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Determination of furosemide with its acyl glucuronide in human plasma and urine by means of direct gradient high-performance liquid chromatographic analysis with fluorescence detection
Preliminary pharmacokinetics and effect of probenecid

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Abstract

Furosemide is metabolized in humans by acyl glucuronidation to the 1-O-glucuronide (Fgluc). Furosemide (F) and the conjugate can be measured directly by gradient high-performance liquid chromatographic analysis without enzymic deglucuronidation. The glucuronide conjugate was isolated by preparative HPLC from human urine samples. Furosemide and its acyl glucuronide were present in plasma. No isoglucuronides were present in acidic urine of a volunteer. Calibration curves were constructed by enzymic deconjugation of samples containing different concentrations of isolated F-acyl glucuronide. The limit of quantitation of F in plasma is 0.007 μg/ml, Fgluc 0.010 μg/ml. The limits of quantitation in urine are respectively: F 0.10 μg/ml, Fgluc 0.15 μg/ml. A pharmacokinetic profile of furosemide is shown, and some preliminary pharmacokinetic parameters of furosemide obtained from one human volunteer are given. Probenecid does not inhibit the formation of the acyl glucuronide of F, but inhibits the renal clearance of both compounds.

1. Introduction

Furosemide, 4-chloro-N-(2-furylmethyl)-5-sulfamoylanthranilic acid, is still one of the most potent diuretics available today. It inhibits the active reabsorption of chloride ions in the ascending limb of the loop of Henle [1,2].

In humans furosemide (F) is metabolized by phase II metabolism into furosemide acyl glucuronide (1-O-glucuronide), as shown in Fig. 1 [1]. The presence of isoglucuronides (2-O-, 3-O-, and 4-O-glucuronide) in urine must be anticipated to result from isomerization of the instable acyl glucuronides in blood of pH 7.4, or in the gut via enterohepatic recirculation [1,3].

Several methods for the analysis of the

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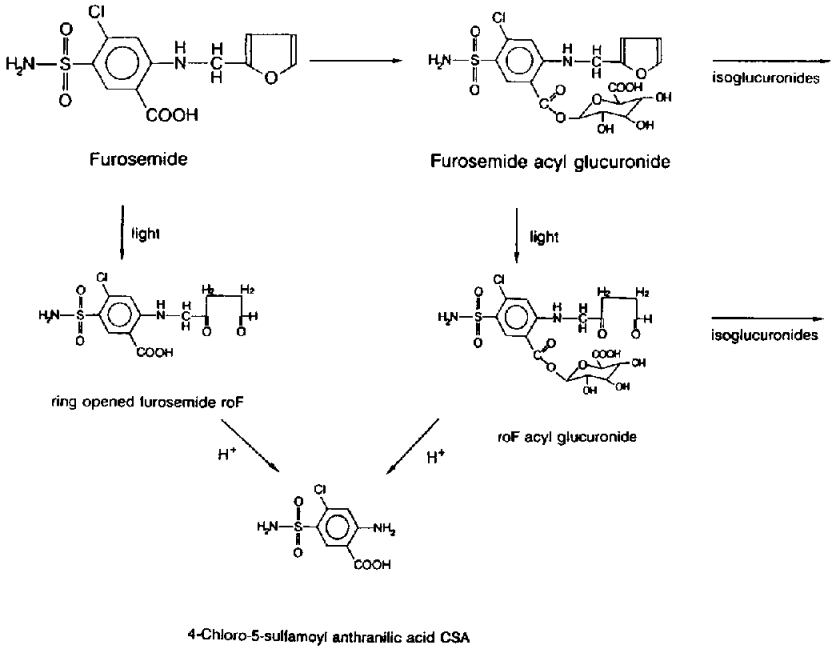

Fig. 1. Structures of furosemide, its metabolite, the open-ring products and hydrolysis product CSA.

furosemide concentration in plasma and urine have been described: radioimmuno assay [4–7], colorimetric detection [8,9], fluorimetric methods [8–11], and a TLC method [12]. To day HPLC analysis of furosemide is the most commonly used method of analysis, however the conjugate is determined after enzymic or alkaline hydrolysis [13–19]. A direct isocratic HPLC analysis of furosemide with its acyl glucuronide was reported [18,20], but a gradient analysis which enables the measurement of all possible metabolites is still lacking.

