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Direct gradient reversed-phase high-performance liquid chromatographic determination of salicylic acid, with the corresponding glycine and glucuronide conjugates in human plasma and urine

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Abstract

A gradient reversed-phase HPLC analysis for the direct measurement of salicylic acid (SA) with the corresponding glycine and glucuronide conjugates in plasma and urine of humans was developed. The glucuronides were isolated by preparative HPLC from human urine samples. The concentration of the glucuronides in the isolated fraction were determined after enzymatic hydrolysis. Salicylic acid acyl glucuronide (SAAG) was not present in plasma. No isoglucuronides were present in acidic and alkaline urine of the volunteer. The limits of quantitation in plasma are: SA 0.2 μg/ml, salicyluric acid (SU) 0.1 μg/ml, salicylic acid phenolic glucuronide (SAPG) 0.4 μg/ml and salicyluric acid phenolic glucuronide (SUPG) 0.2 μg/ml. The limit of quantitation in urine is for all compounds 5 μg/ml. Salicylic acid acyl glucuronide is stable in phosphate buffer pH 4.9 during 8 h at 37°C; thereafter it declines to 80% after 24 h. The subject's urine was therefore acidified by the oral intake of 4 × 1.2 g of ammonium chloride/day. With acidic urine, hardly any salicylic acid is excreted unchanged (0.6%). It is predominantly excreted as salicyluric acid (68.7%).

1. Introduction

Salicylic acid (SA), released from its precursor acetylsalicylic acid, is widely used for its analgesic and antipyretic effects [1]. Salicylic acid is extensively metabolized and the metabolites are excreted in the urine [1]. Salicylic acid, as an *ortho*-hydroxy substituted benzoic acid, is mainly conjugated to glycine, forming salicyluric acid (SU). Conjugation with glucuronic acid can take place at the phenolic hydroxy group leading to salicylic acid phenolic glucuronide (SAPG; ether glucuronide), and at the carboxylic group leading to salicylic acid acyl glucuronide (SAAG; ester glucuronide). SU is also further conjugated with glucuronic acid to form salicyluric acid phenolic

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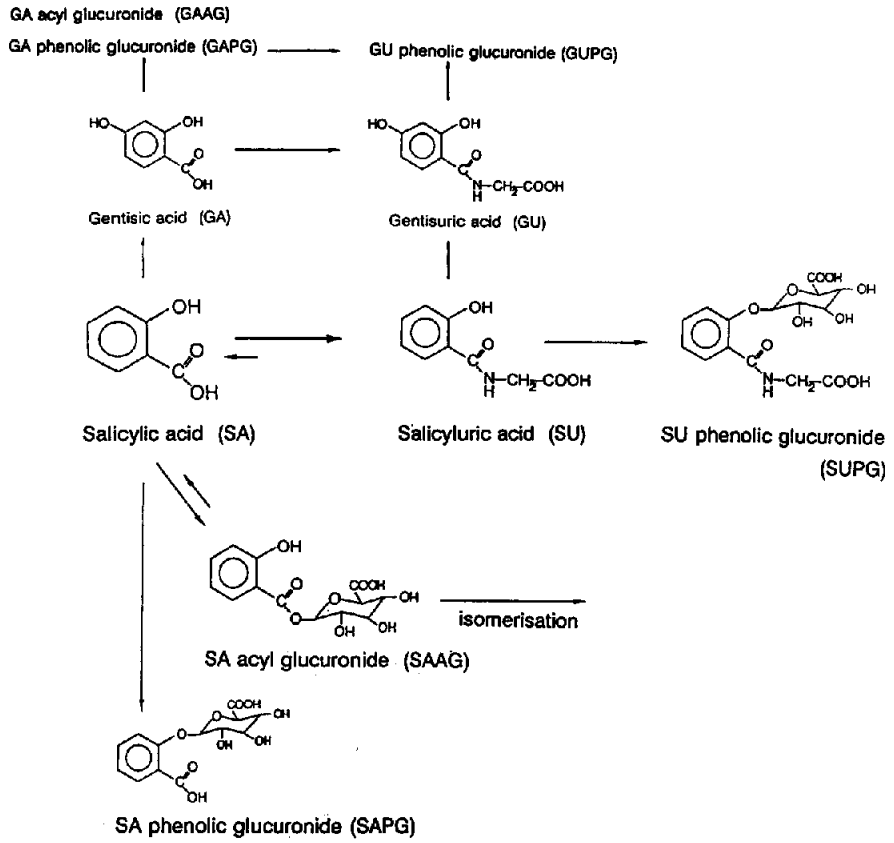

