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## Note

# Solid-phase extraction of furosemide from plasma and urine and subsequent analysis by high-performance liquid chromatography

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Furosemide is a valuable diuretic for the treatment of patients suffering from severe fluid retention associated with renal, hepatic or cardiac disease. Because the drug induces diuresis predominantly by acting at the luminal surface of the nephron, urinary furosemide excretion is the best correlate of activity. Sufficient information delineating the normal response to furosemide is available, but data concerning the determinants of abnormal response in several disease states are still limited and sometimes conflicting [1,2]. Evidently, a sensitive and reliable analytical method to determine furosemide in plasma as well as urine is a prerequisite to correlate diuretic activity with drug kinetics under various conditions.

Previous methods for the quantitative determination of furosemide in plasma and urine employed ultraviolet spectrophotometry and spectrophotofluorometry [3,4], thin-layer chromatography (TLC) with radioactivity and spectrophoto(fluoro)metric detection [5–8], gas chromatography (GC) with electron-capture detection [9] or high-performance liquid chromatography (HPLC) with spectrophoto(fluoro)metric detection [10–21].

For the sample clean-up some of the HPLC methods used methanol [13,21] or acetonitrile [12,15,16] to precipitate the protein. The advantages of this procedure are speed and ease, but especially in the case of methanol the disadvantage is incomplete precipitation of the plasma protein All the other methods used liquid–liquid extraction procedures for the sample clean-up With

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this technique the final organic phase contains not only endogenous substances that are soluble in the solvent but also some water-soluble compounds, since none of the extraction solvents used (diethyl ether, chloroform and methylene chloride) is absolutely immiscible with the aqueous phase [22]. The fact that liquid–liquid extraction is not the most suitable procedure for the extraction of furosemide is demonstrated best by the method of Uchino et al. [20] Although they used three laborious extraction steps to clean up their samples, they still found two endogenous peaks near the furosemide peak, as can be seen in their chromatogram of plasma.

To circumvent these difficulties we developed a new extraction procedure, which is based on the selective adsorption of furosemide on the surface of a reversed-phase silica ($C_{18}$ Bond Elut) column This solid-phase extraction method has also the advantages of short handling time, no need to evaporate the solvent after extraction and no need to dissolve the residue in an appropriate solvent before injection into the HPLC system

## EXPERIMENTAL

### *Reagents and materials*

Furosemide and 4-chloro-5-sulphamoylanthranilic acid were kindly supplied by Hoechst (Frankfurt, F.R G ) and desmethylnaproxen by Syntex (Palo Alto, CA, U.S.A ), $\beta$-glucuronidase was obtained from Sigma (St Louis, MO, U S A ). Methanol, potassium dihydrogenphosphate, disodium hydrogenphosphate dihydrate, orthophosphoric acid, sodium hydrogencarbonate, citric acid monohydrate, potassium hydroxide and urea were all of analytical grade (Merck, Darmstadt, F.R.G.). Vac Elut manifold and Bond Elut $C_{18}$ columns (1 ml capacity, to which a 4-ml Bond Elut reservoir was attached using a Bond Elut adaptor) were manufactured by Analytichem International (Harbor City, CA, U.S A ).

### *Biological fluids*

Human blood plasma stored at $-20°C$ was obtained from the local blood bank. Human urine was collected from a male donor shortly before analysis. From one young healthy male volunteer (27 years old, 80 kg) blood and urine were collected after oral administration of a 40-mg furosemide tablet after an overnight fast

### *Apparatus and chromatographic conditions*

A Hewlett-Packard HP1090 high-performance liquid chromatograph equipped with a variable-volume injector and an autosampler was used. The stainless-steel column (150 mm$\times$ 4.6 mm I.D ) was packed with LiChrosorb RP-18, particle size 5 $\mu$m (Merck) The oven temperature was 40°C and the

injector volume was 10 μl. Furosemide and desmethylnaproxen (internal standard) were measured with a Model 3000 fluorescence spectrophotometer (Perkin-Elmer), the excitation wavelength was 275 nm and the emission wavelength 400 nm (the excitation and emission slits were both 10 nm). The signal was processed by an HP 3393A (Hewlett-Packard) computer integrator. The mobile phase, methanol–0.02 *M* phosphate buffer pH 3 0 (48 52), was delivered at a rate of 1 ml/min, the resulting pressure being 15.3 MPa

