Journal of Chromatography, 163 (1979) 315—321
Biomedical Applications

CHROMBIO. 341

Note

High-performance liquid chromatographic assays for furosemide in plasma and urine

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(Received January 23rd, 1979)

Fluorometric [1, 2], gas chromatographic [3], thin-layer chromatographic [4, 5] and high-performance liquid chromatographic (HPLC) [6, 7] assays are available for the determination of the diuretic furosemide in biological fluids. All require prior derivatization and/or extraction. In addition, one HPLC assay [6] uses a mobile phase containing 0.02 *M* chloride which can damage the stainless-steel fittings on the instrument. A third HPLC assay has been described [8]. It has the advantage of not requiring any extraction or derivatization steps. The disadvantages of this method relate to the fact that no internal standard is used and to the necessity of bringing samples into an acid pH range (i.e., 2.5) for fluorometric measurement. As pointed out by the authors, low pH apparently leads to a degradative breakdown of furosemide. Moreover, the lower limit of detection for this assay is only 1 mg/l [8].

The purpose of the present investigation was to develop an assay for furosemide that could be used in our bioavailability/pharmacokinetic studies and that could also be used for routine monitoring of furosemide levels in adult, children and neonate patients. For these reasons it was imperative that we develop an assay capable of rapidly but reproducibly measuring low levels of furosemide in small volumes of plasma and urine. We have developed two different HPLC assays. The two methods differ in their mobile phase, internal standards, and methods of detection. Neither method requires prior extraction and/or derivatization of the plasma or urine samples. Both methods are rapid, sensitive and accurate.

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EXPERIMENTAL

Apparatus

We used a high-pressure liquid chromatograph (Model ALC/GPC 244; Waters Assoc., Milford, Mass., U.S.A.), characterized by a constant solvent flow at working pressures up to 420 kg/cm^2. This model includes a U6-K universal injector and a dual-channel fixed-wavelength, ultraviolet absorption detector. The instrument was fitted with a 30 cm × 3.9 mm I.D. μBondapak C_{18} reversed-phase column, particle size 10 μm (Waters Assoc.). The chromatograph was operated isocratically at a flow-rate of 2 ml/min, at ambient temperature. For Method 1 the wavelength of detection was fixed at 280 nm. For Method II the wavelengths of detection were fixed at 254 and 280 nm. A dual-pen recorder was used (Omniscribe Model A5211-1; Houston Instruments, Austin, Texas, U.S.A.). Chart speed was 2.5 cm/min and full-scale response was 1 mV.

Reagents

Chemicals. The furosemide, Lot RW 1793, was obtained from Hoechst-Roussel (Sommerville, N.J., U.S.A.), sodium cephalothin Lot 96123 was obtained from Eli Lilly (Indianapolis, Ind., U.S.A.), sodium phenobarbital, Lot 63453, was obtained from Merck (Rahway, N.J., U.S.A.).

The methanol (glass-distilled, Burdick and Jackson Labs., Muskegon, Mich., U.S.A.), acetonitrile, Analytical Reagent (Mallinckrodt, St. Louis, Mo., U.S.A.) and distilled water (glass-redistilled and stored in glass) were filtered through a 0.45-μm filter (HAWPO 4700 and FHLPO 4700; Millipore Corp., Bedford, Mass., U.S.A.) before use. All other chemicals were ACS reagent grade or better.

Mobile phase. Method I: The mobile phase consisted of methanol—0.01 *M* sodium acetate, pH 5.0 (35:65), prepared by mixing 350 ml of methanol with 650 ml of water, adding 0.6 ml of glacial acetic acid, and adjusting the pH of the solution to 5.0 with 4 *M* sodium hydroxide. Method II: The mobile phase consisted of acetonitrile—0.01 *M* sodium acetate, pH 5.0 (25:75), prepared by mixing 250 ml of acetonitrile with 750 ml of water, adding 0.6 ml glacial acetic acid, and adjusting the pH of the solution to 5.0 with 4 *M* sodium hydroxide. These mobile phases are degassed under vacuum before use.

Biological fluids. Human plasma that had been stored at −20° for four weeks was obtained from the blood bank of the University of California, San Francisco. The plasma was thawed to room temperature before use. Human urine from a male donor was collected daily.

Urine was collected from a normal male volunteer given 40 mg of furosemide by intravenous injection. Blood from the same patient was collected in a heparinized tube (Vacutainer; Becton-Dickinson, Rutherford, N.J., U.S.A.). It was centrifuged (3000 *g*, 4 min) and the plasma fraction was decanted. These plasma and urine samples were stored at −20° before analysis.