Fig. 1. Molecular structures of salicylic acid and its possible metabolites.

glucuronide (SUPG). Fig. 1 shows the metabolic pathways of salicylic acid and the possible metabolites [1–5]. Acyl glucuronides are known for their capacity to isomerize and hydrolyze into salicylic acid when the pH of the medium is 7 or higher [6–9].

For a proper elucidation of the pharmacokinetics of salicylic acid, a HPLC method able to separate all metabolites, should be available. A first attempt was made by Imhoff *et al.*, measuring SAPG, but not the SAAG [10]. Shen *et al.* developed a suitable isocratic HPLC method for salicylic acid and its metabolites and re-investigated the pharmacokinetics [11,12]. It was shown that the acyl glucuronide was stable at pH 5 and unstable at pH 7–8; however in the kinetic study the urinary pH of the volunteers was left uncontrolled, leading to large fluctuations in the renal excretion of salicylic acid and SAAG [12]. To demonstrate that the isomerization and hydrolysis of salicylic acid acyl glucuronide will take place in alkaline urine and possibly affects the renal excretion of salicylic acid, a direct gradient HPLC method was developed for salicylic acid with the glycine and glucuronide conjugates.

2. Experimental

2.1. Chemicals

Salicylic acid and sodium salicylate were obtained from De Onderlinge Pharmaceutische Groothandel OPG (Utrecht, Netherlands). Gentisic acid was obtained from Janssen Chimica (Beerse, Belgium), hippuric acid from Sigma (St. Louis, MO, USA), and salicyluric acid from Merck-Schuchardt (Hohenbrunn, Munich, Germany). Ammonium chloride (Ammonchlor) was

obtained from Südmedica (Munich, Germany). Sodium bicarbonate was obtained from the hospital pharmacy.

Acetic acid, and orthophosphoric acid were of analytical grade and obtained from Merck (Darmstadt, Germany). All other reagents were of p.a. quality and obtained from Merck. Acetonitrile Fisons was obtained from Betron (Rotterdam, Netherlands).

Salicylic acid acyl glucuronide, salicylic acid phenolic glucuronide and salicyluric acid phenolic glucuronide were isolated and identified by enzymic hydrolysis in human urine after the intake of 1000 mg salicylic acid. β-Glucuronidases were obtained from Sigma.

2.2. Gradient HPLC analysis

The HPLC system consisted of a Spectra-Physics SP 8775 autosampler (Spectra-Physics, Eindhoven, Netherlands), an SP 8800 ternary HPLC pump, a Kratos Spectroflow 783 UV detector (Separations, Hendrik Ido Ambacht, Netherlands), and an SP 4290 integrator. A two-column system was used. The first column (15 cm × 4.6 mm I.D.) was packed with Cp Spher C_8 (particle size 8 μm) (Cat. No. 950748, Chrompack, Middelburg, Netherlands), and was followed by a second column (25 cm × 4.6 mm I.D.) packed with Spherisorb 5 ODS, particle size 5 μm (Chrompack, Cat. No. 28812).

The mobile phase consisted of a mixture of acetonitrile (solvent A), and 6 g orthophosphoric acid with 1 ml glacial acetic acid in 1 l distilled water (solvent B). At $t = 0$ the mixture consisted of 1.0% A and 99% B, which in 35 min linearly changed to 35% A and 65% B (v/v). At $t = 35$, the system was changed to its initial state within 3 min and was left for 2 min to equilibrate ($t = 40$ min total time). The flow-rate was 1.5 ml/min. An injection loop of 20 μl was used. Detection of salicylic acid and conjugates was effected at 236 nm. The assay was carried out at room temperature. Retention times and capacity factors are given in Table 1.

