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IMPROVED METHOD FOR THE ANALYSIS OF FUROSEMIDE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Modifications of existing rapid high-performance liquid chromatographic procedures for the determination of furosemide in plasma were made in order to achieve greater sensitivity. To a small volume of plasma was added an internal standard structurally related to furosemide. Then, following previously described procedures, acetonitrile was added to precipitate the proteins and the clear supernatant was separated. However prior to injection of the supernatant the pH and composition of the sample were adjusted. This modification of the sample enabled an injection volume of up to 300 µl of the supernatant to be injected onto the chromatographic column. The effluent was monitored spectrofluorimetrically. A standard linear calibration curve with a mean precision of ± 4.4% was obtained for plasma samples containing 20—900 ng/ml of furosemide. Two structurally related compounds were used as internal standards in the furosemide assay.

INTRODUCTION

Furosemide is one of the most commonly used orally effective diuretics. A number of high-performance liquid chromatography (HPLC) methods [1—7] have been published for the analysis of furosemide in plasma. Two of the most recent HPLC procedures [6, 7] offer increased rapidity and simplicity over previously available methods. Although these characteristics are highly desirable, each has limitations. Lin et al. [6] proposed two HPLC methods with UV detection. Method I had the disadvantages of using an unstable internal standard, sodium cephalothin, and of accepting the potential of column pressure buildup due to injection of sample supernatant obtained from the incomplete methanolic precipitation of plasma proteins. Method II involves a more time-consuming evaporation step, requires a dual-wavelength detector, and utilizes a structurally unrelated internal standard, sodium pheno-

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barbital. In the procedure described by Nation et al. [7], no internal standard was used and a sensitivity limit of only 100 ng/ml was established. The method described here which is based primarily on those of Nation et al. [7] and Lin et al. [6] is simple, rapid, specific and sensitive for the analysis of furosemide in plasma specimens.

EXPERIMENTAL

Reagents and materials

Furosemide (I) and 2-amino-4-chloro-5-sulfamoylanthranilic acid (IV) were purchased from the United States Pharmacopeia (Rockville, MD, U.S.A.). The two internal standards that were used in this study, N-benzyl-4-chloro-5-sulfalmoylanthranilic acid (II) and 3-butylamino-4-phenoxy-5-sulfamoylbenzoic acid (III), were obtained as gifts (Fig. 1). All other chemicals used

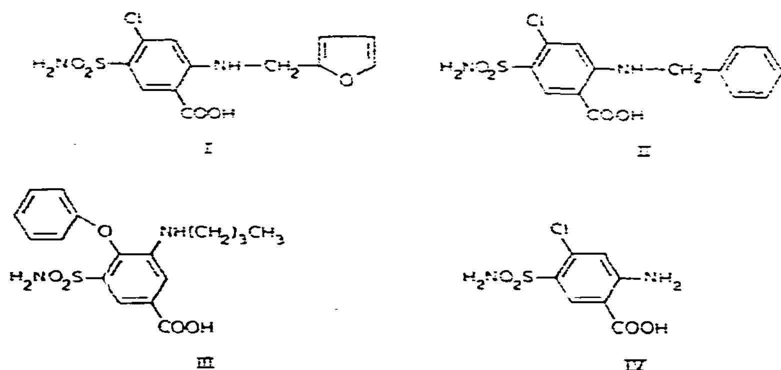

Fig. 1. Chemical structures of compounds used. Compound I is furosemide, 4-chloro-N-furfuryl-5-sulfamoylanthranilic acid; II, an internal standard, is a phenyl analogue of furosemide, N-benzyl-4-chloro-5-sulfamoylanthranilic acid; III, an alternative internal standard, is 3-butylamino-4-phenoxy-5-sulfamoylbenzoic acid; IV is 2-amino-4-chloro-5-sulfamoylbenzoic acid.

were of reagent grade. Acetonitrile was of HPLC grade and was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Lasix (furosemide) injection was obtained from Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.).

Instrumentation

The modular high-performance liquid chromatograph consisted of a Waters Assoc. Model 6000A constant flow pump, a Waters Assoc U6K injector, a Schoeffel Instrument Model FS 970 fluorescence detector and a Varian Model 9176 strip chart recorder. The column was a μBondapak C_{18} prepacked stainless-steel column (30 cm × 4.6 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.). A Waters Assoc. in-line precolumn filter and a Brownlee Labs. (Santa Clara, CA, U.S.A.) precolumn (3 cm × 3.9 mm I.D.) packed with a C_{18} 10-μm reversed-phase material were used to remove interfering materials from the sample prior to passage onto the analytical column. A Waters Assoc. Model 440 UV detector was also used in the study of the degradation product of furosemide.