Acyl glucuronides are unstable in alkaline media (pH > 7.0); therefore urine must be kept acidic at pH 5.0 (already inside the body) to prevent hydrolysis of the possibly formed acyl glucuronides [3,21–24].

The aim of this investigation was (a) to develop a simple and direct gradient HPLC analysis of furosemide and its glucuronide conjugates in plasma and urine, and (b) to study the human pharmacokinetics of furosemide and the effect of probenecid in pilot experiments.

2. Experimental

2.1. Chemicals

Furosemide and 4-chloro-5-sulfamoylanthranilic acid (CSA; batch Sch 127-1) were obtained from Hoechst (Amsterdam, Netherlands; Frankfurt-Hoechst, Germany). Lasix (40 mg tablets) and probenecid (Benemid, 500 mg tablets) were obtained from the hospital pharmacy. All other reagents were of p.a. quality and obtained from Merck (Darmstadt, Germany).

Furosemide acyl glucuronide was isolated and identified in human urine after intake of 80 mg furosemide. ß-Glucuronidases were obtained from Sigma (St. Louis, MO, USA).

2.2. Gradient HPLC analysis

The HPLC system consisted of a Spectra Physics SP 8775 autosampler (Spectra Physics, Eindhoven, Netherlands), a Spectra Physics SP 8800 ternary HPLC pump, a Hitachi 1050 fluo-

rescence detector (Merck, Amsterdam, Netherlands), and a Spectra Physics SP 4290 integrator. The column was Cp Spherisorb ODS 5 μm, 250 mm × 4.6 mm I.D. (Chrompack, Bergen op Zoom, Netherlands) with a guard column 75 mm × 2.1 mm I.D., packed with pellicular reversed-phase (Chrompack Cat. No. 28653). The mobile phase was a mixture of acetonitrile and 0.5% orthophosphoric acid (98%) pH 2.1. At $t = 0$, the mobile phase was acetonitrile–phosphoric acid (5:95, v/v). During the following 30 min the mobile phase changed linearly until it attained a composition of 41:59 (v/v). At 35 min ($t = 35$) the mobile phase was changed within 5 min to the initial composition, followed by equilibration during 2 min. The flow-rate was 1.2 ml/min. Fluorescence detection was achieved at 345 nm excitation and 405 nm emission wavelength.

The capacity factors of furosemide, products with an opened ring, CSA and the acyl glucuronides are given in Table 1.

2.3. Sample treatment

Plasma samples (100 μl) were deproteinized with 100 μl acetonitrile, centrifuged at 3000 *g* for 5 min, and 20 μl of the supernatant were injected onto the column. During the first day of the human experiments, 100 μl of plasma sample were processed immediately upon receipt of furosemide in order to detect the presence of the acyl glucuronide of furosemide. The plasma samples were injected one by one manually onto the column.

Urine samples were diluted 1:1 with water and 20 μl were injected onto the column.

2.4. Isolation of furosemide acyl glucuronide

The peak in the chromatograms that was assumed to be the metabolite of furosemide was isolated by means of preparative HPLC.

The preparative Gilson HPLC consisted of a Gilson 302 sample pump (Gilson, Meyvis, Bergen op Zoom, Netherlands), two 305 Gilson gradient pumps, a 811 B Dynamic mixer, a Kratos 757 UV detector (Separations, Hendrik Ido Ambacht, Netherlands), an LKB 2211 superrac (LKB, Woerden, Netherlands), and a BD7 recorder (Kipp and Zonen, Delft, Netherlands). The column was a C_8 8-μm, 250 mm × 10 mm I.D., Rainin Dynamax 60 Å column (Meyvis).