2.3. Sample treatment

Plasma samples (200 μl) were deproteinized with 200 μl of 0.33 *M* perchloric acid, mixed thoroughly, and centrifuged at 2600 *g* for 10 min. The clear supernatant was directly injected onto the column. Urine samples (100 μl) were diluted with 0.9 ml of 0.2 *M* phosphate buffer (pH 5), mixed, and injected onto the column.

Table 1
Retention times and capacity factors of salicylic acid and its metabolites

Compound[a]	t_R (min)	k'
t_0	2.6	
SUPG	15.57	4.99
SAPG	17.63	5.78
Hippuric acid[b]	18.23	6.01
Gentisic acid (GA)	18.98	6.30
SAAG	21.45	7.25
Salicyluric acid (SU)	23.37	7.99
Salicylic acid (SA)	32.09	11.34

[a] PG = phenolic glucuronide, AG = acyl glucuronide.
[b] Endogenous compound.

2.4. Isolation of the acyl glucuronides

The peaks in the chromatogram which showed a pharmacokinetic behaviour (*i.e.* time dependent concentration) of metabolites of salicylic acid were 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, 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).

At $t = 0$, the mobile phase consisted of 1% acetic acid in water and acetonitrile (99:1, v/v) which thereafter changed linearly in 24 min to 70:30 (v/v). The retention time of salicylic acid acyl glucuronide was 21 min, of salicylic acid phenolic glucuronide 17 min, and of salicyluric acid phenolic glucuronide 15 min. Concentration of the trapped sample was carried out by an IKA rotavapor (Janke and Kunkel, Staufen, Ger-

many) equipped with a Trivac vacuum pump (Leybold-Heraeus, Woerden, Netherlands).

The urine was treated as follows: to urine (pH 5) was added 20 g/l Celite 545 and the mixture was filtered (filter type GS 0.22 μm, Millipore, Etten-Leur, Netherlands) and injected onto the column.

2.5. Deconjugation of the glucuronides

Deglucuronidation was carried out with 100 μl of urine, 100 μl of β-glucuronidase, and 800 μl of 0.2 *M* (Na_2H/KH_2)phosphate buffer at 37°C for 2 h.

Four different glucuronidase enzymes (A–D) were used:

(A) β-Glucuronidase type B1 100 000 U/ml (Bovine liver, Sigma, USA, Cat. No. G-0251) and phosphate buffer pH 5.0;

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

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

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

2.6. Calibration curves

The fractions of acyl-, and phenolic glucuronide of salicylic acid and salicyluric acid isolated from human urine by preparative HPLC, were deconjugated by enzyme system D (see above) at 37°C for 0.5 h. The increase in the concentrations of salicylic acid represented the concentration of the specific conjugate in the isolated fraction with the help of the following formula:

$$[\text{SAgluc}] = d[\text{SA}] \times M_{\text{SAgluc}}/M_{\text{SA}}$$

where d[SA] is the difference in concentration of salicylic acid before and after deconjugation, and M is relative molecular mass ($n = 4$). This was used as a standard solution with known concentration. A similar formula is used for the measurement of the concentration of salicyluric acid phenolic glucuronide in the standard solutions ($n = 4$).

Calibration curves for salicylic acid, salicylic phenolic-, and acyl glucuronide, salicyluric acid and salicyluric acid phenolic glucuronide were made by adding known quantities of the compound to blank human plasma and urine. The correlation coefficients of the calibration curves of all compounds in plasma and urine were better than 0.9992.

2.7. Stability

The stability of the isolated glucuronides of salicylic acid and salicyluric acid was tested as follows:

(A) In buffer. Four-ml volumes of solutions of SAPG, SAAG and SUPG (100 μg/ml) were brought to pH 4.9 and pH 7.4 with phosphate buffer, and incubated at 37°C for 24 h. At regular time intervals a 100-μl sample was taken and 900 μl of 0.2 *M* phosphate buffer pH 5.0 was added. The samples were mixed and injected onto the column.

(B) In plasma. A 2-ml volume of plasma of pH 7.4 (1900 μl of blank plasma spiked with 100 μl of standard solution) was incubated at 37°C for 24 h. At regular time intervals a sample was taken and the reaction stopped by deproteinizing with 100 μl of 0.33 *M* perchloric acid. After centrifuging for 10 min at 2600 *g* the supernatant was injected onto the column.

