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Note

Direct thin-layer densitometric determination of pharmacological concentrations of furosemide in plasma and urine

IB STEINESS*, JOHANNES CHRISTIANSEN and EVA STEINESS

Rigshospitalet and Department of Pharmacology, University of Copenhagen, Copenhagen (Denmark)

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Furosemide is one of the most commonly used diuretics. Therapeutic doses vary enormously, from 20 mg in elderly patients suffering from congestive heart failure to one or more grams in for instance uraemic patients and some patients with the nephrotic syndrome.

The clinical effect is easily registered by the diuretic response and since furosemide is widely non-toxic, monitoring of the plasma concentrations is not used in daily clinical treatment. However, measurements of furosemide concentrations in plasma and urine are necessary in studies of the renal effect in different pathophysiological states.

Several methods are available for estimation of furosemide in biological fluids. Both the original colorimetric [1] and the fluorometric methods [2—4] have the disadvantage that they are non-specific and estimate both furosemide and its pharmacologic inactive metabolite 4-chloro-5-sulfamyolanthranilic acid (CSA). The gas chromatographic method has a high specificity [5] but is very time-consuming and has a very low analytical capacity. A specific direct thin-layer fluorometric method with a very high sensitivity has recently been described but only for plasma measurements [6]. The method described needs 1000 μl of plasma for duplicate measurements.

During the last few years we have used a direct thin-layer densitometric assay based on a colour reaction with Ehrlich's reagent for estimation of furosemide in both plasma and urine. It seemed preferable to use the same method for both plasma and urine in order to minimize errors, because a direct comparison

*To whom correspondence should be addressed, at the following address: Medical Department P 2132, Rigshospitalet, Blegdamsvej, DK-2100 Copenhagen Ø, Denmark.

of the concentrations in plasma and urine is required for determination of the renal clearance of the drug. Since we have been interested in studying renal furosemide clearance in anaemic uremic patients, it was further desirable to reduce the amount of plasma needed.

MATERIALS AND METHODS

Apparatus

A Zeiss spectralphotometer with thin-layer chromatographic TLC-scanning equipment KM 3 (Carl Zeiss, Oberkochen, G.F.R.) linked to a Servogor Sb RE 646 recorder (Goerz Electro, Vienna, Austria) was used. The apparatus was equipped with two photomultipliers for the simultaneous measurement of remission and transmission.

Chemicals

All reagents were of guaranteed reagent grade and were used without further purification. Furosemide and CSA were obtained by courtesy of Hoechst Pharmaceuticals (Frankfurt/M, G.F.R.).

The composition of Ehrlich's reagent: 1 g of *p*-dimethylaminobenzaldehyde dissolved in 50 ml of hydrochloric acid (25%) and 50 ml of ethanol (96%).

TLC plates

Pre-coated silica gel 60 glass plates without fluorescent indicator, 20 × 10 cm, with a layer thickness of 0.25 mm (Merck, Darmstadt, G.F.R.) were used. Before chromatography the plates were washed with acetone for 15 min and dried for 15 min at 80°.

Standard solutions

Furosemide (100 mg) was dissolved in 100 ml of ethanol (99%). This solution was diluted with distilled water. Standards with known amounts of furosemide were prepared from furosemide-free pooled plasma and urine spiked with 20 µl solution per ml plasma or urine. Standard solutions were prepared once a month.

Extraction

Samples were stored deep-frozen until analysis. A 250-µl aliquot of plasma acidified with 50 µl of hydrochloric acid (3 *M*) or 150 µl of urine acidified with 75 µl of phosphate buffer (0.2 *M*, pH 2.0) was extracted with 1.5 ml of chloroform for 5 min using a mechanical shaker. The two phases were separated by centrifugation for 5 min at 3000 *g*. 1.2 µl of the organic phase was transferred to a conical glass tube and evaporated to dryness under a stream of nitrogen at 40°. Residuals were redissolved in 100 µl of methanol and the evaporation to dryness was repeated. The residue was dissolved in 10 or 20 µl of ethylene chloride—methanol (3:1) and 5 µl of this extracted material was spotted under nitrogen onto the TLC plate.

Thin-layer chromatography

TLC was conducted with the exclusion of light in a tank lined with Whatman

No. 2 chromatography paper. The solvent was chloroform—methanol—glacial acetic acid (89:6:2.5). In this solvent furosemide has a R_F value of 0.25—0.29, CSA of 0.11.

