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A SIMPLIFIED ASSAY OF FUROSEMIDE IN PLASMA AND URINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A simplified high-pressure liquid chromatographic method for determination of furosemide in plasma and urine has been developed using a fluorometric detector directly coupled to the column effluent. The method includes an ether extraction from acidified biologic samples. The mobile phase used for chromatography on a reversed-phase column (C_{18} hydrocarbon permanently bonded to silica particles) is sufficiently acidic to induce fluorescence of furosemide. The methylester of furosemide is employed as an internal standard. The sensitivity is 0.1 and 0.25 μg per ml plasma and urine, respectively. The applicability to pharmacokinetic studies of furosemide is shown.

INTRODUCTION

Furosemide is an extremely potent diuretic agent, the major use of which is in acute or chronic renal failure, congestive heart failure and liver cirrhosis [1, 2]. The earliest methods for analysis of furosemide were based on spectrophotometry [3] or spectrophotofluorometry [4] following extraction of serum and urine with organic solvents. These methods suffered from interference by the hydrolytic product, 4-chloro-5-sulfamoylanthranilic acid (CSA).

In order to improve the specificity, high-pressure liquid chromatographic (HPLC) methods have subsequently been developed [5—7] one of which employs fluorometry for quantification of the column effluent after acidification of individual fractions [7].

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The present paper describes an HPLC method utilizing a column packed with C_{18} hydrocarbon phase permanently bonded to very small silica particles (μBondapak). This type of chromatography obviates monitoring of salt concentrations and pH which is required when ion-exchange columns are used [5, 7]. Our method does not require acidification of individual fractions collected from the column effluent because the mobile phase is sufficiently acidic to render furosemide fluorescent. This allows continuous monitoring of the column effluent by fluorometry. Furthermore, the use of an internal standard improves accuracy of quantification.

MATERIALS AND METHODS

All analyses were performed utilizing a Waters Assoc. high-performance liquid chromatograph. A Model M6000 solvent delivery system, a Model U6K injector and a C_{18} μBondapak column (300 mm × 3.9 mm I.D.; particle size 10 μm) were used to perform the chromatographic separation. Furosemide and the internal standard, the methyl ester of furosemide, were measured with a fluorometer (Fluoro-Monitor, American Instr. Co.) employing a 70-μl Suprasil quartz flow cell, a Corning No.7-60 primary filter, a Kodak No.2A secondary filter and a General Electric No.F474•BL lamp. The mobile phase, methanol—water—glacial acetic acid (34:46:3), was delivered at a rate of 2.0 ml/min. The system was operated at room temperature.

Blood samples were collected in heparinized glass tubes and centrifuged to separate the plasma. Urine was collected in glass bottles without preservative. All samples were stored in the dark at −20°.

To 1 ml of plasma or urine were added 10—20 μg of the internal standard (IS) (4-chloro-N-furfuryl-5-sulfamoyl-anthranilic acid methyl ester, the methyl ester of furosemide), 0.1 ml of 5 *N* HCl, 0.5 ml distilled water and 10 ml of diethyl ether. The samples were shaken in 13-ml glass tubes with Teflon®-lined screw caps for 5 min and centrifuged for 5 min at approximately 800 *g*. 7—8 ml of the organic phase were transferred to a clean glass tube and the solvent evaporated to dryness with a gentle stream of dry nitrogen. The residue was dissolved in 0.5 ml of distilled water of which 40—100 μl were injected onto the column.

The compounds were quantified by comparing peak height ratios of furosemide and the internal standard from plasma or urine to peak height ratios of known concentrations of furosemide and the internal standard added to drug free of plasma or urine.

The internal standard was prepared by methylation of furosemide with ethereal diazomethane [8]. Diethyl ether (1 ml) saturated with diazomethane was added to approximately 5 mg of furosemide dissolved in 1.0 ml of methanol. This mixture was left at room temperature for 15 min. The solvent was evaporated with the aid of a gentle stream of dry nitrogen at room temperature. The residue was dissolved in 1.0 ml of methanol and the internal standard was isolated from the unreacted furosemide and byproducts by chromatography as described above. Identity of the methyl ester of furosemide was confirmed by mass spectrometry.

In urine samples of subjects treated with furosemide we discovered a peak

in addition to furosemide, the internal standard and CSA. Because the glucuronide had previously been described as a metabolite of furosemide [9], we incubated urine with a glucuronidase containing preparation (Gluculase®; Endo Lab., Garden City, N.Y., U.S.A.) at pH 5.5 and 37° for 24 h.