The mobile phase consisted of 1% acetic acid in water and acetonitrile (80:20, v/v) during the first minute and subsequently changed linearly in 15 min to 65:35 (v/v). The flow-rate was 1.2 ml/min.

The retention time of furosemide 1-O-acyl glucuronide is 5.4 min.

Concentration of the sample was carried out by a IKA rotavapor (Janke and Kunkel, Staufen, Germany) equipped with a Trivac vacuumpump (Leybold-Heraeus, Woerden, Netherlands).

2.5. Deconjugation of the acyl glucuronide

Deglucuronidation was carried out with 200 μl of human urine containing furosemide and acyl glucuronide, 100 μl of ß-glucuronidase and 200 μl of 0.2 *M* (Na_2H/KH_2)phosphate buffer at 37°C for 2 h.

Four different ß-glucuronidase enzymes (A–D) were tested:

(A) 100 000 U/ml ß-glucuronidase type B1 (Bovine liver, Sigma, St. Louis, MO, USA, Cat. No. G-0251) and phosphate buffer pH 5.0.

(B) 107 200 U/ml ß-glucuronidase type H2 (*Helix pomatia*, Sigma, Cat. No. G-0876) and phosphate buffer pH 5.0.

(C) 100 000 U/ml ß-glucuronidase type LII (lyophilized powder from limpets *Patella vulgata*, Sigma, Cat. No. G-8132) and phosphate buffer pH 3.8.

(D) 20 000 U/ml ß-glucuronidase type VIIA (*Escherichia coli*, Sigma, Cat. No. G-7646) and phosphate buffer pH 6.8.

2.6. Calibration curves

Samples containing different concentrations of furosemide acyl glucuronide, isolated from human urine by preparative HPLC, were deconjugated by enzyme system C.

The increase in the concentration of furosemide (aglycon) represented the concentration of the conjugate furosemide acyl glucuronide. A calibration curve was constructed with the following formula:

$$[\text{F-gluc}] = d[\text{F}] \cdot M_{\text{F-gluc}}/M_{\text{F}}$$

were $d[\text{F}]$ is the difference in concentration of furosemide before and after deconjugation and M is relative molecular mass ($r = 0.999$).

The concentration of the isolated furosemide acyl glucuronide was determined and the sample was used as stock solution.

The calibration curve for furosemide and furosemide acyl glucuronide in plasma was constructed by spiking blank human plasma samples with known concentrations of the compound ($r = > 0.9998$). Calibration curves for furosemide and furosemide acyl glucuronide in urine were constructed by spiking blank urine samples with known concentrations of the compounds ($r > 0.9994$).

2.7. Stability

The stability of furosemide and its acyl glucuronide in water in the light was tested as follows: a stock solution of 1.0 mg/ml furosemide and 1.1 mg/ml furosemide acyl glucuronide were exposed during one week to UV light. Samples were taken twice a day.

The stability of furosemide and its acyl glucuronide in urine and buffer at different pH values was tested as follows: three samples of two ml of urine were brought to pH 2.1, 3.8, 5.0, 7.4, and pH 8.0 and incubated at 37°C for 24 h. At regular time intervals (1–2 h) a 100-μl sample was taken, and the reaction stopped with 900 μl 0.01 M H_3PO_4. From this mixture, 20 μl were injected onto the column.

The stability of furosemide and furosemide acyl glucuronide in the autosampler in water and 0.01 M H_3PO_4 was tested during 24 h in the dark. Samples were taken every 0.5 h and injected onto the column.

2.8. Isomerization of the acyl glucuronides

Isolated furosemide acyl glucuronide was subjected to hydrolysis and isomerization in a phosphate buffer of pH 2.1, 3.8, 5.0, 7.4 and pH 8.0 during 24 h at 37°C. The formation of isoglucuronides was followed by analysis of samples taken every hour.

