Journal of Chromatography, 174 (1979) 469–473

CHROM. 11,779

Note

Measurement of furosemide by high-performance liquid chromatography

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(Received February 7th, 1979)

Furosemide is a potent diuretic agent widely used in the treatment of disease states complicated by sodium retention. A variety of different assay methods are available for measuring furosemide[1–6], but most have problems related to specificity or sensitivity, or are too complicated for use in detailed pharmacokinetic studies. Recently, Carr *et al.*[7] described a method for measurement of furosemide which has many advantages over the previous methods. Using high-performance liquid chromatography (HPLC) and an internal standard, they have developed a sensitive and selective method which has improved accuracy and precision. We have concurrently developed an HPLC method using a different internal standard which has five times the sensitivity and significantly reduces the time required for chromatography when compared with the method of Carr *et al.*[7]

EXPERIMENTAL

Standards

Standard solutions of both furosemide and the internal standard, N-benzyl-4-chloro-5-sulfamoylanthranilic acid (both gifts from Hoechst, Frankfurt/M, G.F.R.) are made in pH 10.2 0.1 *M* carbonate–bicarbonate buffer. Standard furosemide concentrations ranged from 0.02 μg to 50 μg per 100 μl buffer. Concentration of the internal standard was 0.5 μg per 100 μl buffer or 10.0 μg per 100 μl buffer, depending on the range of concentrations expected in unknown samples. New solutions of both compounds were made every 2 weeks and stored at 4°.

Extraction

The extraction procedure shown in Fig. 1 is a simple two-step process involving an acid extraction into diethyl ether and back extraction into aqueous sodium hydroxide. A 0.5 μg/100 μl aliquot of internal standard is pipetted into a culture tube (13 × 100 mm) with a PTFE-lined screw cap to which are added 0.1–1.0 ml plasma or urine and 2.0 ml water. Next, the plasma or urine is acidified with 150 μl of 4 *M* HCl. Five ml of diethyl ether are then added to the tube and the

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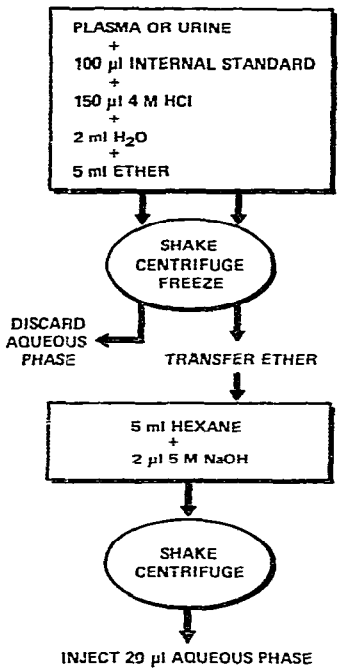

Fig. 1. Flow diagram showing the extraction procedure.

mixture is allowed to shake on a Labquake shaker for 10 min followed by centrifugation at relative centrifugal force of 1100 *g* for 5 min. The tube is immersed in a dry ice–acetone bath and the aqueous phase frozen. The ether phase is then poured into a tube with an elongated cone at its base which contains 2 µl 5 *M* NaOH (measured with a 10-µl Hamilton syringe) and 5 ml *n*-hexane. This mixture is allowed to shake 10 min and centrifuged for 5 min. The entire aqueous phase (now consisting of *ca.* 20 µl) is then sampled from the tube with a 25-µl Hamilton syringe. One µl of 5 *M* acetic acid is also drawn into the syringe, and the total of 21 µl is injected into the chromatograph.

Chromatographic conditions

A Varian dual-pump high pressure liquid chromatograph fitted with a Rehodyne loop injector and a Varian CH-10 reversed-phase column is used for the separation. The solvent mixture is 0.5% acetic acid in acetonitrile–water (30:70). The flow-rate is 60 ml/h which results in a column input pressure of 170 atm.

