificity and precision for the determination of FD in human biological fluids, and which would be suitable for a pharmacokinetic study following a low dose of FD.

EXPERIMENTAL

Drugs and reagents

Furosemide, 4-chloro-5-sulfamoyl anthranilic acid (CSA) and piretanide [4-phenoxy-3-(1-pyrrolidinyl)-5-sulfamoyl benzoic acid], which was used as internal standard (I.S.), were kindly supplied by Hoechst Japan (Tokyo, Japan). CSA has been reported as a major breakdown product of FD [3, 9], and its pharmacological activity was weak. In this study, therefore, the interference of CSA with the peak of FD was checked. The chemical structures of these drugs are shown in Fig. 1.

Retard capsules of FD (Eutensin®) in a sustained-release dosage form were obtained from Hoechst Japan, and contained 40 mg of FD. Lyophilized serum (Con Sera® "Nissui") was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). All the other solvents and reagents used were of reagent grade, and were obtained from Wako (Tokyo, Japan).

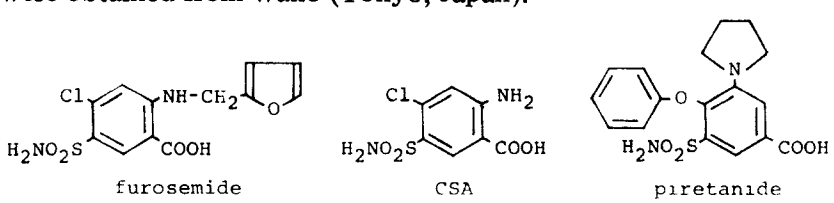

Fig. 1. Chemical structures of furosemide, 4-chloro-5-sulfamoyl anthranilic acid (CSA) and piretanide.

Extraction of FD from biological samples

To 0.5 ml of plasma, plasma water, 50 times-diluted urine or ascites fluid in a 50-ml glass-stoppered centrifuge tube were added 6 μg of internal standard in 100 μl of ethanol, 2 ml of H_3PO_4—KH_2PO_4 solution (1 *M*, pH 1.5) and 11 ml of methylene chloride. The mixture was vigorously shaken with a mechanical shaker for 15 min, then centrifuged in a KN-70 centrifuge (Kubota Seisakusho, Tokyo, Japan) at 1630 *g* for 5 min. Nine millilitres of lower organic phase were transferred to another tube, and were added with 2.5 ml of 0.001 *M* sodium hydroxide. The mixture was vigorously shaken for 15 min, then separated by centrifugation. Two millilitres of upper aqueous phase were transferred to another tube, mixed with 2 ml of H_3PO_4—KH_2PO_4 solution (1 *M*, pH 1.5), and extracted with 11 ml of methylene chloride once again. Ten millilitres of lower organic phase were transferred to another tube and evaporated to dryness using a rotary vacuum evaporator at room temperature. The dried residue was dissolved in 100 μl of ethanol (Super Special Grade, Wako) and 20 μl of this solution were injected into the HPLC column.

Light-protecting containers were used throughout this extraction procedure.

Chromatography

The HPLC apparatus used in this study was composed of a Shimadzu liquid chromatograph LC-3A and a Shimadzu spectrofluorophotometer RF-500 (Shimadzu, Kyoto, Japan). An excitation wavelength of 268 nm and an emission wavelength of 410 nm were used for the detection of FD. The mobile phase consisted of 65 vols. of ethanol and 35 vols. of $HClO_4$—$NaClO_4$ solution (0.02 *M*, pH 2.0), and was degassed before use. The flow-rate of the mobile phase was 0.2 ml/min (pressure approximately 70 kg/cm^2). The column was packed with 10-μm spherical porous particles of styrene—divinylbenzene (Hitachi Gel® 3011, Hitachi Seisakusho, Hitachi, Japan) in a stainless-steel column (50 cm × 2 mm I.D.). The column temperature was maintained by a column jacket connected to a water bath at 30° C.