Furosemide was obtained from Hoechst-Roussel (Somerville, N.J., U.S.A.) and CSA from the U.S.P.C. (Rockville, Md., U.S.A.). Glass-distilled methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All other chemicals were ACS reagent grade obtained from commercial suppliers.

RESULTS AND DISCUSSION

Figs. 1A and 2A show chromatograms of blank plasma and urine, respectively, and demonstrate the lack of interfering compounds. Figs. 1B and 2B show the same biological samples spiked with the internal standard and with furosemide at concentrations of 0.25 and 0.5 μg furosemide per ml, respectively, demonstrating that these levels can be readily distinguished from the background.

The retention times of pure internal standard, furosemide and CSA were 12, 6 and 1.7 min, respectively. The retention times of these compounds were not altered when they were extracted from plasma. Thus, CSA will not interfere with furosemide analysis. Standard curves plotting peak height ratio of internal standard to furosemide on the y-axis and concentration of furosemide

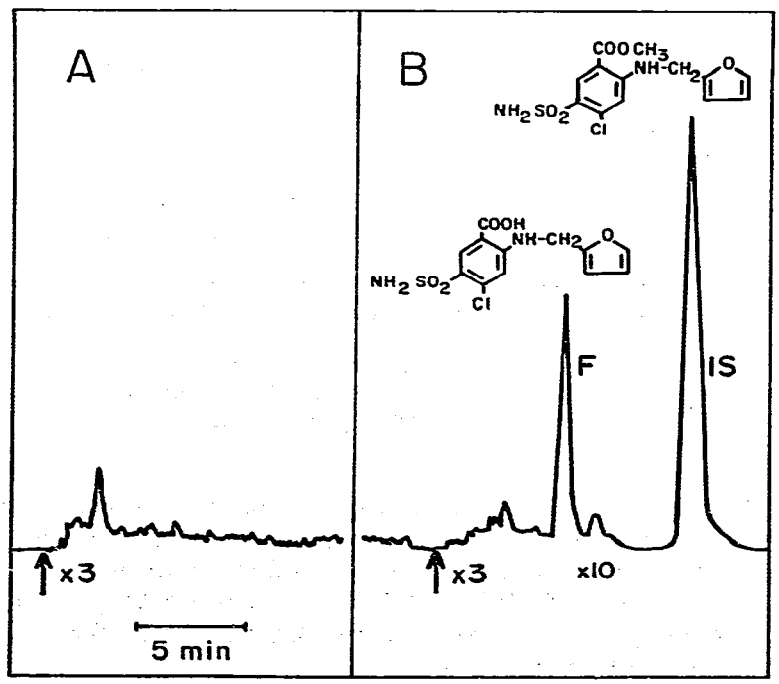

Fig. 1. Chromatograms of blank plasma (A) and the same plasma spiked (B) with furosemide (F) at a concentration of 0.25 μg/ml and the internal standard (IS). The arrows indicate time of injection. The sensitivity of the fluorometer is indicated by ×3 and ×10, ×3 being the more sensitive.

