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THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF FUROSEMIDE AND 4-CHLORO-5-SULFAMOYL ANTHRANILIC ACID IN PLASMA AND URINE

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SUMMARY

A method is described for the assay of furosemide based on thin-layer chromatography and measurement of fluorescence directly on the plates. Conditions are specified for stabilizing fluorescence over the time of measurement. As little as 10 ng can be accurately measured and fluorescence is linear up to 160 ng. The metabolite or decomposition product 4-chloro-5-sulfamoyl anthranilic acid is well separated and measured quantitatively in the procedure. Application of the method to human plasma and urine is demonstrated.

INTRODUCTION

Furosemide is a widely used diuretic which is effective by oral or parenteral administration. It may now be marketed by any registered pharmaceutical manufacturer who obtains approval of the Food and Drug Administration. One of the requirements for approval is proof of bioavailability of the finished product, which requires measurement of plasma or urine concentrations in normal human subjects following a single low dose of the drug. Thus, there is a need for measurement of low levels of furosemide in plasma and urine. In addition, since it is primarily removed from the body by renal excretion [1], there is a need to assess plasma levels in patients where renal function is impaired. It is important that an assay of furosemide involves separation of 4-chloro-5-sulfamoyl anthranilic acid (CSA), since this has been identified in samples from patients given furosemide, and has been thought to be both a product of metabolism and of in vitro hydrolysis [2].

Furosemide has been measured in biological fluids by spectrofluorometry [3—5], gas chromatography [6], liquid chromatography [7—12] and thin-layer chromatography (TLC) [13—16]. Several of these methods require

lengthy extraction procedures or large sample volumes because of the low concentrations found in plasma [3—6, 8, 10]. Other methods involve derivatization of the drug in the extract prior to measurement [6], thus extending the complexity of the assay. Some of the procedures do not separate the drug from the metabolite or decomposition product and may yield misleading results [3—5, 9]. One of the TLC methods [13] uses radioactive furosemide and the bands, corresponding to furosemide and CSA, are scraped from the plates for counting. Similar scraping of the plates is used for a spectrofluorometric method on thin layers [14]. A direct densitometric assay measures the color of the spots on the plates after reacting the drug with Ehrlich reagent [15]. The most recent TLC method [16] measures fluorescence of the drug and CSA with a spectrodensitometer directly on the plates. Applied to plasma containing furosemide and CSA, it deproteinates small volumes with methanol and brings them onto the chromatoplates. The failure to separate the drug and the metabolite from plasma proteins constitutes a troublesome weakness.

The present paper describes a method for extracting furosemide quantitatively, separating the furosemide and CSA by TLC, stabilizing the fluorescence on the plates and measuring the fluorescence directly by densitometry. The method was evaluated with spiked plasma and urine samples and by application to samples from volunteers and patients given furosemide.

EXPERIMENTAL

Materials and reagents

The TLC plates used were 20 × 20 cm Silica Gel 60 (E. Merck, Darmstadt, G.F.R.). Hamilton microliter syringes were used for spotting.

All solvents were A.C.S. spectranalyzed grade and the diethyl ether was distilled in glass prior to use. Phosphate buffer, 0.2 M (pH 2.2) was prepared from 0.2 M phosphoric acid and potassium dihydrogen phosphate.

The mobile phase consisted of chloroform—methanol—glacial acetic acid (89:6:5). The moisturizing solution for the plates contained 45 ml propyleneglycol, 130 ml water and 25 ml glacial acetic acid.

Samples of furosemide powder and 40-mg tablets were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.); CSA reference standard was purchased from U.S. Pharmacopeial Convention (Rockville, MD, U.S.A.).

Stock solutions of furosemide and CSA were prepared to contain 100 μg/ml in methanol. These were diluted with water to spike plasma and urine and diluted with methanol to prepare standard solutions for the plates. Aqueous solutions were prepared fresh daily and methanol solutions were stored in the refrigerator.

Apparatus

TLC plates were divided into twenty 1-cm channels with a scoring device (SDA 320, Schoeffel Instrument). Samples of extracts and standards were applied simultaneously to the channels by means of an automatic spotter (Multi-Spotter, Analytical Instrument Specialities).

A scanning spectrodensitometer with fluorescent attachment was used for measurement of fluorescence on the plates (Model SD 300-1 UV-VIS Spectro-

densitometer, Schoeffel Instrument). This was attached to an integrator (M-2 Calculating Integrator, Perkin-Elmer) and a recorder (Honeywell OPTS 102 110). The system was used in the fluorescence mode with irradiation at 275 nm and an ultraviolet exclusion filter in the emission detector.