2.9. Limits of quantitation

The limits of detection of furosemide and furosemide acyl glucuronide in water and quantitation in plasma and urine were determined at a signal-to-noise ratio of 3, and are shown in Table 2.

2.10. Recovery

Recovery of furosemide and its acyl glucuronide from plasma was compared with that from standards in Na_2HPO_4 buffer of pH 7.4

2.11. Subjects

One human subject (I, male, 50 year of age, 92 kg body weight) took 80 mg furosemide orally (Lasix) after an overnight fast. One month later 1 gram probenecid (Benemid, MSD, Haarlem, Netherlands) was taken after an overnight fast, one hour later followed by the intake of 80 mg furosemide. The study had the approval of the hospital ethics committee and informed consent was obtained from the volunteer.

2.12. Sampling

Blood samples were drawn at regular time intervals after administration during 2 days by means of fingertip puncture with Monolet lancets (Monoject, St. Louis, MO, USA). After centrifuging plasma samples were stored at −20°C until analysis.

Urine was collected upon untimed voiding. The total time of sample collection was 48 h. Urinary pH was kept acidic (pH 5.0–5.5) by the oral intake of 1 gram ammoniumchloride q.i.d.

(Ammonchlor, Südmedica, Munich, Germany). Four urine samples of 5 ml of each void were immediately stored at −20°C until analysis. The urine samples were cumulatively collected during 8 h, stored and protected from UV light during the collection period and thereafter at −20°C pending the preparative isolation of furosemide acyl glucuronide.

2.13. Pharmacokinetics

The pharmacokinetic parameters were calculated using the MediWare computer package [25].

3. Results and discussion

Fig. 2 shows the chromatograms of an actual human plasma and urine sample after oral administration of 80 mg furosemide. The chromatograms show the presence of the acyl glucuronide of furosemide in plasma and urine; no acyl isoglucuronides of furosemide were observed. Table 1 shows the retention times, capacity factors of drug and metabolites and group contributions to the retention behaviour. Table 2 shows the detection and quantitation limits of furosemide and its metabolite.

Fig. 3 shows that furosemide acyl glucuronide was unstable in plasma at 37°C and pH 7.4. The $t_{1/2}$ of decomposition was 2.5 h. Plasma samples were processed as quickly as possible, protected from UV light, and manually injected onto the column. Furosemide acyl glucuronide was stable in urine and buffer at pH < 6.0 and unstable at pH 6.8 and pH 8.0 (Fig. 3). Furosemide is stable in buffers of pH 3.8, 5.0, 6.0, 6.8 and 8.0 during 48 h. Urine samples of pH 5 and kept in the dark were stable in the autosampler of the HLPC during 24 h. A stability test showed that furosemide acyl glucuronide is stable over a 30-min period at pH 7.4. Fig. 3 additionally shows the chromatogram obtained from a sample taken at $t = 11$ h and pH 8.0 and the degree of isomerization of isolated furosemide acyl gluc-

Table 1
Retention times and capacity factors of furosemide and its glucuronide conjugate

Compound	t_R	k'	Ratio k'		
	(min)		gluc./aglycon	light	-furanoyl
t_0	2.09				
CSA	12.00	4.74			0.37
roF acyl gluc	17.49	7.37	0.83	0.70	
F isogluc	20.23	8.68			
roF	20.57	8.84		0.69	
F isogluc	21.25	9.17			
F isogluc	21.92	9.49			
F isogluc	22.49	9.76			
Furosemide acyl glucuronide	24.22	10.59	0.83		
Furosemide	28.77	12.77			

Acyl glucuronide = 1-O-glucuronide; F = furosemide; roF = furanoyl open-ring furosemide. CSA = 4-chloro-5-sulfamoylanthranilic acid; isoglucuronide = 2-O-glucuronide, 3-O-, and 4-O-glucuronide. Glucuronidation reduces the capacity factor of furosemide by a factor 0.83, ring opening of the furanoylring reduces the capacity factor of furosemide by a factor 0.70, and loss of the furanoylring by a factor 0.37.