Ascertainment of chromatographic peak of FD by mass spectrometry

Eight or nine tubes each containing 1 ml of FD solution 20 μg/ml in 0.01 *M* sodium hydroxide were extracted using the method described above. The dried residue was dissolved in 100 μl of ethanol, and 25 μl of this solution were injected into the HPLC column. One millilitre of mobile phase corresponding to the FD peak was collected in a 50-ml centrifuge tube just after passage through the cell of the detector, and this manipulation was repeated about thirty times. Thirty millilitres of the solution thus obtained were added to 1 ml of 1 *M* sodium hydroxide solution to prevent hydrolysis of FD, and the mixture was evaporated to dryness using a rotary vacuum evaporator at about 30° C. The dried residue was dissolved in 2 ml of 0.01 *M* sodium hydroxide, mixed with 2 ml of H_3PO_4—KH_2PO_4 solution (1 *M*, pH 1.5) and extracted with 11 ml of methylene chloride. The extracted residue was dissolved in 0.5 ml of ethanol, and this solution was subjected to mass spectrometry.

A JMS-300 mass spectrometer equipped with a multiple-ion detector and JMS-2000 data processing system including a JEC-980B computer (Jeol, Tokyo, Japan) were used in this study. Mass spectra were obtained by direct

inlet probe in electron-impact mode. The scanning mode for measurement of mass spectra was as follows: ionization energy, 70 eV; ionization current, 0.3 mA; scan speed, *m/z* 0 to 800 in 5 sec. The ion-source temperature was maintained at 210° C. The obtained data were stored directly on a disc and displayed on a cathode ray tube or printed out on a graphic printer.

Calibration curves

To prepare the plasma sample, 25 mg of FD were dissolved in 0.1 *M* sodium hydroxide in a 25-ml volumetric flask. An aliquot of this solution was diluted with reconstituted lyophilized serum in distilled water to produce a standard solution of the desired concentration (0, 10, 25, 50, 100, 250, 500 or 1000 ng per 0.5 ml). The other biological samples were prepared for calibration curves in the same manner. Thus, the 1 mg/ml FD solution in sodium hydroxide was diluted with diluted urine (× 50) or FD-free ascites fluid to produce standard solutions of the desired concentrations. Distilled water was used instead of plasma water, because a large volume of FD-free plasma water was difficult to obtain from normal volunteers. Furthermore, the extraction recovery of FD from plasma water was equal to that from distilled water in preliminary tests. These samples were analysed by the method described above. The calibration curve for each biological sample was obtained by plotting the peak height ratio (FD/I.S.) against the concentration of FD in each spiked sample.

Check for interference by coadministered drugs

The present method was checked for interference by drugs frequently used together with FD in the treatment of patients with ascites and/or oedema. Their prescriptions were used to search for these drugs, which were allopurinol, spironolactone, canrenone, diazepam, digitoxin, digoxin, dipyridamole, hydralazine, indomethacin, α-methyldopa, prednisolone, propranolol, reserpine, aspirin (salicylic acid), compound preparations of vitamins of the B group (Vitaneurin®-50, Takeda Chemical Industries, Osaka, Japan), and warfarin.

Sample collection

Three normal subjects were orally administered 40 mg of FD in a retard capsule at 09:00 a.m. once a day. All subjects were shown by physical and laboratory examinations to be in good physical condition. They consented to participate in this study after the aim and protocol were explained. Blood samples were drawn through an indwelling cannula at the times indicated in Fig. 4, until 12 h after administration; the plasma was immediately separated from whole blood by centrifugation. Urine samples were collected at the times shown in Fig. 4 until 24 h after the administration of FD.

Ascites fluid was collected by abdominal paracentesis from a decompensated cirrhotic patient with intractable ascites. The patient was intravenously administered 20—60 mg of FD for the treatment of ascites, but the ascites failed to decrease.