Seventeen samples were spotted onto each plate. Ten to seven of the unknown urine or plasma samples were applied onto one plate together with the appropriate reference spots from standard solutions.

Staining

After chromatography the spots of furosemide were coloured in situ by the following procedure: The TLC plates were dipped in Ehrlich's reagent—ethanol (99%)—hydrochloric acid (25%) (20 ml:40 ml:40 ml) for 7 sec and excess of reagent was removed by pressing the wet layer onto filter paper No 617. The TLC plates were then immediately placed in an oven at 50° for 10 min. To ensure uniform heating of the TLC plate it was placed on a 3 mm thick copper plate. After heat treatment the TLC plates were left at room temperature for 15 min before scanning, to ensure full development of the spots, which then remain stable for at least one week.

Measurement and quantitation

The monochromator was set at 480 nm and the remission/transmission ratio used was 100:25. The scanning speed was 120 mm/min. Measurements on the plate can be taken either in the direction of the solvent flow or perpendicular to the solvent flow across the furosemide spots. The latter procedure is much less time-consuming and gives the same results as the former.

The amount of furosemide was calculated by comparison of the peak areas (integrator counts) for samples and standards. The standard curve was fitted with a polynomium of the form $y = ax^b$ where y is the peak area and x is the concentration. The correlation coefficients obtained were 0.99 or better.

RESULTS AND DISCUSSION

Plasma and urine from subjects who had not ingested furosemide showed a variable but always negligible peak in the TLC scan (Fig. 1). As shown in Fig. 2 furosemide is separated from the metabolite CSA. After a single administration of furosemide CSA could not be detected in either plasma or urine. The TLC scan from an uraemic patient given 1000 mg furosemide orally, showed no interfering substances and no CSA in the chromatogram (Fig. 3).

The lower limit for reliable quantification of furosemide in plasma and in urine was 0.1 μg/ml. Reproducibility studies were performed on plasma and urine samples spiked with furosemide and the results are outlined in Table I. The areas of the standards differed from day to day and to calculate the reproducibility the different areas were calculated in per cent of the area of 8 μg/ml (plasma) or 10 μg/ml (urine) of the furosemide standard. The coefficient of variation of day-to-day estimations decreased with increasing furosemide concentrations (Table II). The recovery of furosemide in plasma was 97.0% (S.D. ± 3.4) and in urine 103.7% (S.D. ± 4.5) and for both plasma and urine independent of the furosemide concentration within the range studied (plasma 0.3—7.0 μg/ml, urine 0.3—9.0 μg/ml).

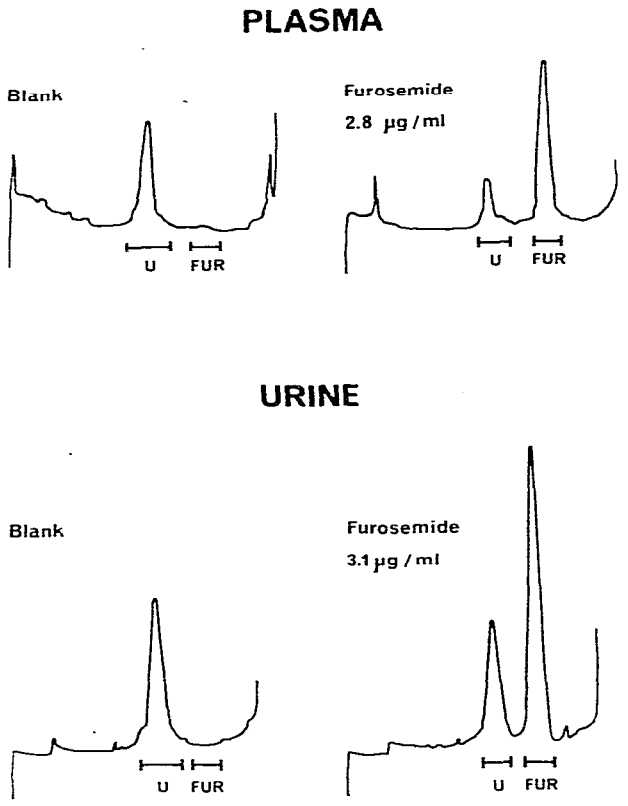

Fig. 1. TLC scans of plasma and urine from patients before and during intraveneous infusion of furosemide. FUR = furosemide, U = unknown substance.