The stability of all compounds in phosphate buffer pH 4.9 was tested in the autosampler for 24 h. Samples were taken every hour and injected onto the column.

2.8. Isomerization of salicylic acid acyl glucuronide

Isolated salicylic acid acyl glucuronide was subjected to hydrolysis and isomerization in a phosphate buffers of pH 4.9 and 7.4 and in blank plasma during 24 h at 37°C. The formation of isoglucuronides was followed by taking and analysing a sample every hour.

2.9. Limits of detection and quantitation

The limits of detection in water and quantitation of salicylic acid and its conjugates in plasma and urine were determined at a signal-to-noise ratio of 3, and are shown in Table 2.

2.10. Subjects

A Caucasian male (90 kg, age 50 years) with normal liver and kidney function volunteered for a pilot study. The study had the approval of the Sint Radboud hospital Ethics Committee. A single oral dose of approximately one gram salicylic acid (as sodium salt) was administered in two gelatine capsules after an overnight fast. In the first experiment the urine was kept acidic by administering 1.2 g ammonium chloride (Ammonchlor) 4 times a day and three weeks later in the second experiment the urine was kept alkaline by administering 3 g sodium bicarbonate 4 times a day.

2.11. Sampling

Blood samples were drawn at regular time intervals by means of fingertip puncture with Monolet lancets (Monoject, St. Louis, MO USA). After centrifugation for 10 min at 2600 *g* plasma samples were stored at −20°C pending analysis.

Urine was collected upon untimed voiding. The total time of sample collection was 60 h. Urinary pH was measured immediately after collection. Of each urine void 3 samples of 5 ml were stored at −20°C pending analysis.

Table 2
Limits of detection and quantitation of salicylic acid and its metabolites[a]

Compound	Detection limit in water (μg/ml)	Quantitation limit (μg/ml)	
		Plasma	Urine
SUPG	0.11	0.2	5
SAPG	0.13	0.4	5
SAAG	0.08	n.d.[b]	5
SU	0.05	0.1	5
SA	0.08	0.2	5

[a] $n = 3$, mean value.
[b] n.d. = not detectable due to hydrolysis.

2.12. Pharmacokinetics

The pharmacokinetic parameters were calculated using the MW/Pharm computer package (Mediware, Groningen, Netherlands) [13].

3. Results

Fig. 2 shows the chromatograms of salicylic acid with its glucuronide conjugates in actual urine samples at pH 5.73 and at pH 8.48 (compound H is endogenous hippuric acid). Fig. 3 shows the chromatograms of salicylic acid with its glucuronide conjugates in an actual plasma sample at pH 7.40. Both in the urine and in the plasma samples no gentisic acid or its glucuronides could be detected in all collected samples during 60 h.

The phenolic(SAPG)- and acyl(SAAG) glucuronides of salicylic acid were identified based on their rate of deconjugation by β-glucuronidase, which is instantaneously for the acyl glucuronides. In addition when the two glucuronides were treated with alkaline (pH 7.4), only the acyl glucuronide is subject to isomerisation and hydrolysis. Fig. 4 shows a chromatogram of salicylic acid acyl glucuronide after 4 h isomerization. SAAG predominantly isomerized to the isoglucuronides and partly hydrolyzed to SA. SAPG and SUPG were stable in buffer and plasma during 24 h. The rate of decline of salicylic acid acyl glucuronide (SAAG) in plasma pH 7.4, buffer of pH 7.4, and buffer of pH 4.9 is shown in Fig. 5. SAAG is stable at pH 4.9 and 37°C during 8 h and declined thereafter slowly to reach 80% of its initial value after 24 h. All compounds were stable in the autosampler for 24 h in phosphate buffer pH 4.9 at room temperature.