*Sample pretreatment*

The experiments were carried out in subdued daylight. Into an amber test-tube, 100 μl of internal standard (1.80 mg per 100 ml methanol) were pipetted and evaporated to dryness under a gentle stream of nitrogen at room temperature. Aliquots of 0.50 ml of plasma (or 0.05 ml of urine and 0 45 ml of 0.075 *M* phosphate buffer pH 6 8) and 1.50 ml of a 50% (w/w) aqueous solution of urea were successively pipetted into the amber test-tube. The tube was vortex-mixed gently for 1 s. After standing for 10 min, 2.0 ml (1 5 ml for urine) of 0.01 *M* potassium citrate buffer (pH 3 0) were added to the test-tube. The tube was again vortex-mixed gently for 1 s. The prepared sample was now ready to be loaded onto the $C_{18}$ solid-phase extraction column.

*Extraction procedure*

Extraction of furosemide and desmethylnaproxen from plasma and urine was achieved by the use of a bonded-silica solid-phase extraction column (Bond Elut $C_{18}$, 1 ml capacity). The column was conditioned prior to use by drawing three column volumes (ca. 3 ml) of methanol followed by a similar volume of 0.01 *M* potassium citrate buffer (pH 5.0) through the column. The prepared sample was loaded onto and drawn through the column. The column was then washed with 10 ml of 0.01 *M* potassium citrate buffer (pH 5 0). The cover of the manifold was then removed and the bluntnose stainless-steel needle of the Vac Elut cover was wiped with a tissue to remove drops of washing solution. The Vac Elut rack, holding a 2-ml glass sampling tube, was placed under the column. Furosemide and desmethylnaproxen were eluted from the column with 1 ml of a mixture consisting of 0.5 ml of methanol and 0.5 ml of an 0.01 *M* aqueous solution of sodium hydrogencarbonate. The eluate collected was measured as described

*Determination of furosemide glucuronide*

Each urine sample was divided into two portions, one of which was used for the determination of furosemide (see *Sample pretreatment*) and the other for determination of furosemide and its glucuronide together

Into an amber extraction tube, 100 μl of internal standard (1 8 mg per 100 ml methanol) were pipetted and evaporated to dryness under a gentle stream of nitrogen at room temperature. Into this test-tube were successively pipetted

0.45 ml of 0.075 *M* phosphate buffer (pH 6.8) containing 500 U of β-glucuronidase and 0.05 ml of urine. The extraction tube was closed with a PTFE-lined screw-cap. The mixture was incubated for 21 h at 37 °C in the dark. After chilling to room temperature, 1.5 ml of a 50% (w/w) aqueous solution of urea were added, and the sample was processed further as described under *Sample pretreatment.*

## RESULTS AND DISCUSSION

### *Chromatography*

Fig. 1 shows typical chromatograms for blank plasma and urine, and for plasma and urine samples spiked with furosemide and the internal standard desmethylnaproxen (DMN). The total run time for each sample was 7 min, in which furosemide and DMN were resolved with retention times of 4.3 and 6.1 min, respectively. Under the same chromatographic conditions the retention time of 4-chloro-5-sulphamoylanthranilic acid (CSA) was 2.0 min; however, no effort was made to retain CSA on the extraction column CSA is no longer considered to be a metabolite of furosemide, but an analytical artefact that can be avoided by appropriate specimen handling [15,18,19]. Using the solid-phase extraction procedure described, no interfering peaks due to endogenous components of human plasma and urine were observed in the chromatograms.