Chromatographic conditions

The mobile phase for the analysis of furosemide consisted of acetonitrile—0.08 *M* aqueous phosphoric acid (37.5 : 62.5) and the flow-rate was 2 ml/min. The mobile phase used in the analysis of IV consisted of acetonitrile—0.08 *M* aqueous phosphoric acid (17 : 83). The flow-rate was 2 ml/min.

Analysis of plasma samples

To 0.2 ml of plasma in a disposable test tube, 15—30 μl acetonitrile containing the internal standard, either II (2.06 ng/μl) or III (1.0 ng/μl) were added and the mixture was mixed for 10 sec. An additional 0.4 ml of acetonitrile was added to precipitate the proteins and the contents were again mixed for 10 sec. The clear supernatant was separated from the precipitate by centrifugation for 10 min at 1085 *g*. To the supernatant were added 100 μl of 0.08 *M* aqueous phosphoric acid and the contents were mixed for 10 sec. From this solution, an aliquot of up to 300 μl was injected onto the HPLC column. The mobile phase consisted of acetonitrile—0.08 *M* phosphoric acid (37.5 : 62.5) and the eluate was monitored with a fluorescence detector. In the analysis of furosemide, the wavelength of excitation was 233 nm and the emission was monitored with a 389-nm cutoff filter. For the analysis of IV, the mobile phase was changed as indicated above and a UV detector (254 nm) was substituted for the fluorescence detector.

Precision

Plasma samples were spiked with known concentrations of furosemide and the samples were analyzed. At each concentration, a set of three or four samples was analyzed. Precision of analysis was determined using the two internal standards in two separate experiments.

Recovery

Known aliquots from solutions containing different concentrations of furosemide were injected onto the high-performance liquid chromatograph, and peak heights were obtained. A straight-line correlation was obtained for the peak height as a function of concentration. Similarly, peak heights obtained from experimentally processed samples gave a straight-line correlation when plotted against concentrations. The ratio of the slopes was used to estimate the percent recovery.

RESULTS AND DISCUSSION

In modifying the presently available methods, the following factors were considered: the detection system, pH of the mobile phase, the volume of the sample to be injected, and the choice of an internal standard. The first factor examined was the HPLC detection system. The fluorimetric detector was selected since furosemide is highly fluorescent. Blair et al. [3] pointed out that the fluorescence of furosemide is pH dependent. Fluorescent properties of furosemide were investigated in buffers of various pH values. The relative fluorescent intensities determined were 100% at pH 1.6, 97% at 2.5 and 81% at 4.6. At pH 5.6 and 6.6 furosemide was not fluorescent. The second

factor of interest was that of mobile phase pH. Since the fluorescence of furosemide is higher at lower pH and the peak for furosemide was found to be sharper at lower pH, the pH of the mobile phase was adjusted to 2.0. The improved chromatographic characteristics observed in this study at pH 2.0 are in agreement with the observation of Lindström [1]. This is understandable since the carboxylic acid group of furosemide has a pK_a of about 3.6 and approximately a pH of 2.0, the ionization of the carboxylic acid group would be minimal and the compound would primarily exist as the undissociated species.

The third factor that was explored was that of the volume of injection. This factor was of interest as a very efficient means to increase the sensitivity of the assay. It was found using the method of Nation et al. [7] that as the volume of injection increased, there was a profound effect on the chromatographic peak characteristics. Band broadening occurred as expected but with injection volumes greater than 50 µl splitting of the peaks also was observed (Fig. 2a). Since a rapid method with a sensitivity limit of 20 ng/ml was desired,