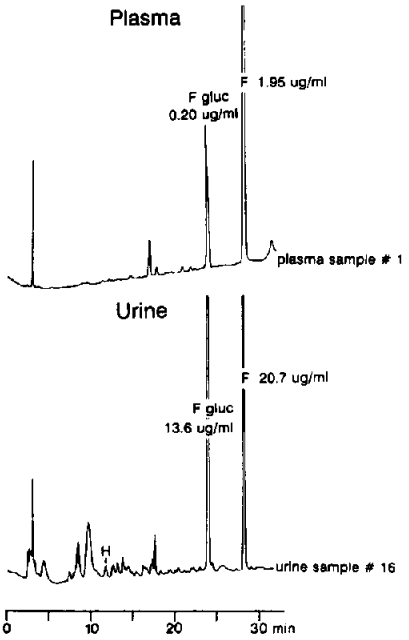

Fig. 2. Chromatograms of an actual human plasma and urine sample containing furosemide and its metabolite. Fluorescence detection at 345 nm excitation and 405 nm emission. No acyl isoglucuronides, nor the open-ring products or hydrolysis product CSA are present. H = hippuric acid.

uronide (1-O-glucuronide) into furosemide acyl isoglucuronides (2-O-, 3-O-, and 4-O-acylglucuronide) and furosemide. This reaction does not proceed at pH 5. Four acyl isoglucuronides could be separated and it was

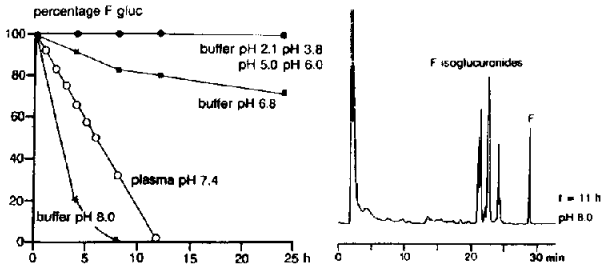

Fig. 3. Stability of furosemide acyl glucuronide in plasma at 37°C and pH 7.4 and in buffer solutions of various pH values. The right panel shows the chromatogram of furosemide and the acyl isoglucuronides in the buffer sample at $t = 11$ h and pH 8.0.

assumed that 4-O-glucuronide had the shortest retention time. The acyl isoglucuronides were glucuronidase resistant [20]. The position of the glucuronide group (2-O-, 3-O-, and 4-O-) of the isoglucuronides has to be identified by LC–mass spectrometry. Rachmel *et al.* demonstrated that the mass spectra of the four isoglucuronides were indistinguishable when a 'soft' ionization technique (FAB) was used [20]. The UV spectra of probenecid isoglucuronides were indistinguishable when recorded with diode-array detection (courtesy Dr. R. Hendriks, Varian, Houten, Netherlands).

Fig. 4 shows the ring opening of the furanoyl ring of furosemide when a solution of furosemide in water is subjected to UV light for 4 days ($t_{1/2}$

Table 2
Recovery and limits of detection and quantitation of furosemide and its conjugate ($n = 5$)

Compound	Detection limit (μg/ml)	Quantitation limit (μg/ml)
Plasma		
Furosemide	0.005	0.007
Furosemide acyl glucuronide	0.010	0.010
urine		
Furosemide	0.005	0.100
Furosemide acyl glucuronide	0.010	0.150
Percentage recovery from plasma ($n = 5$)		
Furosemide	91.50 ± 5.10%	
Furosemide acyl glucuronide	85.52 ± 4.73%	

Detection limit of the analyte in water; quantitation limit of the analyte in the biological matrix.