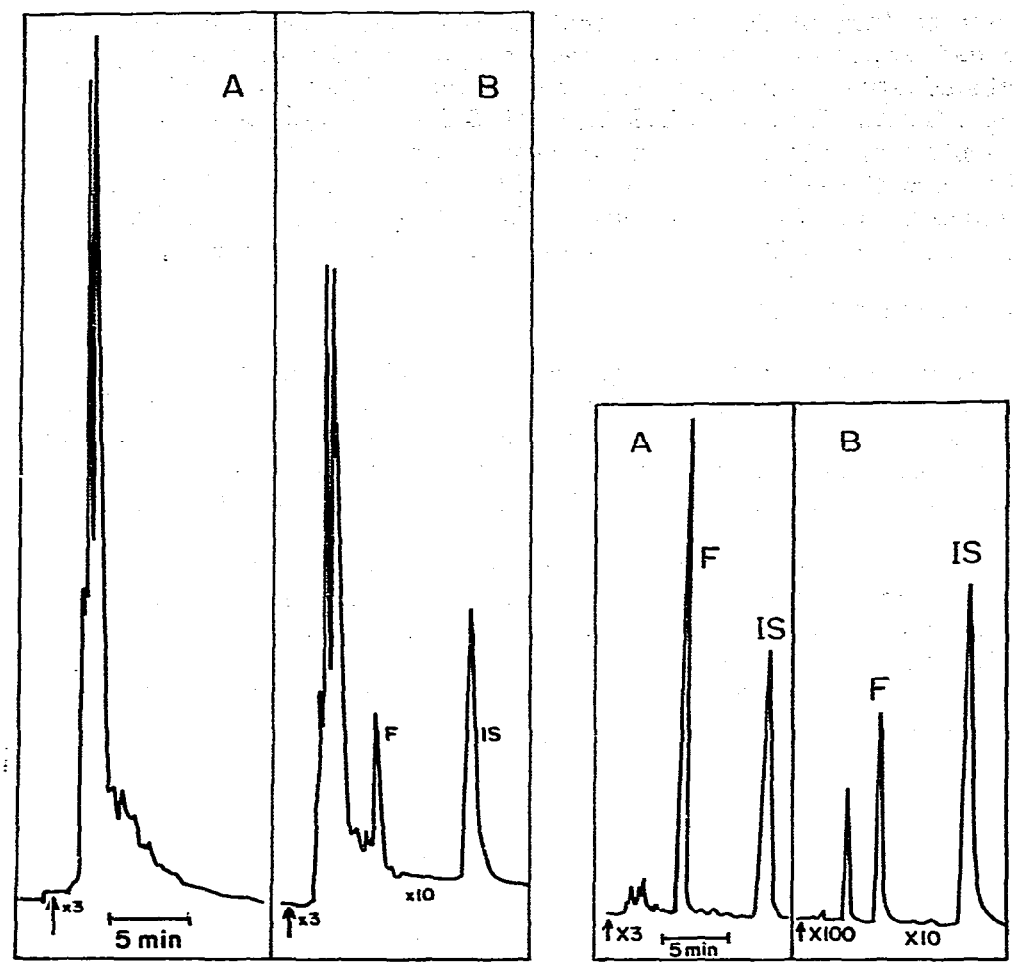

Fig. 2. Chromatograms of blank urine (A) and the same urine spiked (B) with furosemide (F) at a concentration of 0.5 μg/ml and the internal standard (IS). Symbols as in Fig. 1.

Fig. 3. Chromatograms of furosemide extracted from plasma (A) and urine (B) from a healthy male volunteer that received an intravenous dose of 80 mg furosemide. Concentrations: plasma, 4.1 μg/ml; urine, 15.2 μg/ml. See text for further explanation.

in samples of urine and plasma spiked with furosemide on the x-axis were linear, went through the origin and had a slope one. Identical curves were obtained for urine. The coefficient of variation for the analysis in urine ($n = 5$) was 5.3, 4.2 and 2.5% at levels of 0.5, 2.5 and 5.0 μg/ml, respectively. The coefficient of variation for analysis of furosemide in plasma was 9, 5.6 and 0.72% at levels of 0.2, 2 and 5 μg/ml, respectively.

Representative chromatograms of plasma and urine of a subject who had received 80 mg of furosemide intravenously are shown in Figs. 3A and B. No major peak other than furosemide and the internal standard could be observed in plasma. In urine an additional peak, clearly separated from furosemide and

CSA, with a retention time of approximately 3.5 min was observed. On incubation of this urine with glucuronidase this peak decreased while the furosemide peak increased (Figs. 4A and B). Thus, this peak may represent a conjugate, possibly the glucuronide of furosemide which has been suggested by Beerman et al. [9].

The applicability of this method to kinetic studies of furosemide is shown in Fig. 5 which depicts the plasma concentration profile of furosemide in a healthy adult volunteer after an intravenous dose of 80 mg furosemide.

The assay was checked for interference by drugs frequently used in patients who are treated with furosemide. For this, blank plasma was spiked with various drugs at concentrations indicated in Table I. Salicylic acid was the only drug tested that gave any interference. Its retention time was somewhat shorter than that of furosemide and hence it can be identified in this method. The level of salicylic acid added was about 20-fold higher than that found in the plasma of patients treated with high doses of aspirin [10].