Detector

A Schoeffel FS970 fluorescence monitor with a quartz cell and a deuterium lamp is used. The excitation monochromator is set at 350 nm and a 389-nm cut-off emission filter is used. The output from the detector is recorded on a Varian Model 9176 recorder.

Calibration

The assay is calibrated by analyzing 1-ml aliquots of control plasma to which

has been added 0.02–5.0 μg of furosemide and 0.5 μg of internal standard or 1.0–50.0 μg of furosemide and 10 μg of internal standard. Control urine samples of 0.1 ml containing 0.5–5.0 μg of furosemide and 0.5 μg internal standard are used for the urine calibration graph.

For each sample, the ratio of furosemide peak height to internal standard peak height is determined. Each ratio is divided by the amount of furosemide in that sample to give a normalized peak-height ratio. The peak-height ratios are plotted against the concentrations to determine the linearity of the extraction and detector response. The normalized peak-height ratios are averaged and the mean value is used to determine the amounts of furosemide in unknown samples. Precision of the assay is estimated by determining the coefficient of variation of the normalized peak-height ratios.

RESULTS AND DISCUSSION

Retention times under the conditions described are 3.0 min for furosemide and 6.5 min for the internal standard. One sample can be chromatographed in 8–9 min. Fig. 2 shows chromatograms of 1.0 ml control plasma (A) and 0.1 ml of control urine (C) as well as 1.0 ml plasma containing 0.2 μg furosemide and 0.5 μg internal standard (B) and 0.1 ml urine containing 0.2 μg furosemide and 0.5 μg internal standard (D). The two compounds are well separated, and there were no significant peaks which interfere with the peaks of interest.

Reproducibility

The reproducibility of the assay was measured by determining the coefficients of variation of five replicate samples of 0.02, 0.05 and 0.50 μg furosemide in 1 ml plasma or 0.1 ml urine. Table I shows coefficient of variation for the calibration graphs and the reproducibility studies. These results indicate that the overall variability of the assay is quite acceptable. The calibration graphs are linear and pass through the origin. The lower limit of sensitivity for the assay is 20 ng extracted from either 1 ml plasma or 0.1 ml urine. This is sufficient sensitivity for use in pharmacokinetic studies of the drug. The reproducibility studies at different concentrations showed low variability for both plasma and urine, even at 20 ng/ml. Reproducibility with varying amounts of plasma or urine was determined by extracting 0.5 μg furosemide from 0.1, 0.2, 0.5 and 1.0 ml of plasma or urine. Reproducibility with different volumes of plasma or urine was essentially the same as the reproducibility with equal volumes and there was no consistent trend in the data.

Efficiency

The efficiency of the extraction procedure was determined by extracting 110 ng (1350 dpm) of generally labelled [^{3}H]furosemide (prepared by New England Nuclear, Boston, Mass., U.S.A., and purified at Syntex Labs., Palo Alto, Calif., U.S.A.) and counting aliquots of each phase in the extraction procedure with a Beckman LS230 liquid scintillation counter. Recovery studies with [^{3}H]furosemide showed that an average of 93% of the drug was extracted into the ether during the first extraction, and 66% of the total was recovered in the aqueous phase after the back extraction.