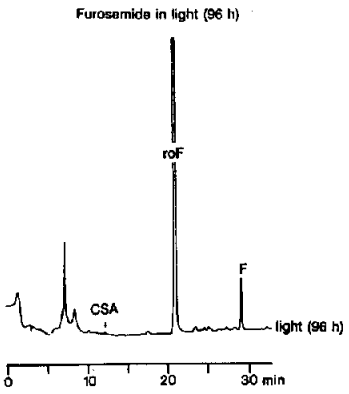

Fig. 4. Chromatogram of a water sample containing furosemide (F) and its open-ring product (roF) after 96 h exposure to UV light (TL light) at room temperature (23°C).

1 day) [26]. When isolated furosemide acyl glucuronide was subjected to UV light, the furanoyl ring opened into the corresponding acyl glucuronide (roF) as shown in Fig. 5 (Fig. 1).

Acid hydrolysis of furosemide by 5 *M* HCl and its acyl glucuronide resulted via the open-ring intermediate in the cleavage of the molecule into levulinic acid and 4-chloro-5-sulfamoylanthranilic acid (CSA).

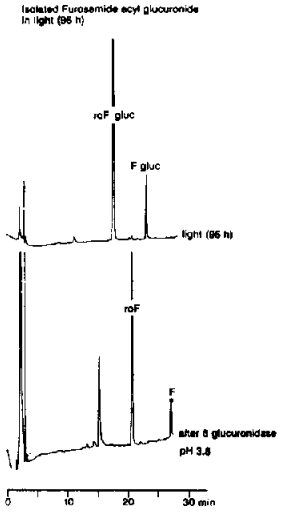

Fig. 5. Chromatogram of a water sample containing isolated furosemide acyl glucuronide and (Fgluc) its open-ring product (roFgluc) after 96 h exposure to UV light (TL light) at room temperature (23°C) and after hydrolysis with ß-glucuronidase.

Tables 3 and 4 show the intra- and inter-day variations of furosemide and its metabolite in plasma and urine respectively.

Fig. 6 shows the plasma concentration–time curve and renal excretion rate–time profiles of furosemide and its acyl glucuronide in one male volunteer.

Table 5 summarizes some pharmacokinetic parameters of furosemide in the human volunteer after two pilot experiments. As low furosemide acyl glucuronide concentrations are found in plasma, due to high renal clearance and the hydrolysis in alkaline medium (pH 7.4), the high concentration of furosemide acyl glucuronide found in control acidic urine may partly be

Table 3
Inter-day and intra-day coefficient of variation (C.V.) of spiked furosemide and conjugate in human plasma ($n = 4$, *in vitro*)

Concentration (μg/ml)	C.V. (%)	
	Inter-day	Intra-day
Furosemide		
2.15	3.02	1.63
1.04	1.60	1.76
0.21	1.16	1.25
Furosemide acyl glucuronide		
1.08	1.19	1.70
0.53	0.27	3.53
0.10	4.08	2.02

Table 4
Inter-day and intra-day coefficient of variation (C.V.) of furosemide and conjugate in human urine ($n = 4$, *in vitro*)

Concentration (μg/ml)	C.V. (%)	
	Inter-day	Intra-day
Furosemide		
10.75	1.71	1.30
1.07	2.01	2.34
0.11	3.72	5.18
Furosemide acyl glucuronide		
5.37	1.28	1.40
0.54	1.55	0.83
0.06	6.79	4.57

Table 5
Some pharmacokinetic parameters of 80 mg furosemide and its conjugate in human