Glucuronidase hydrolysis of the glucuronides by all four β-glucuronidase systems tested was completed after 2 h. Enzyme system D, with

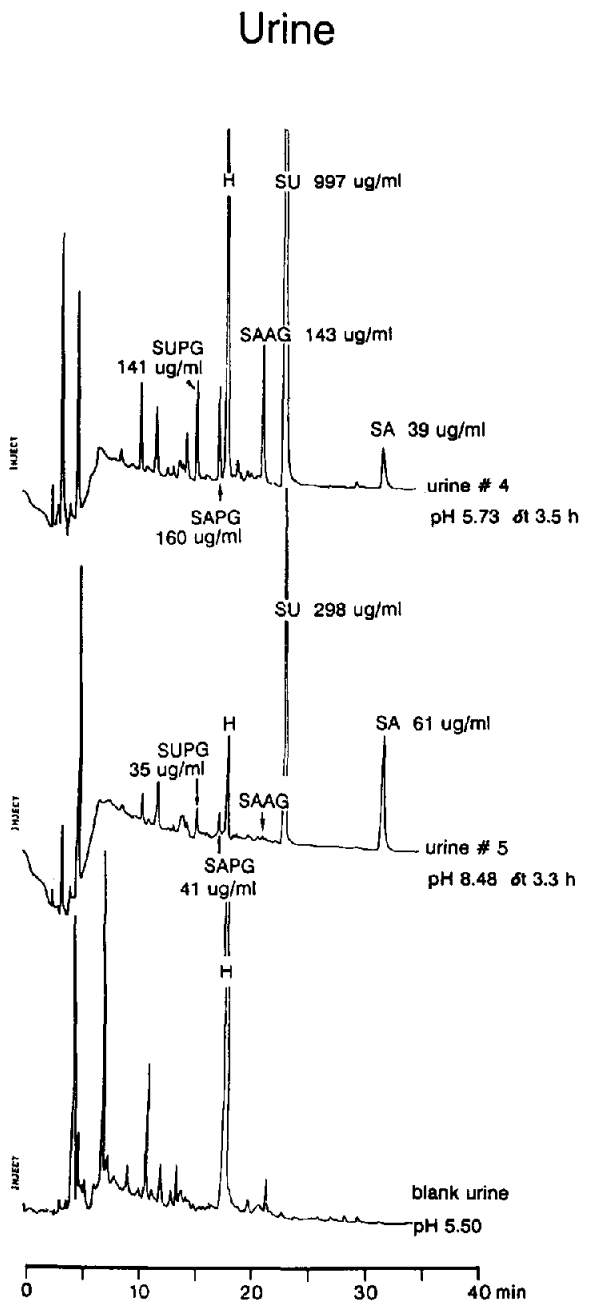

Fig. 2. Chromatograms of acidic urine sample No.4 (pH 5.73), alkaline urine sample No.5* (pH 8.48) and a blank urine sample of the same volunteer after oral administration of 1382 mg sodium salicylate (1183 mg salicylic acid) (No.5* taken from the second experiment in which the same volunteer took 1465 mg sodium salicylate and the urine was kept alkaline; pH 8.32 ± 0.19). H is endogenous hippuric acid.

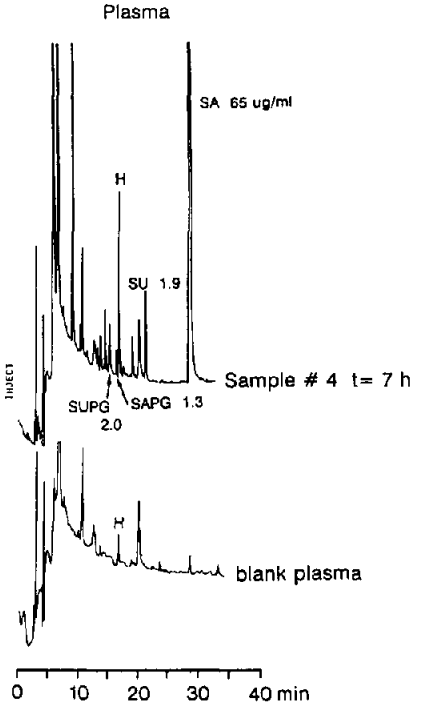

Fig. 3. Chromatograms of plasma sample No.4, and a blank plasma sample of the same volunteer after oral administration of 1382 mg sodium salicylate. H is hippuric acid.

Escherichia coli type VIIA, was chosen over the other systems because of the cleaner chromatograms. The intra-day and inter-day variations of salicylic acid, the glucuronides and glycine conjugates are shown in Tables 3 and 4 respectively.