Procedure

Method I. Plasma: Add 20 μl of a 68 mg/l aqueous solution of sodium cephalothin, the internal standard, to 200 μl of plasma. Shake well, add 200 μl of methanol and shake to mix. Centrifuge for 15 min at 10,000 *g* to pre-

cipitate the proteins. Pour the supernatant into a 4-ml glass tube. Inject 25—30 μl of these solutions onto the chromatograph. Set the 280 nm detector at 0.01 a.u.f.s. Operate the chromatograph at a flow-rate of 2.0 ml/min at room temperature using methanol—sodium acetate (35:65) as the mobile phase. Retention times for sodium cephalothin and furosemide are 6 and 8 min, respectively.

Urine: Add 20 μl of the 68 mg/l aqueous sodium cephalothin solution to 200 μl of urine. Shake well to mix. Inject directly onto the chromatograph. The chromatographic conditions are identical to those described above for plasma.

Method II. Plasma: Add 20 μl of a 150 mg/l aqueous solution of sodium phenobarbital, the internal standard, to 200 μl of plasma. Shake well, add 400 μl of acetonitrile and shake to mix. Centrifuge at 3000 *g* for 10 min to precipitate the protein. Pour the supernatant into a 4-ml test tube. Evaporate under nitrogen at ambient temperature to a volume of about 150 μl. Add 30 μl of the mobile phase, mix and inject onto the chromatograph. Injection volumes of 15—20 μl were satisfactory at 0.01 a.u.f.s. at both 280 and 254 nm. Operate the chromatograph at a flow-rate of 2.0 ml/min at room temperature using the acetonitrile—sodium acetate (25:75) mobile phase. Retention times were 5 and 7 min, respectively, for furosemide and sodium phenobarbital. A dual-channel ultraviolet absorption detector must be used to monitor simultaneously the furosemide at 280 nm and the sodium phenobarbital at 254 nm.

Urine: Add 50 μl of the 150 mg/l aqueous sodium phenobarbital solution to 200 μl of urine. Inject directly onto the chromatograph. Volumes of 10 to 15 μl were satisfactory when the sensitivity of the detector was set at 0.01 a.u.f.s. at both 280 and 254 nm. All other chromatographic conditions were identical to those described above for plasma.

With both methods standard curves are prepared by adding furosemide and the appropriate internal standard to plasma or urine. The concentration of furosemide in samples is determined by comparing the furosemide/internal standard peak height ratios to standard curves of peak height ratios versus furosemide concentration. With all curves, we made a straight-line fit of the data by least squares linear regression analysis using the PROPHET system, a specialized computer resource developed by the Chemical/Biological Information Handling Program of the National Institutes of Health.

RESULTS AND DISCUSSION

We have developed two HPLC methods for quantitating furosemide in plasma and urine. Both are rapid, sensitive and accurate. Neither requires extraction and/or derivatization. Fig. 1 shows a chromatogram for the quantitation of furosemide in urine using Method I. The retention times for sodium cephalothin and furosemide are 6 and 8 min, respectively. The peak height ratio indicates the furosemide concentration is 3.6 mg/l. For Method I standard curves were constructed by adding known amounts of furosemide and sodium cephalothin, the internal standard, to urine and plasma and plotting the peak height ratio versus concentration of furosemide in mg/l. Over a