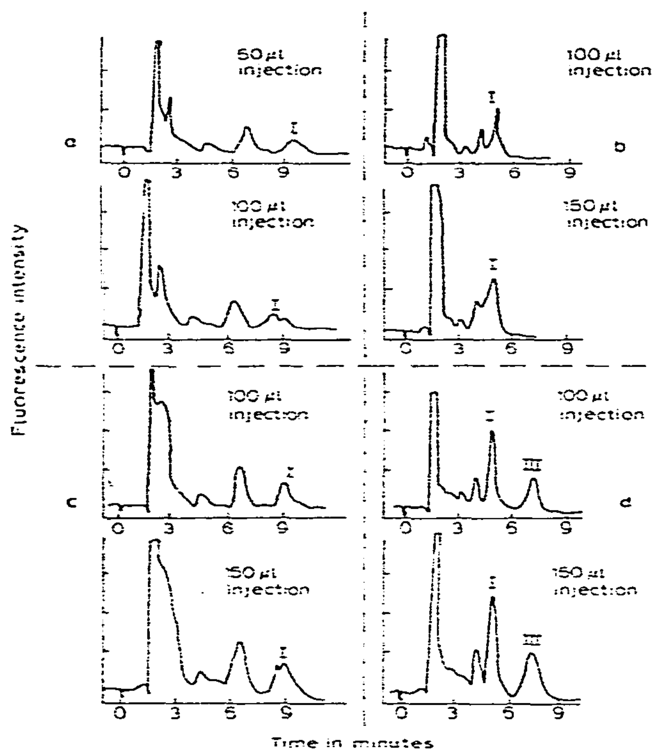

Fig. 2. HPLC traces showing effects of (1) increased concentration of phosphoric acid in mobile phase and (2) addition of phosphoric acid to plasma samples on the maximum volume of injection without loss of acceptable chromatographic characteristics. Chart speed 2 cm/min; fluorescence sensitivity range 0.2 µA. (a) Mobile phase and assay procedure of Nation et al. [7]; volumes of injection 50 and 100 µl. (b) Mobile phase modifications as suggested in the present paper and assay procedure of Nation et al. [7]; volumes of injection 100 and 150 µl. (c) Mobile phase of Nation et al. [7] and assay procedure as suggested in the present paper; volumes of injection 100 and 150 µl. (d) Mobile phase and assay procedure as suggested in the present paper; volumes of injection 100 and 150 µl.

it was necessary to be able to increase the volume of injection. Modifications of the composition of mobile phase and sample were explored to achieve this goal. At first the molarity of the phosphoric acid in the mobile phase of Nation et al. [7] was increased. Aliquots of the same spiked plasma that had been assayed and reported in Fig. 2a were then re-assayed. Marked improvement in the chromatograms was observed. Volumes up to 100 μl of the sample supernatant could be injected while still maintaining satisfactory peak shape (Fig. 2b). To study the effect of sample composition on the chromatograms, the mobile phase of Nation et al. [7] was again used. This time, however, phosphoric acid was added to the plasma supernatant prior to injection. Again aliquots of the same plasma as studied in Fig. 2a and b were used. Marked improvement was observed in the chromatograms and the injection volume could also be increased to 100 μl (Fig. 2c). The effect of combining the above two changes on the assay of another aliquot of the same plasma is evident in Fig. 2d. In this case at least 150 μl of sample could be injected with excellent chromatographic characteristics. Although the maximum volume of injection does depend on the plasma assayed, at least 150 μl of all plasma supernatants studied to date have been successfully injected. It must be emphasized that since peak width varies with volume of injection, the volume of injection must be held constant for all samples and standards.

The last factor examined was the choice of the internal standard. The use of a proper internal standard is essential for any precise assay. An ideal internal standard should have similar chromatographic detection properties, similar solubility properties and similar ionic properties and a molecular weight close to the compound to be analyzed. Two compounds with similar structure to that of furosemide were utilized as internal standards. Both II and III are highly fluorescent and have reasonable retention times. The availability of two possible internal standards increased the flexibility of the method. Hence, if plasma samples contain other substances, which may interfere with one of the internal standards, the other internal standard may be used.

The assay was evaluated using compound II as the internal standard and demonstrated good precision (average C.V. of 4.5%) (Table I). The standard curve was linear (r = 0.997) over a range of 20—900 ng/ml. HPLC traces of processed blank plasma sample and a sample containing 35 ng/ml of furosemide are presented in Fig. 3b. Similarly using compound III as the internal standard, furosemide concentrations as low as 20 ng/ml in plasma could be determined. The method is precise (average C.V. of 3.2%) and linear (r = 0.998). HPLC traces of processed blank plasma sample and a sample containing 50 ng/ml of furosemide are presented in Fig. 3a.