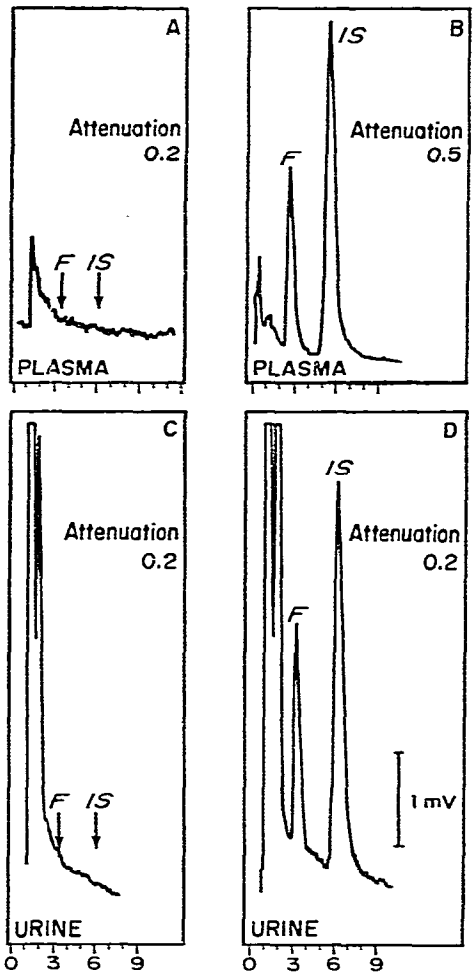

Fig. 2. Chromatograms of plasma and urine extracts. Arrows show where furosemide (arrow farthest to the left) and the internal standard would elute if they were present. (A) 1.0 ml of control plasma; (B) 1.0 ml of plasma containing 0.02 μg of furosemide and 0.5 μg internal standard; (C) 0.1 ml of control urine; (D) 0.1 ml of urine containing 0.2 μg furosemide and 0.5 μg internal standard.

Interference by other drugs

Several blank plasma samples spiked with various drugs were taken through the analysis to see whether or not they caused interference. These drugs, which seemed likely to be given coincidentally with furosemide, were propranolol, digoxin, hydralazine, methyldopa, quinidine, NAPA, prazosin, mixiletene and disopyramide.

Of the drugs which were checked for interference in the assay, prazosin, NAPA and quinidine were found to have similar retention times and to fluoresce under these conditions. However, at normal therapeutic concentrations they are not extracted from plasma using the procedure described above.

TABLE I

SUMMARY OF DATA FOR CALIBRATION GRAPHS AND REPRODUCIBILITY STUDIES

	Furosemide (μg)	*Internal standard (μg)*	*Sample size (ml)*	*n**	*Coefficient of variation (%)*
Plasma					
Calibration graphs	0.02– 5.0	0.5	1.0	7	4.2
	1.0 –50.0	10.0	1.0	5	4.3
Reproducibility studies	0.02	0.5	1.0	5	8.0
	0.05	0.5	1.0	5	7.5
	0.5	0.5	1.0	5	1.3
	0.5**	0.5	0.1–1.0	4	3.8
Urine					
Calibration graphs	0.02– 5.0	0.5	0.1	7	7.4
	1.0 –50.0	10.0	0.1	5	4.4
Reproducibility studies	0.02	0.5	0.1	5	7.7
	0.05	0.5	0.1	5	4.6
	0.5	0.5	0.1	5	0.8
	0.5**	0.5	0.1–1.0	4	3.2

* Number of replicates in each study.
** Reproducibility with varying volumes.

ACKNOWLEDGEMENTS

This study was supported by National Institutes of Health Grant GM22209. Dr. Blaschke is a Burroughs Wellcome Scholar in Clinical Pharmacology, and recipient of a Research Career Development Award (GM00407) from the National Institutes of Health.

We gratefully acknowledge the help of Dr. Howard Parneff of Syntex Laboratories in purifying the labelled furosemide and Linda Halloran for preparation of the manuscript.

REFERENCES

1 F. Andreasen and P. Jakobsen, *Acta Pharm. Toxicol.*, 35 (1974) 49.
2 A. D. Blair, A. W. Forrey, B. T. Meijsèn and R. E. Cutler, *J. Pharm. Sci.*, 64 (1975) 1334.
3 B. Lindström, *J. Chromatogr.*, 100 (1974) 189.
4 B. Lindström and M. Molander, *J. Chromatogr.*, 101 (1974) 219.
5 M. L. MacDougall, D. W. Shoeman and D. L. Azarnoff, *Res. Commun. Chem. Pathol. Pharmacol.*, 10 (1975) 285.
6 E. Mikkelson and F. Andreason, *Acta Pharm. Toxicol.*, 41 (1977) 254.
7 K. Carr, A. Rane and J. C. Frölich, *J. Chromatogr.*, 145 (1978) 421.