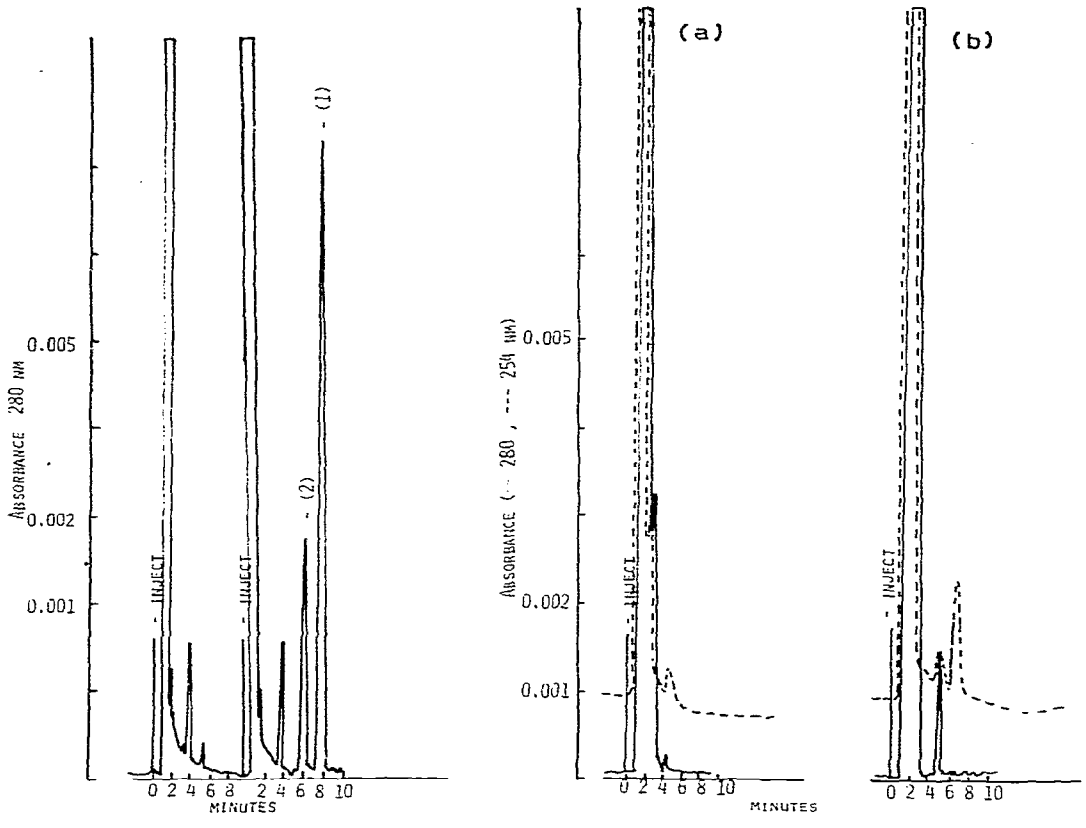

Fig. 1. Chromatograms developed using Method I of (a) blank urine and (b) urine with added furosemide (1) and sodium cephalothin (2). The peak height ratio indicates a furosemide concentration of 3.6 mg/l.

Fig. 2. Chromatograms developed using Method II of (a) blank plasma, (b) plasma with added furosemide, peak at 5 min on 280 nm; and sodium phenobarbital, peak at 7 min on 254 nm. The peak height ratio indicates a furosemide concentration of 0.82 mg/l.

period of 20 days we constructed eight plasma standard curves. With 37 points the regression line for plasma was $y = (0.093 \pm 0.002)\,x + (0.012 \pm 0.045)$ with a coefficient of variation of the slope of 2% and a correlation coefficient of 0.99. With urine, we constructed three standard curves over 20 days. With 15 points the regression line for urine was $y = (0.089 \pm 0.002)\,x + (0.044 \pm 0.039)$ with a coefficient of variation of the slope of 2% and a correlation coefficient of 0.99. For both plasma and urine the concentration of furosemide ranged from 1.8 to 36 mg/l.

Fig. 2 shows a chromatogram for the analysis of furosemide in plasma using Method II. The retention times of furosemide and sodium phenobarbital are 5 and 7 min, respectively. The peak height ratio indicates that the furosemide concentration is 0.82 mg/l. For Method II standard curves were constructed by adding known amounts of furosemide and sodium phenobarbital, the internal standard, to urine and plasma and plotting the peak height ratios of furosemide to sodium phenobarbital against the concentra-

tion of furosemide in mg/l. Over a period of twelve days we constructed six plasma standard curves. With 50 points the regression line for plasma was $y = (1.161 \pm 0.046)\ x + (0.016 \pm 0.052)$ with a coefficient of variation of the slope of 4% and a correlation coefficient of 0.99. With urine we constructed five standard curves over a period of two months. With 34 points the regression line for urine was $y = (0.456 \pm 0.008)\ x + (0.030 \pm 0.042)$, with a coefficient of variation of the slope of 2% and a correlation coefficient of 0.99. For plasma the concentration of furosemide ranged from 0.081 to 2.45 mg/l and for urine from 0.205 to 10.25 mg/l.

Preliminary stability studies with 10 mg/l and 2 mg/l of furosemide in plasma were performed over a period of 20 days using Method I. Concentrations of furosemide in plasma were obtained by comparing the furosemide/sodium cephalothin peak height ratios with those of a standard curve obtained the same day. The results (Table I) show that furosemide can be stored frozen in plasma for at least three weeks.

For both methods, furosemide was detected at 280 nm. This wavelength is quite close to the absorption maximum of furosemide which is 275 nm. The internal standard, sodium cephalothin, for Method I was also detected at 280 nm. However, for Method II the sodium phenobarbital was detected at 254 nm. We do not suspect any interference from endogenous substances using either method because under either set of conditions, all the extraneous peaks have retention times of less than 4 min (Figs. 1 and 2).

The two compounds chosen as internal standards are therapeutic agents which may be administered to patients receiving furosemide. If a patient is receiving one of these drugs, then the alternate method and internal standard may be used. Under the unlikely circumstances that a patient is receiving phenobarbital, cephalothin and furosemide, it is still possible to use sodium phenobarbital as the internal standard. Since the measurement of this compound is carried out at a wavelength different from that for furosemide, higher concentrations of sodium phenobarbital may be used while decreasing the sensitivity of the detector at 254 nm, thus swamping out the relatively low plasma levels of phenobarbital usually observed in patients.