Although the addition of a small amount of 0.08 M aqueous phosphoric acid to the processed samples prior to injection simplified the procedure, the stability of furosemide had not been previously established under such acidic conditions (pH = 2). This is essential since recently Smith et al. [8] demonstrated by HPLC that furosemide is partially degraded to IV under the strongly acidic experimental conditions that were used by Perez et al. [9]. In order to investigate whether decomposition of furosemide occurred under the experimental conditions of this method, a number of plasma samples spiked with different concentrations of furosemide were processed as de-

TABLE I

ASSAY VALIDATION

Internal standard	Theoretical concentration (ng/ml)	Experimental concentration (ng/ml)		Coefficient of variation (%)
		Mean	Range	
Compound II	25	26	25— 28	6.4
(n = 4)	45	44	42— 45	3.3
	90	88	85— 92	3.8
	150	151	143—161	5.0
	300	296	283—304	3.5
	500	498	479—510	2.7
	700	694	663—739	5.1
	900	908	854—959	6.0
Compound III	20	20	19— 20	1.4
(n = 3)	50	49	47— 51	3.5
	100	92	87— 97	5.7
	150	159	152—166	4.1
	200	201	193—205	3.5
	300	297	295—301	1.2

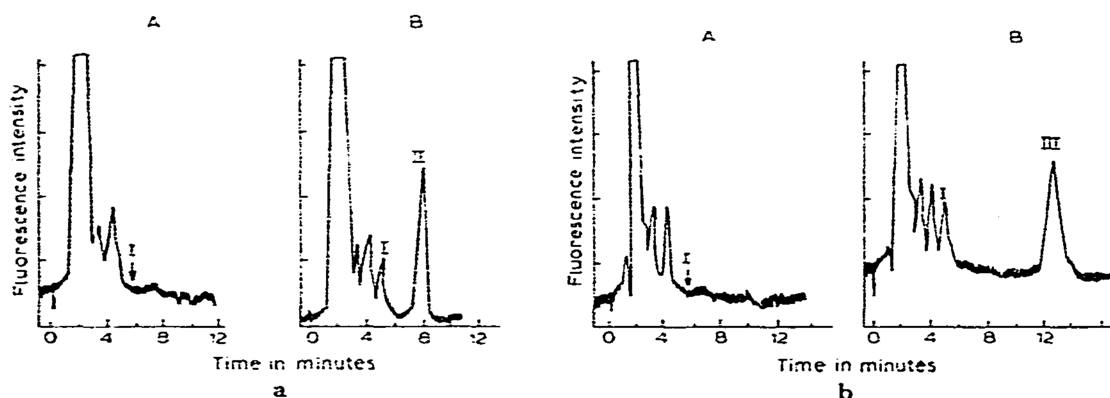

Fig. 3. (a) HPLC traces of (A) blank plasma and (B) of plasma containing 35 ng/ml of I and 150 ng/ml of II. (b) HPLC traces of (A) blank plasma and (B) plasma containing 50 ng/ml of I and 75 ng/ml of III. Chart speed 0.5 cm/min; fluorescence sensitivity range 0.2 μA; injection volume 300 μl.

scribed under Experimental, and the samples were left up to 4 h or more under the mild acidic conditions of this procedure. Analysis of these samples for the presence of IV did not show the presence of this degradation product.

To demonstrate the usefulness of this method, results from the analysis of plasma samples obtained from a healthy volunteer who had ingested a 40-mg solution of furosemide are shown in Fig. 4. The method is being used in pharmacokinetic studies and the results will be reported later.

In summary, modifications have been made to existing methods that have resulted in a more sensitive assay procedure while maintaining the desired rapidity.

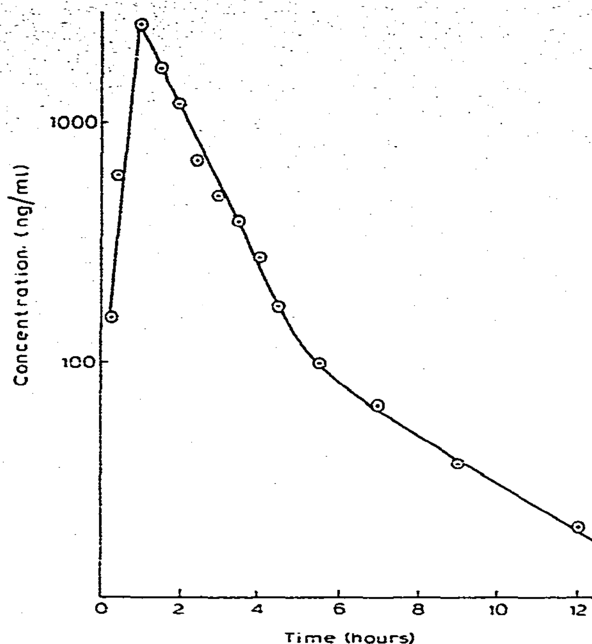

Fig. 4. Plasma concentration of furosemide in a healthy volunteer following ingestion of a 40-mg solution.

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