Parameter	value	
Subject	I	I[a]
Gender	male	male
Body weight (kg)	90	90
Furosemide		
Biovailability	0.60	0.45
C_{max} (μg/ml)	1.89	3.80
t_{max} (h)	0.70	2.97
$t_{1/2\alpha}$ (h)	1.0	–
$t_{1/2\beta}$ (h)	2.5	2.4
MRT (h)	2.1	5.8
AUC (mg/l h)	4.43	17.45
Furosemide acyl gluc.		
C_{max} (μg/ml)	0.21	0.39
t_{max} (h)	0.78	3.84
$t_{1/2\alpha}$ (h)	0.5	–
$t_{1/2\beta}$ (h)	1.6	2.6
MRT (h)	2.70	7.43
AUC (mg/l h)	0.39	3.78
Furosemide		
Total body clearance (l/h)	10.94	2.06
Volume of distribution (l)	18.70	7.09
Renal clearance (ml/min)		
Furosemide	166 ± 53	50.0 ± 58.6
Furosemide acyl gluc	399 ± 191	110 ± 71.1
Maximal urine flow (ml/min)	22	21
Percentage of the dose excreted (%)		
Furosemide	45.5	31.0
Furosemide acyl glucuronide	14.1	13.7
total	59.6	44.7
Percentage proteinbinding (in vitro; n = 5)		
Furosemide	98.5 ± 1.1	
Furosemide acyl glucuronide	98.9 ± 0.7	
Urinary pH kept acidic (between pH 5.0 and 5.5)		

[a] + 1000 mg probenecid.
Acyl glucuronide = 1-O-glucuronide;– = not present

formed by the kidney [23,27]. A similar phenomenon was observed for probenecid [28–30], nalidixic acid [31] and indomethacin [23]. In contrast, the presence of acyl isoglucuronides in urine was reported for naproxen and O-desmethylnaproxen [3].

Previously it has been hypothesized that if probenecid and a particular drug/compound were glucuronidated in the human kidney, probenecid might be able to interfere with or inhibit the glucuronidation of the concomitantly administered drug [27]. Furosemide itself shows high renal clearance values, due to active tubular secretion [1,2]. Probenecid co-medication did

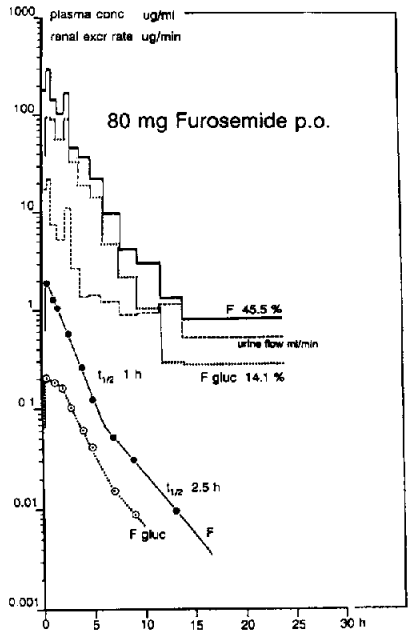

Fig. 6. Plasma concentration–time curve and renal excretion rate–time profiles of furosemide (F), furosemide acyl glucuronide (Fgluc), in a human volunteer after an oral dose of 80 mg of furosemide. The urine flow (ml/min)–time curve shows the diuretic effect of furosemide and its acyl glucuronide.

not inhibit the glucuronidation of the parent drug as shown in Table 5, but only inhibited the active tubular secretion of both furosemide and its acyl glucuronide, thus reducing the renal clearance values. The results of a full pharmacokinetic study will be published elsewhere.

4. Conclusion

The analysis of furosemide and its metabolite in human plasma and urine samples can be easily performed with gradient HPLC with fluorescence detection. In plasma and urine the parent drug and the acyl glucuronide could be detected. The discrepancy between the low concentration of furosemide acyl glucuronide in plasma and the high concentration in urine gives rise to the hypothesis that the conjugation of this compound may take place in part in the human kidney.