Procedure

To 0.1—0.5 ml of plasma or 0.05—0.25 ml of urine in a 15-ml screw-cap centrifuge tube with PTFE cap liner, are added 0.2 ml of phosphate buffer and 2 ml of diethyl ether, and the tube is rotated in a suitable mechanical rotator for 10 min. The tube is removed and centrifuged for 5 min to separate the phases. The ether layer is transfered to a conical 15-ml glass tube, the aqueous layer is extracted a second time with 2 ml of diethyl ether and the combined extracts are evaporated to dryness at 35°C under a stream of nitrogen. The residue is dissolved in 100 μl methanol and one or more 20-μl portions are spotted on the TLC plate. Along with each set of samples, at least two methanol standards are applied to the same plate. The plates are protected from light and developed in a glass tank with a solvent-saturated atmosphere. In this sytem the R_F value is 0.29—0.30 for furosemide and 0.13—0.15 for CSA. The solvent front is allowed to move about 15 cm, the plate is removed and the solvent allowed to evaporate. The plate is then dipped in moisturizing solution, contained in a suitable dip tray, placed in an oven at 35°C for 10 min, removed and allowed to stand at room temperature for 30 min. The plate is then scanned to record the fluorescence and integrate peak areas, making sure that fluorescence has stabilized before proceeding by reading the first channel at 2-min intervals until peaks are constant.

Amounts of furosemide and CSA are calculated by comparison of peak areas of samples to those of known standards on the same plate.

Linearity of response, recovery and application

To determine the range of linearity of response, standard solutions of furosemide and CSA were spotted on the plates, developed and measured.

Recovery was determined by assaying samples of plasma and urine spiked with varying concentrations of furosemide and CSA, then comparing the areas obtained with standard solutions spotted directly on the plate.

This method was applied to samples of plasma and urine of patients and volunteers and was found to be sensitive enough for measurement following a single 40-mg dose of furosemide.

RESULTS AND DISCUSSION

The solvent mixture used for the development of the TLC plates yielded clear separation of furosemide and CSA, as is demonstrated in Fig. 1. Standard curves of furosemide were linear in the range of 0—160 ng, but non-linear above 160 ng. For CSA the fluorescence—concentration curve was somewhat lower than that of the parent compound and was linear up to 200 ng per spot. These curves are shown in Fig. 2.

The presence of acid in the development fluid greatly enhanced the fluorescence of furosemide on the plates. This is demonstrated in Fig. 3, where
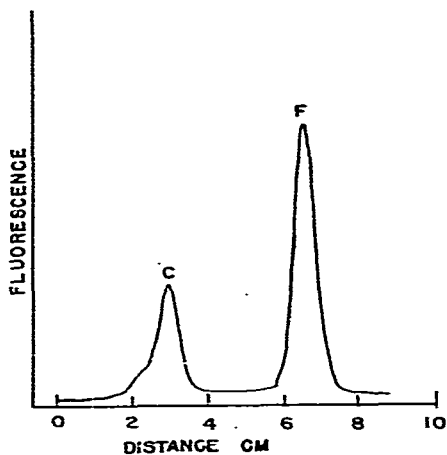
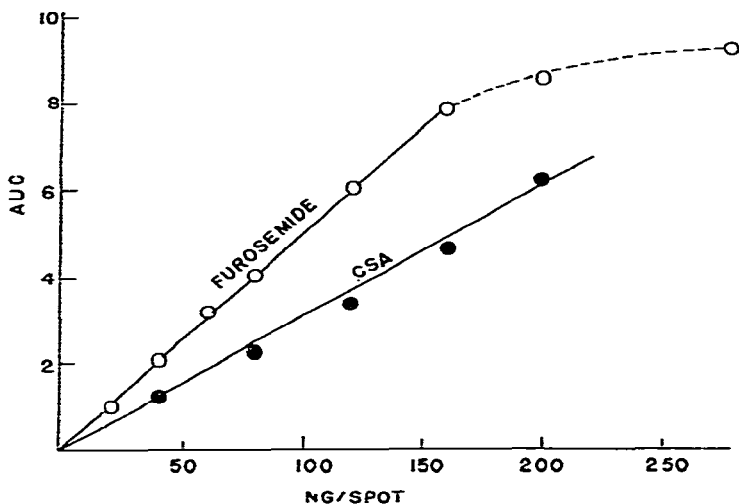Fig. 1. Separation of furosemide (F) and CSA (C) isolated from spiked human plasma.

Fig. 2. Relationship of area under the curve (AUC) to concentration of furosemide and CSA.

peak areas of standards are shown following development in an acidic fluid (chloroform—methanol—glacial acetic acid, 89:6:5) and an alkaline fluid (sec.-propanol—butyl acetate—water—ammonium hydroxide, 50:30:15:5) [13].