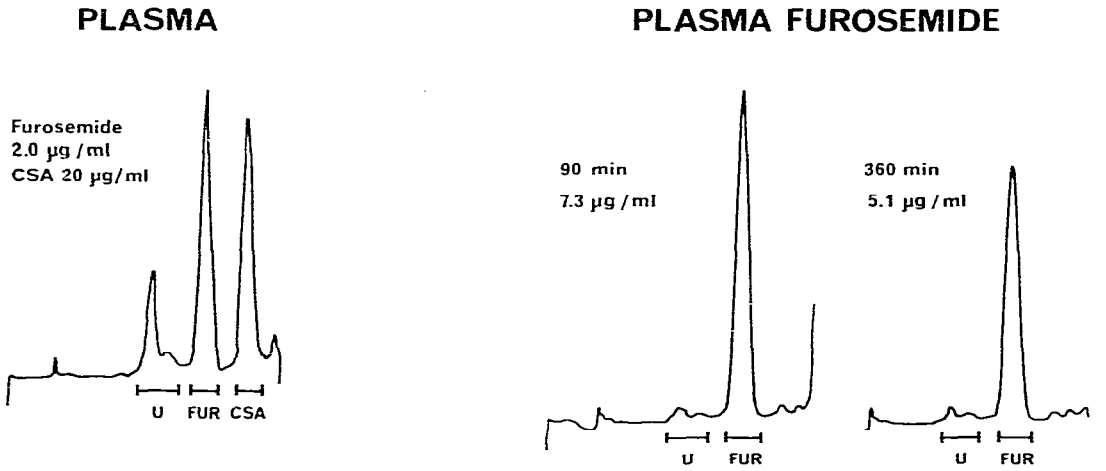

Fig. 2. TLC scan of plasma spiked with furosemide (FUR) and CSA. U = unknown substance.

Fig. 3. TLC scans of plasma from a uraemic patient (creatinine clearance 0.1 ml/sec) 90 and 360 min after oral administration of 1000 mg furosemide (FUR). U = unknown substance.

TABLE I

REPRODUCIBILITY OF THE ANALYSIS

Sample	Amount of furosemide added (μg/ml)	Area $\bar{x}$*	S.D.	S.E.M.
Plasma	0	1.53	0.39	0.11
	0.1	3.32	0.44	0.13
	0.25	5.85	0.49	0.14
	0.5	9.49	0.82	0.24
	1.0	17.70	0.69	0.20
	2.0	32.01	1.56	0.41
	4.0	57.71	2.39	0.69
Urine	0	3.05	0.30	0.09
	0.1	3.89	0.28	0.09
	0.25	5.05	0.25	0.08
	0.5	6.39	0.19	0.06
	1.0	9.65	0.45	0.14
	4.0	27.95	1.21	0.38
	10.0	10.07	2.68	0.85

*n = 12 for plasma; n = 9 for urine.

TABLE II

DAY TO DAY VARIATIONS AT DIFFERENT FUROSEMIDE CONCENTRATIONS

Single determinations.

Sample	Concentration of furosemide (μg/ml)	Coefficient of variation (%)	n
Plasma	0.38	9.1	10
	0.97	6.8	12
	7.20	4.0	10
Urine	0.19	9.0	8
	1.87	6.2	10
	9.26	3.6	7

So far no interfering compounds have been found in samples from patients undergoing multi-drug therapy.

CONCLUSION

A convenient and adequately sensitive direct thin-layer densitometric method has been developed for measurements of furosemide in both plasma and urine. The method is specific which is a necessary requirement for clinical pharmacological studies of furosemide.

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REFERENCES

1 P. Hajdú and A. Häussler, Arzneim.-Forsch., 14 (1964) 709.
2 A. Häussler and P. Hajdú, Arzneim.-Forsch., 14 (1964) 710.
3 A.W. Forrey, B. Kimpel, A.D. Blair and R.E. Cutler, Clin. Chem., 20 (1974) 152.
4 F. Andreassen and P. Jakobsen, Acta Pharmacol. Ther., 35 (1974) 49.
5 B. Lindström and M. Molander, J. Chromatogr., 101 (1974) 219.
6 M. Schäfer, H.E. Geissler and E. Mutschler, J. Chromatogr., 143 (1977) 636.