The plasma concentration–time curves and the renal excretion rate–time profiles of salicylic acid, its acyl glucuronide, the phenolic glucuronide, salicyluric acid, salicyluric acid phenolic glucuronide after oral administration of 1382 mg sodium salicylate (= 1183 mg salicylic acid) and acidic urine conditions are shown in Fig. 6.

The renal excretion rate–time profiles of the parent drug and its metabolites show a terminal half-life of 3 h. Approximately 90% of the administered dose is excreted in the urine, predominantly as salicyluric acid (68.7%) and to a lower extent the glucuronides SAPG (4.1%), SAAG (6.0%) and SUPG (5.2%). The unconju-

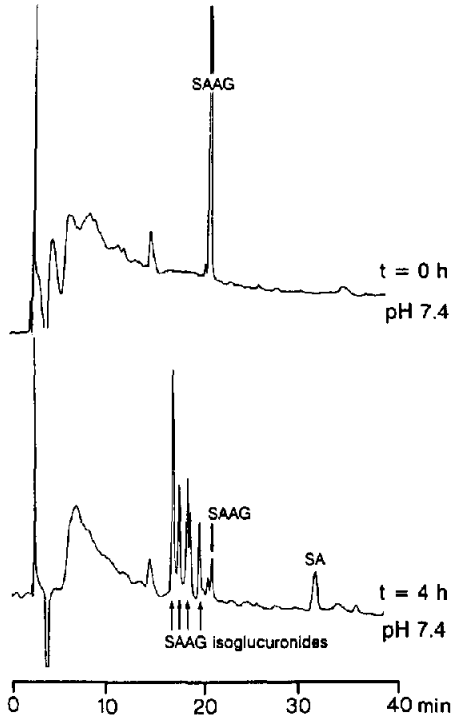

Fig. 4. Chromatograms of isolated salicylic acid acyl glucuronide at the start ($t = 0$ h) and after 4 h ($t = 4$ h) of the process of acyl isomerisation in a phosphate buffer of pH 7.4.

gated compounds are excreted only for a small fraction of the dose: salicylic acid (0.6%), Some pharmacokinetic parameters of salicylic acid are summarized in Table 5.

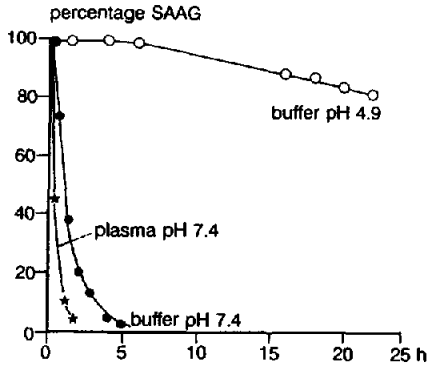

Fig. 5. Stability of the acyl glucuronide of salicylic acid (SAAG) in plasma with pH 7.4 and in phosphate buffer with pH 4.9 and 7.4 respectively.

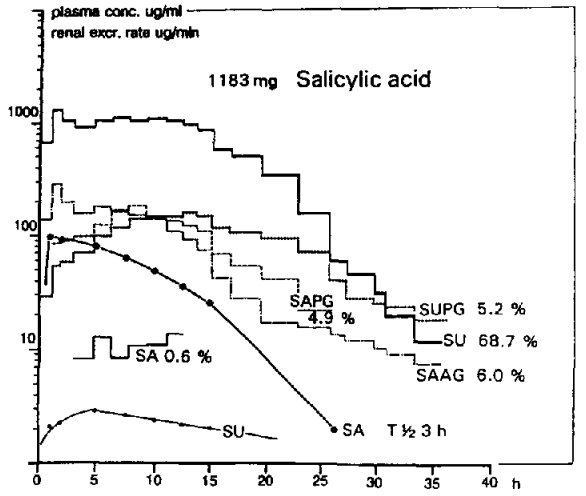

Fig. 6. Plasma concentration–time curves and renal excretion rate–time profiles of salicylic acid (SA), its acyl glucuronide (SAAG), salicylic acid phenolic glucuronide (SAPG), and salicyluric acid (SU), in a volunteer with acidic urine conditions after an oral dose of 1382 mg sodium salicylate (= 1183 mg salicylic acid).