Early in the development of the assay it was recognized that fluorescence of furosemide on the plates decreased in intensity with time, due to loss of moisture and that the decrease was more pronounced with alkaline fluids than with acidic ones. To minimize the fading of fluorescence, a moisturizing solution was selected which maintained moisture and acidity on the plates and yielded stable readings over the time required to scan all the channels on a given plate. This is illustrated in Fig. 4.

Recoveries of furosemide and CSA are shown in Table I. For furosemide it is 100.4 ± 3.2% from plasma and 101.6 ± 4.7% from urine; for CSA it averaged 97.3 ± 5.2% from plasma and 102.0 ± 1.0% from urine. The procedure as written is accurate for samples containing 0.5 μg/ml of furosemide, but much
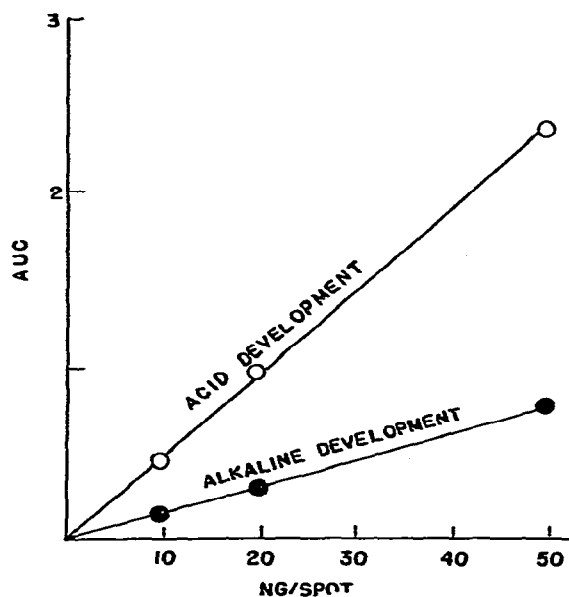
Fig. 3. Relationship of area under the curve (AUC) to concentration of furosemide in acidic (o) and alkaline (•) solvents.
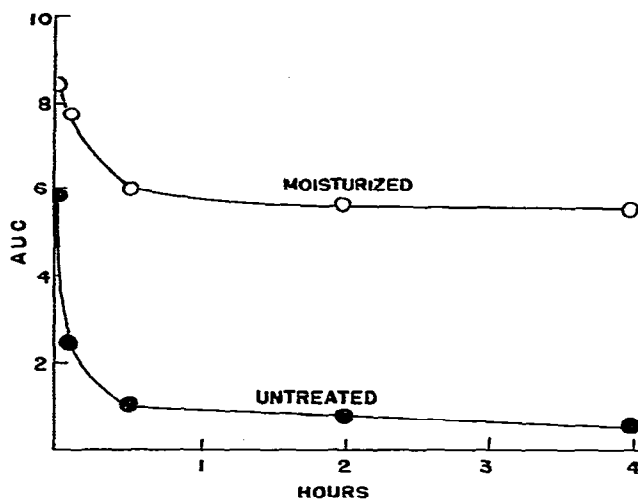

Fig. 4. Relationship of area under the curve (AUC) to concentration of furosemide on moisturized (o) and untreated (•) plates.

lower concentrations can be measured by dissolving the extraction residues in smaller volumes of solvent, since as little as 10 ng on a plate gives sufficient fluorescence. For example, by using 0.5 ml plasma, dissolving the extracted residue in a small volume of methanol and transferring it quantitatively to the plate, a concentration of 20 ng/ml in the sample could be accurately measured.

We have applied our method to plasma samples where the patient was given an intravenous (i.v.) dose of furosemide and plasma samples were taken at intervals up to 3 h. The results are illustrated in Fig. 5. It is seen that the

TABLE I

RECOVERIES OF FUROSEMIDE AND CSA FROM SPIKED HUMAN PLASMA AND URINE

Plasma			Urine		
Added (μg/ml)	Recovered (μg/ml)	Percent recovery ± C.V.	Added (μg/ml)	Recovered (μg/ml)	Percent recovery ± C.V.
*Furosemide**					
0.5	0.48	96.0 ± 2.1	1.0	1.11	111.0 ± 3.4
1.0	1.04	104.0 ± 8.2	2.5	2.48	99.0 ± 2.1
2.5	2.42	97.0 ± 4.0	5.0	4.89	98.0 ± 1.0
5.0	5.21	104.0 ± 0.6	10.0	9.99	100.0 ± 2.1
10.0	10.12	101.0 ± 7.1	20.0	20.02	100.0 ± 0.5
CSA**					
1.0	0.92	92.0 ± 0.2	2.0	2.08	104.0 ± 3.9
2.0	1.81	92.0 ± 1.3	4.0	4.09	102.0 ± 2.4
4.0	4.07	102.0 ± 2.4	8.0	8.14	102.0 ± 1.0
8.0	8.24	103.0 ± 1.8	16.0	15.95	100.0 ± 0.8

*Each value represents four samples.
**Each value represents three samples.

plasma levels drop quite rapidly to a low level at first and then drop much more slowly subsequently. No CSA was detected in these or other patient plasma samples.