Plasma samples for separation of plasma water were collected from a normal volunteer after intravenous administration of FD, and from patients with liver

cirrhosis and/or renal disease who received FD for the treatment of ascites or oedema. The plasma water was separated from plasma by ultracentrifugation. According to the methods of Tanimura et al. [14] and Nishihara et al. [15], two 3-ml aliquots of plasma were pipetted into a length of curved seamless cellulose acetate tubing (Type 20/32, 130 mm long; Visking, U.S.A.) with both open ends held tightly by a silicone rubber stopper against the inner wall of a glass vessel. The glass vessel holding the tubing inside was centrifuged (Hitachi 20 PR-5) at 1630 *g* at 37 ± 0.5°C for about 40 min. Five hundred microlitres of ultrafiltrate (i.e. plasma water) were used for the determination of FD.

All biological samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

The extraction and HPLC procedures for the determination of FD in biological samples were set following the most desirable chromatographic and detection conditions. Methylene chloride for extraction of FD was contaminated with smaller amounts of endogenous substances present in biological fluids than are other organic solvents such as acetonitrile, choroform, diethyl ether and ethyl acetate. Furthermore, the basic extraction step reduced the quantities of endogenous substances extracted with methylene chloride from urine. As the fluorescence intensity of FD was most stable in ethanol and became greater with increasing acidity of the solution, the mobile phase was composed of 65 vols. of ethanol and 35 vols. of $HClO_4$—$NaClO_4$ solution (0.02 *M*, pH 2.0). The column was prepared with Hitachi Gel® 3011

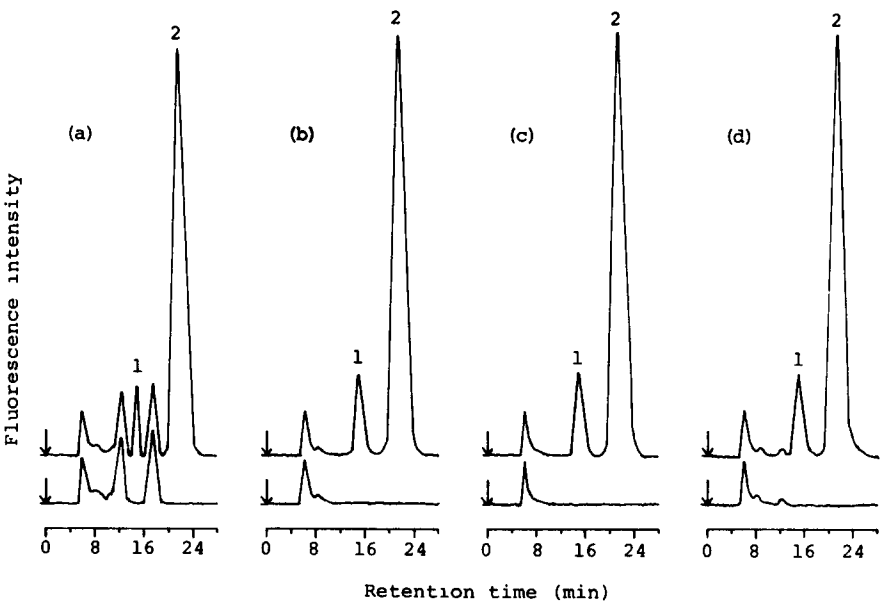

Fig. 2. Upper chromatograms obtained from 0.5 ml of (a) plasma, (b) plasma water, (c) urine and (d) ascites fluid from normal subjects or patients, spiked with 50 ng of furosemide and 6 μg of piretanide as the internal standard. Lower chromatograms obtained from drug-free (a) plasma, (b) plasma water, (c) urine and (d) ascites fluid. Peaks: 1 = furosemide, 2 = piretanide. Chromatographic conditions were as described in Experimental.

using the mobile phase because the degree of swelling of the gel was considerably influenced by the composition of the mobile phase. The column used in this study seems to be remarkably stable, since no changes in chromatographic properties were noticed after about 2000 injections of biological samples.