5. References

[1] L.Z. Benet, *J. Pharmacokin. Biopharm.*, 7 (1979) 1.
[2] M. Hammarlund-Udenaes and L.Z. Benet, *J. Pharmacokin. Biopharm.*, 17 (1989) 1.
[3] T.B. Vree, M. van den Biggelaar-Martea and C.P.W.G.M. Verwey-van Wissen, *J. Chromatogr.*, 578 (1992) 239.
[4] B. Beerman, E. Dalén, B. Lindström, and A. Rosén, *Eur. J. Clin. Pharmacol.*, 9 (1975) 51.
[5] B. Calesnick, J.A. Christensen and M. Richter, *Proc. Soc. Exp. Biol. Med.*, 123 (1966) 17.
[6] J.D. Wallin, P. Ryals and N. Kaplowitz, *J. Pharmacol. Exp. Ther.*, 200 (1977) 52.
[7] G.J. Yakatan, D.D. Maness, J. Scholler, W.M.J. Novick and J.T. Doluisio, *J. Pharm. Sci.*, 65 (1976) 1456.
[8] P. Hajdú and A. Häusler, *Arzneim. Forsch.*, 14 (1964) 709.
[9] A. Häusler and P. Hajdú, *Arzneim. Forsch.*, 14 (1964) 710.
[10] R.A. Branch, C.J.C. Roberts, M. Homeida and D. Levine, *Br. J. Clin. Pharmacol.*, 4 (1977) 121.
[11] A.W. Forrey, B. Kimpel, A.D. Blair and R.E. Cutler, *Clin. Chem.*, 20 (1974) 152.
[12] E. Mikkelsen and F. Andreasen, *Acta Pharmacol. Toxicol.*, 41 (1977) 254.
[13] F. Andreasen, C.K. Christensen, F.K. Jakobsen and C.E. Mogensen, *Acta Pharmacol. Toxicol.*, 49 (1981) 223.
[14] K. Carr, A. Rane and J.C. Frölich, *J. Chromatogr.*, 145 (1978) 421.
[15] A.L.M. Kerremans, Y. Tan, *C.A.M. van Ginneken and F.W.J. Gribnau*, J. Chromatogr. 229 (1982) 129.
[16] A. Matsuura, T. Nagayama and T. Kitagawa, *J. Chromatogr.*, 617 (1993) 339.
[17] F.G.M. Russel, Y. Tan, J.J.M. van Meijel, F.W.J. Gribnau and C.A.M. van Ginneken, *J. Chromatogr.*, 496 (1989) 234.
[18] D.E. Smith, E.T. Lin and L.Z. Benet, *Drug Metab. Dispos.*, 8 (1980) 337.
[19] T.B. Vree, E. van der Kleijn, Ch.F. Gusdorf and J.G.M. Zum Vörde Sive Vörding, *Pharm. Weekbl.*, 118 (1983) 121.
[20] A. Rachmel, G.A. Hazelton, A.L. Yergey and D.J. Liberato, *Drug Metab. Dispos.*, 13 (1985) 705.
[21] M. Faed, *Drug Metab. Rev.*, 15 (1984) 1213.
[22] T.B. Vree TB and E.W.J. Beneken Kolmer, *Pharm. Weekbl. [Sci].*, 14 (1993) 83.
[23] T.B. Vree, M. van den Biggelaar-Martea, C.P.W.G.M. Verwey-van Wissen, *J. Chromatogr.*, 616 (1993) 771.
[24] T.B. Vree, E.W.J. van Ewijk-Beneken Kolmer and F.J.M. Nouws, *J. Chromatogr.*, 579 (1992) 131.
[25] J.H. Proost and D.K.F. Meyer, *Comput. Biol. Med.*, 22 (1992) 155.
[26] L.F. Fieser and M. Fieser, *Organic Chemistry*, 3rd ed., Reinhold, New York, 1956, p. 796.

[27] T.B. Vree, Y.A. Hekster and P.G. Anderson, *Ann Pharmacother.*, 26 (1992) 1421.
[28] T.B. Vree, E.W.J. Beneken Kolmer, E.W. Wuis and Y.A. Hekster, *Pharm. Weekbl. [Sci].*, 14 (1992) 325.
[29] T.B. Vree, E.W.J. van Ewijk-Beneken Kolmer, E.W. Wuis, Y.A. Hekster and M.M.M. Broekman, *Pharm. World. Sci.*, 15 (1993) 197.
[30] T.B. Vree, M. van den Biggelaar-Martea, E.W.J. van Ewijk-Beneken Kolmer and Y.A. Hekster, *Pharm. World Sci.*, 15 (1993) 98.