Results on urine samples are shown in Fig. 6 where data were plotted as cumulative amounts excreted over nearly 13 h. In these and urine samples from other patients small amounts of CSA were consistently found.

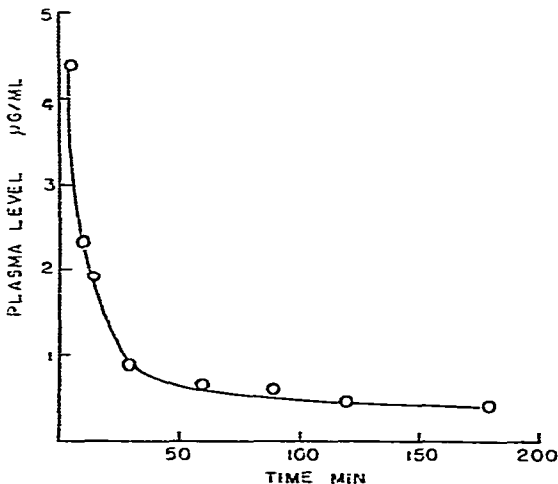It was noted that no CSA has been detected in any plasma sample but it has

Fig. 5. Plasma levels (μg/ml) vs. time (min) of furosemide in a patient on i.v. dose of furosemide.
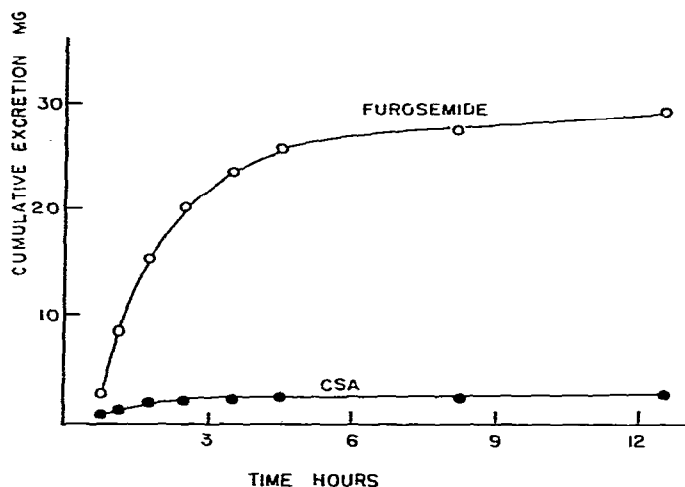
Fig. 6. Cumulative excretion of furosemide (○) and CSA (●) over time in a subject after a 40-mg oral dose of furosemide.

been found in all urine samples. To be certain that CSA was not being formed by chemical reaction in the assay process, urine samples spiked with furosemide were assayed, but no CSA was found. These facts suggest that CSA is a metabolite of furosemide which is filtered from the plasma as rapidly as it is formed and thus is found in urine only.

This method has advantages over previous ones in that it provides for separation and sensitive measurement of both the drug and the metabolite in concentrations that are present in biological fluids for several hours after a small dose. Thus it will afford improved measurement following single-dose administration of the drug as is required in bioavailability studies. Since each TLC plate serves for as many as fifteen samples (and two or three standards) this method is convenient for the assay of large numbers of samples.

This method has advantages over a similar TLC method [16] where preparation of samples for application to TLC plates involves simply addition of methanol to the plasma, centrifugation and filtration of the supernatant. The developing solvent was chloroform—ethyl acetate—formic acid, and the plates were air-dried for at least 3 h, then sprayed with a mixture of ehtyleneglycol and citric acid solution. Tests of this method in our laboratories revealed several problems and we were unable to reproduce the results. It was quickly recognized that substantial amounts of the drug were lost in the methanol protein precipitation procedure and it was suggested that this may be due to plasma protein binding. Further tests with diluted plasma did not show a clear relationship between plasma protein concentration and percentage of drug loss. These tests showed that the recovery of furosemide fluctuated within a wide range of 22.7—79.1% with a mean of 55.6% and standard deviation of ± 12.3, based on 79 samples. Also, the fluorescence was consistently lower with this developing solvent than with the system used in our procedure. Thus, there was still need for an assay which would yield more reproducible results.

ACKNOWLEDGEMENTS

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