Typical chromatograms of FD and each blank obtained from biological samples are shown in Fig. 2. The retention times of FD and internal standard were 15.4 and 21.4 min, respectively. Since the retention time of CSA was 7 min, it did not interfere with the analysis of FD. Further, the blank chromatograms showed that no interference would occur with endogenous substances. The purity of the chromatographic peak of FD was ascertained by mass spectrometry. The results are shown in Fig. 3. The sample obtained from HPLC separation showed a mass spectrum identical to that of authentic FD.

The extraction recoveries of FD from biological fluids were determined at four different concentrations. As shown in Table I, the mean extraction recovery was about 95% for plasma and about 100% for plasma water, urine and ascites fluid. The coefficients of variation were less than 5%.

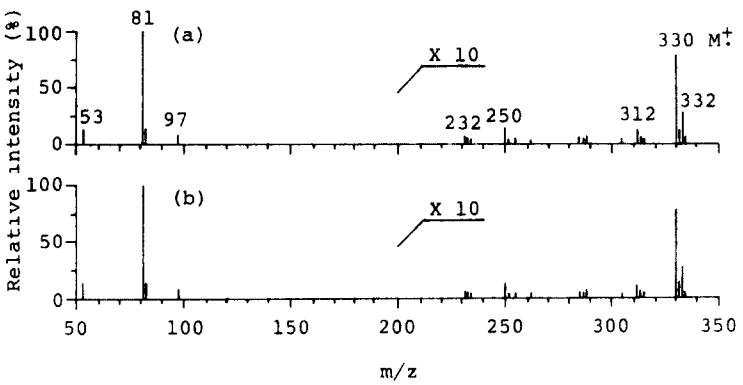

Fig. 3. Electron-impact (70 eV) mass spectra of (a) authentic furosemide and (b) sample from the present HPLC method obtained using a direct inlet system. Mass spectrometric conditions were as described in Experimental.

TABLE I

MEAN EXTRACTION RECOVERIES OF FUROSEMIDE FROM SEVERAL BIOLOGICAL SAMPLES

Each value represents mean ± S.D. of five experiments.

Added (ng)	Extraction recoveries (%)			
	Plasma	Urine	Plasma water	Ascites fluid
10	95.3 ± 3.9	100.4 ± 4.7	100.8 ± 3.2	102.1 ± 2 5
50	94.6 ± 4.1	105.3 ± 1.4	99.1 ± 2.8	99.2 ± 2.9
250	96.4 ± 1.6	100.9 ± 1.3	99.5 ± 2.8	103 5 ± 4.2
500	96.7 ± 2.7	101.3 ± 2.1	102.8 ± 3.3	100.6 ± 2.5
Mean	95.6	102.0	100.6	101.4

The calibration curves of FD for plasma, plasma water, urine and ascites fluid were linear over the FD concentration range 10—1000 ng per 0.5 ml, and the lines passed through the origin. The detection limit of FD in each biological fluid was 10 ng per 0.5 ml with a signal-to-noise ratio of about 10:1.

To examine the reproducibility of this method, a single dose of 40 mg of FD in a retard capsule was orally administered to a normal subject, and five 0.5-ml plasma samples at each sampling time were analysed. The results were satisfactory, as shown in Table II.

The interference by coadministered drugs is shown in Table III. Most drugs were completely removed by the first extraction step with methylene chloride under acidic conditions and/or by chromatographic separation, and only reserpine interfered. This, however, was removed by additional extraction with the basic aqueous solution.

TABLE II

REPRODUCIBILITY OF FUROSEMIDE (FD) MEASUREMENT AT EACH SAMPLING TIME AFTER ORAL ADMINISTRATION OF 40 mg OF FD IN A RETARD CAPSULE TO A NORMAL SUBJECT

Sampling time (h)	Plasma concentration of FD (ng/ml)	
	Mean ± S.D. (n = 5)	C.V. (%)
3	67.6 ± 2.8	4.1
6	205.2 ± 3.3	1.6
12	51.8 ± 2.0	3.9

TABLE III

INTERFERENCE BY COADMINISTERED DRUGS

Concomitant drug	Interference*		
	Acidic extraction	Chromatographic separation	Acidic + basic extraction
Allopurinol	—		
Spironolactone	—		
Canrenone	—		
Diazepam	—		
Digitoxin	—		
Digoxin	—		
Dipyridamole	+	—	
Hydralazine	+	—	
Indomethacin	—		
α-Methyldopa	—		
Prednisolone	—		
Propranolol	—		
Reserpine	+	+	—
Aspirin (salicylic acid)	+	—	
Vitaneurin®-50	—		
Warfarin	—		

*— indictes lack of interference.