With urine samples, where no precipitation is necessary, analyses can be performed in less than 10 min per sample. In the case of plasma, centrifugation after protein precipitation is necessary because direct injections of plasma onto the chromatograph result in increases in operating pressure caused by the build up of proteins at one end of the column. In Method I after 15 min of centrifugation, analyses can be performed in less than 10 min. In

TABLE I

THE EFFECT OF STORAGE ON FUROSEMIDE CONCENTRATION

Furosemide (mg/l)	Time (days)					
	0	1	2	6	10	20
10	9.95	—	10.00	10.10	9.75	9.60
2	2.10	2.00	1.95	2.00	1.95	1.95

Method II 10 min of centrifugation and 10 min of evaporation are required after which analyses can be performed in less than 10 min.

Fig. 3 shows the plasma levels of furosemide (left axis) vs. time and the urinary excretion rate (right axis) vs. time curves obtained after intravenous administration of 40 mg of furosemide to a male volunteer. The terminal plasma elimination half-life of furosemide in this volunteer is 70 min similar to that seen in other volunteers [9].

Chronologically, we developed Method I first. It has the advantage of using a single fixed wavelength for detection of both the furosemide and the internal standard. It is also rapid, sensitive and accurate. However, we noticed some problems with the stability of sodium cephalothin. That is, the internal standard solutions had to be prepared fresh daily and could not be stored for even a few days. We had also noticed that precipitation of the plasma proteins by acetonitrile gave more complete precipitation than methanol. We discovered that with methanol precipitation column pressure built up fairly rapidly and peaks started spreading, thus, decreasing the column life-time. We also wanted to increase the sensitivity of the assay. Therefore, we developed Method II. The acetonitrile gives more complete protein precipitation. The acetonitrile—sodium acetate mobile phase also gives lower pressure at the same flow-rate than the methanol—sodium acetate mobile phase. Both of these factors contribute to longer column life-times. We could also increase the sensitivity by over twenty-fold since the furosemide peaks were sharper.

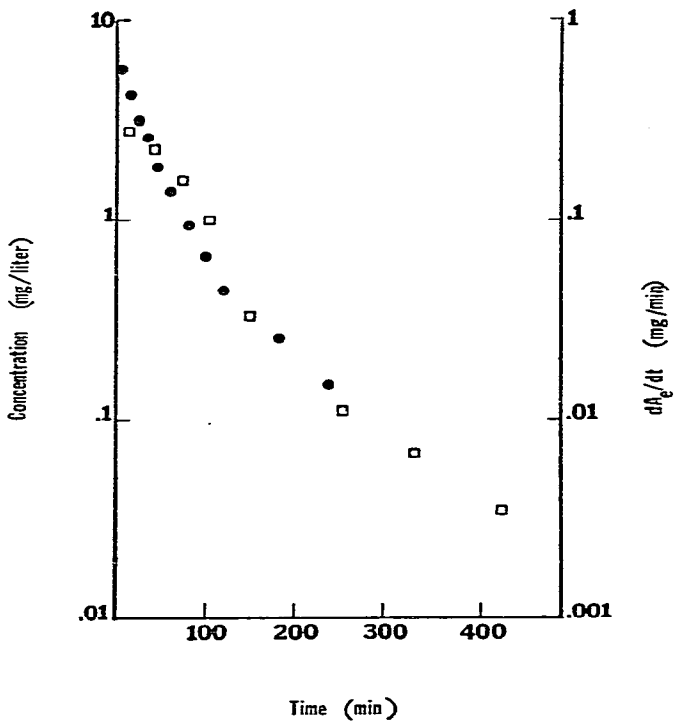

Fig. 3. Plasma concentration (left axis, •) versus time curve and urinary excretion rate (right axis, □) versus time curve following intravenous administration of 40 mg of furosemide to a male patient.

Thus, Method II is as rapid and accurate as Method I but is more sensitive. Method II has the disadvantage of requiring a dual-channel detector in order to measure furosemide at 280 nm and sodium phenobarbital at 254 nm. Because of its greater sensitivity we routinely use Method II. However, for investigators with only a single-channel detector, Method I is a rapid, accurate and sufficiently sensitive method for routine clinical monitoring of furosemide in biological fluids.

ACKNOWLEDGEMENTS

This investigation was supported in part by NIH grants AM 20884 and GM 16496. We wish to thank Hoechst-Roussell for the furosemide and Eli Lilly for the sodium cephalothin.

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