### *Calibration curves*

The furosemide concentration in a sample was determined by comparing the peak-height ratio of furosemide to internal standard (I.S.) with a standard curve of peak-height ratio versus furosemide concentration. Whenever samples containing furosemide were measured a standard curve was generated by adding different amounts of furosemide to blank plasma or blank urine and analyzing them by the procedure described. A linear relationship was found between the peak-height ratio of furosemide to DMN ($y$) and the plasma furosemide concentration ($x$), as given by the equations $y=0.004812x-0.00095$ ($r=0.9998$, $n=4$) for the plasma furosemide concentration range 0.010–0 100 μg/ml (I.S $=0.36$ μg/ml) and $y=0.4817x-0.00781$ ($r=0.9998$, $n=6$) for the plasma furosemide range 0.1–2.5 μg/ml (I.S. $=3.6$ μg/ml). For urine the equa-

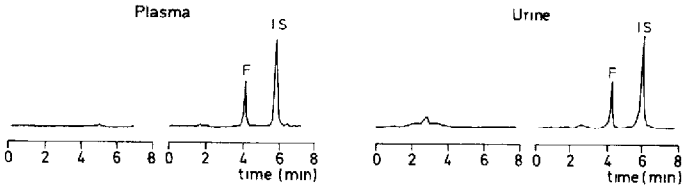


Fig 1 Chromatograms obtained for blank plasma and urine and for samples spiked with 0 100 μg/ml furosemide (F) and 0 360 μg/ml DMN (internal standard, I S )

tion was $y=0.04983x-0.00211$ ($r=0.9999$, $n=7$) for the urine furosemide concentration range 0.5–25 μg/ml (I.S = 36.0 μg/ml).

*Recovery*

Overall recovery with the extraction procedure was determined by comparing the peak heights of furosemide and DMN obtained after injection of non-extracted standard solutions with peak heights obtained after injection of extracted plasma and urine containing equal concentrations of both compounds. The absolute recovery of furosemide and DMN is complete and independent of the concentration with a coefficient of variation (C.V.) less than 4.4% (Table I). Taking the extensive plasma protein binding of furosemide (>98%) into consideration [15], the quantitative recovery indicates that the protein must have been effectively denaturated by the treatment with the urea solution.

*Sensitivity and precision*

The lower limit of detection (four times baseline noise) of the method described was 10 ng/ml for both plasma and urine (5 ng in 0.5 ml). Table II shows the precision of furosemide measurement in plasma and urine. The intra-assay C.V. varied between 3.2 and 1.8% in plasma and between 2.9 and 1.1% in urine over a furosemide concentration range of 0.021–52.5 μg/ml.

*Applicability*

Various drugs that are often concurrently administered with furosemide were examined for their possible interference with the assay (Table III). Plasma

TABLE I

RECOVERY OF FUROSEMIDE AND DESMETHYLNAPROXEN FROM PLASMA AND URINE

For all concentrations, $n=5$

| Sample | Compound | Concentration (μg/ml) | Recovery (%) | C.V. (%) |
|---|---|---|---|---|
| Plasma | Furosemide | 0.100 | 99 | 3.0 |
| | | 5.25 | 101 | 2.8 |
| | | 52.5 | 94 | 2.6 |
| | DMN | 0.360 | 99 | 2.4 |
| | | 12.5 | 103 | 1.8 |
| | | 125.0 | 97 | 2.6 |
| Urine | Furosemide | 0.100 | 96 | 3.0 |
| | | 5.25 | 102 | 2.8 |
| | | 52.5 | 100 | 2.6 |
| | DMN | 0.360 | 99 | 2.4 |
| | | 12.5 | 100 | 1.8 |
| | | 125.0 | 101 | 2.6 |

TABLE II

PRECISION OF FUROSEMIDE ANALYSIS IN SPIKED PLASMA AND URINE SAMPLES

For all concentrations, $n = 5$

| Concentration ($\mu$g/ml) | Coefficient of variation (%) | |
|---|---|---|
| | Plasma | Urine |
| 0 021 | 3 2 | 2 9 |
| 0 053 | 1 8 | 1 4 |
| 0 105 | 2 4 | 1 7 |
| 0 210 | 0 4 | 0 3 |
| 1 05 | 2 6 | 3 1 |
| 5 25 | 1 6 | 2 4 |
| 52 5 | 1 8 | 1 1 |