Plasma of patients treated chronically with the drugs listed in Table II was analyzed before and after spiking with furosemide and the IS. There were no interfering peaks at the expected retention time of either furosemide or the internal standard before spiking and the recovery of the added furosemide was

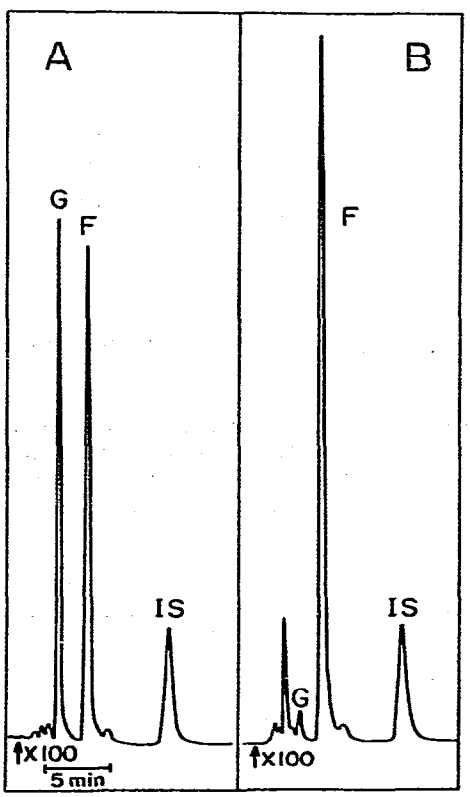

Fig. 4. Chromatograms of urine before (A) and after (B) incubation with Gluculase. IS= internal standard; F=furosemide; G=urinary metabolite of furosemide.

TABLE I

DRUGS ADDED IN VITRO TO PLASMAS CONTAINING FUROSEMIDE AND THE INTERNAL STANDARD AND TESTED FOR INTERFERENCE IN THE ANALYSIS OF FUROSEMIDE

—Indicates lack of interference.

Drug	Concentration (μg/ml)	Interference
Phenobarbital	50	—
Carbamazepine	50	—
Carbamazepine--10, 11-epoxide	12	—
Salicylic acid	1100	+
Guanethidine	0.08	—
α-Methyldopa	10	—
Hydralazine	40	—
Propranolol	0.5	—

quantitative as shown in Table II. Thus, neither the drugs nor their metabolites interfere at therapeutic dosage levels.

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TABLE II

MEASURED PLASMA CONCENTRATION OF FUROSEMIDE AFTER SPIKING WITH 0.5 μg/ml OF FUROSEMIDE

The plasma was obtained from patients treated chronically with therapeutic doses of the drugs listed below. Blanks of each sample were run prior to spiking; no interfering peaks were found in the blanks at the retention times of furosemide or the internal standard.

Drug	Furosemide recovered (μg/ml)
α-Methyldopa	0.52
Guanethidine	0.48
Hydralazine	0.46
Phenobarbital	0.48
Aspirin	0.52
Propranolol	0.46
Carbamazepine	0.46
Carbamazepine-10,11-epoxide	0.46

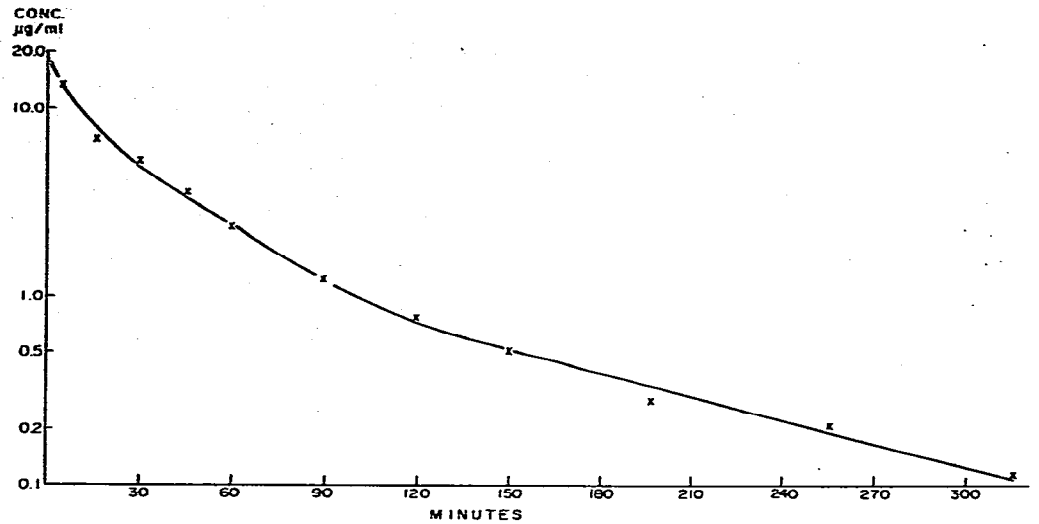

Fig. 5. Plasma concentration profile of furosemide (F) in a healthy adult male volunteer after an intravenous dose of 80 mg furosemide given at time 0.

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