The present method was applied to the determination of FD in several clinical studies. The time courses of mean plasma concentration and cumulative urinary excretion of FD after oral administration of 40 mg of FD in a retard capsule to three normal subjects are shown in Fig. 4. The plasma concentration of FD increased slowly to reach a maximum value (about 200 ng/ml) at about 6 h, and decreased gradually to approach about 30 ng/ml at 12 h. Furosemide was excreted in urine at an approximately constant rate for 10 h; the urinary excretion of FD during 24 h was about 8 mg, corresponding to 20% of the given dose.

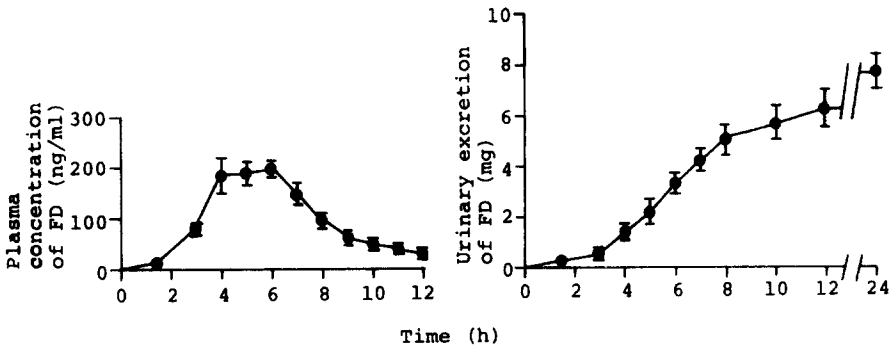

Fig. 4. Time courses of plasma concentration (left) and cumulative urinary excretion (right) of furosemide (FD) after oral administration of 40 mg of FD in retard capsules to three normal volunteers. Each value indicates the mean ± S.E. obtained from three samples.

TABLE IV

BINDING OF FUROSEMIDE (FD) TO PLASMA PROTEIN IN A NORMAL SUBJECT AND PATIENTS

The binding of FD to plasma protein is calculated using the equation

$$\text{Binding of FD to plasma protein (\%)} = \frac{\text{plasma concentration of FD} - \text{plasma water concentration of FD}}{\text{plasma concentration of FD}} \times 100.$$

	Concentration of FD (ng/ml)		Binding of FD to plasma protein (%)
	Plasma	Plasma water	
Normal subject	830.5	20.1	97.6
Liver cirrhosis	351.4	29.4	91 6
Renal disease	5702.0	391.0	93.1

In studies of the disposition of FD in patients with liver cirrhosis, the measurement of FD concentration in ascites fluid was particularly interesting. We applied this technique to ascites fluid from a patient with liver cirrhosis. The concentration of FD in the ascites fluid was 84.6 ng/ml and that in plasma collected simultaneously was 351.4 ng/ml.

The measurement of FD in plasma water at usual therapeutic plasma levels was required [16] since previous studies [17—21] for the binding of FD to plasma protein have been carried out by adding FD to plasma in vitro. Therefore, we applied this technique to plasma water from a normal subject, a

patient with liver cirrhosis and another patient with renal disease after the administration of FD; the results are shown in Table IV. The percentage of FD bound to plasma protein in the normal subject was higher than in the patients.

In conclusion, a sensitive, specific and precise method for the determination of FD in human biological fluids by HPLC was established. The method would be particularly useful for pharmacokinetic study following low doses of FD in retard capsule in normal subjects, as well as in patients with a variety of diseases. Such applications will be reported elsewhere.

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