TABLE III

SOME DRUGS OFTEN COADMINISTERED WITH FUROSEMIDE THAT DO NOT INTERFERE WITH THE ASSAY

Samples were obtained from patients treated with therapeutic doses of the drugs listed

| | |
|---|---|
| Acetylsalicylic acid | Isosorbide dinitrate |
| Aluminium hydroxide | Metoprolol |
| Amiodarone | Nifedipine |
| Beclomethasone | Paracetamol |
| Calcitriol | Promethazine |
| Captopril | Ranitidine |
| Cimetidine | Salbutamol |
| Digitoxin | Salicylates |
| Digoxin | Temazepam |
| Diltiazem | Tolbutamide |
| Ipratropium | Triamterene |

and urine from patients under treatment with therapeutic doses of the particular drugs were processed by the solid-phase extraction method, and the chromatograms were checked for any interfering peaks at the retention times for furosemide and DMN. None of the drugs investigated interfered with the quantitative determination of furosemide, showing its suitability for use in routine drug-monitoring studies.

Monitoring of furosemide levels has been recommended in patients with impaired renal function and in premature infants because of the risk of ototoxicity. For plasma collected from infants, volumes of 50 $\mu$l or less, brought to a volume of 0.50 ml with blank plasma, can be used. Starting from 50 $\mu$l the detection limit will be 100 ng/ml (5 ng in 50 $\mu$l).

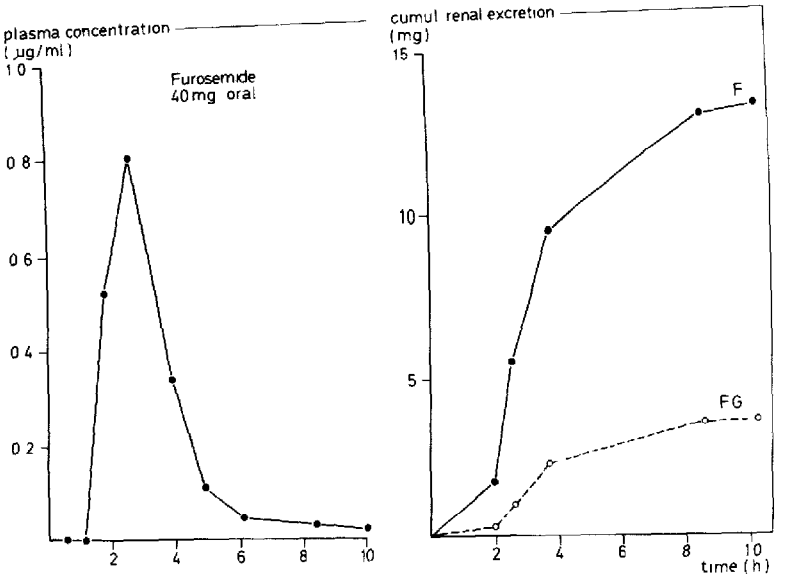


Fig 2 Plasma concentration of furosemide and cumulative renal excretion of furosemide (F) and furosemide glucuronide (FG) after ingestion of a 40-mg tablet by a healthy male volunteer

Fig. 2 shows a typical example of the method applied in a pharmacokinetic study Plasma and urine samples from a healthy male volunteer following oral administration of a 40-mg furosemide tablet were analysed. A peak plasma level of 0.80 µg/ml was measured at 2.30 h. After 10 h, 13.18 mg of furosemide and 3.67 mg of furosemide glucuronide were recovered from urine, corresponding to a total recovery of 42% of the dose. The results are in good agreement with reported values [15,18]

## CONCLUSION

A new extraction procedure based on solid-phase extraction is presented. It provides a rapid, selective and reliable method for the clean-up of furosemide from biological fluids The procedure yields final samples with fewer endogenous substances than are usually obtained using liquid–liquid extraction methods. The recovery of furosemide is complete in spite of the high degree of binding to plasma proteins. Drugs commonly coadministered with furosemide do not interfere, and the detection limit is as low as 10 ng/ml (5 ng in a 0.50-ml sample). This makes the method particularly useful for detailed pharmacokinetic and pharmacodynamic studies in various disease states and for routine monitoring of furosemide levels in small volumes of plasma collected from infants.

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