4. Discussion

4.1. HPLC

Salicylic acid was measured initially by spectrophotometric methods [14,15] which lack sufficient specificity to establish the kinetics of the parent drug and its metabolites. HPLC analysis of salicylic acid and its unconjugated metabolites is the method of choice when the kinetics of the metabolites has to be investigated [16–23]. The isocratic HPLC method of Shen *et al.* [11] uses a 30-cm phenyl column, which enabled the separation of all compounds and conjugates in 16 min. In this short time also all endogenous water soluble waste compounds elute from the column such as their compound X, which must have been hippuric acid. However, the longer the retention time, the better salicylic acid and its conjugates may be separated from the endogenous compounds.

A direct gradient HPLC analysis of the glucuronide conjugates of salicylic acid was not previously reported. The presented method with isolation of the conjugates, followed by deconju-

Table 3
Inter-day and intra-day coefficients of variation of salicylic acid and its metabolites in plasma[a]

Compound	Concentration (μg/ml)	Intra-day			Inter-day		
		Mean (μg/ml)	Precision (C.V., %)	Accuracy (C.V., %)	Mean (μg/ml)	Precision (C.V., %)	Accuracy (C.V., %)
SUPG	1.01	0.86	4.3	14	0.82	5.5	19
	2.01	1.73	3.8	14	1.73	6.6	14
SAPG	0.42	0.55	6.9	30	0.55	9.5	31
	0.83	0.70	5.4	15	0.74	15.7	11
SAAG[b]	–	–	–	–	–	–	–
SU	1.35	1.16	3.3	14	1.12	5.6	17
	2.69	2.28	2.9	15	2.28	2.4	15
SA	1.11	1.01	3.2	8.5	1.01	6.0	8.5
	56.8	58.5	4.5	3.0	54.7	4.8	3.7
	114	115	1.7	1.2	113	2.9	0.9

[a] plasma samples with known concentrations. *In vitro*, $n = 5$.
[b] Not present in plasma.

Table 4
Inter-day and intra-day coefficients of variation of salicylic acid and its metabolites in urine[a]

Concentration (μg/ml)	C.V. (%)	
	Inter-day	Intra-day
SUPG		
32.5	5.4	2.0
50.2	3.4	1.1
111	3.6	0.5
SAPG		
20.1	5.7	2.1
70.8	5.8	2.0
105	3.4	2.3
165	4.5	0.4
SAAG		
6.02	9.7	1.7
188	5.6	1.4
465	3.6	1.7
SU		
143	7.9	1.8
416	9.2	1.0
1061	4.3	0.4
1248	3.0	1.3
SA		
12.5	9.0	2.5
60.6	6.3	2.0
155	4.3	2.0
267	3.3	0.6

[a] samples with unknown concentration, concentration measured. *In vivo*, n = 5.

gation to prepare stock solutions with known concentrations, in order to construct calibration curves of the glucuronide, is an extention of those already published by our group [9,24–28]. The 30-cm phenyl column and isocratic solvent of Shen *et al.*, and the present method with gradient elution over a 40-cm $C_8 + C_{18}$ column may be considered as alternatives for a good separation of the water soluble conjugates of salicylic acid.

4.2. Stability

Salicylic acid acyl glucuronide is unstable at alkaline pH [6,11]. The instability of acyl glucuronides can easily be recognized when urine, containing these glucuronides, is kept at slightly alkaline pH and room temperature. Alkaline acyl glucuronide hydrolysis results in an increase of free aglycone as shown in Fig. 4. SAAG was not present in plasma because of the pH 7.4. The compound in the plasma chromatogram in Fig. 3 at the retention time of SAAG did not show a pharmacokinetic behaviour and could not be identified as SAAG by means of alkaline isomerisation and hydrolysis or hydrolysis by β-glucuronidase. The renal excretion of salicylic acid increases with increasing urinary pH [28], which in part is caused by hydrolysis of

Table 5
Some pharmacokinetic parameters of salicylic acid and its conjugates in a healthy caucasian male volunteer under acidic and alkaline urine conditions

Parameter	Value	
	pH 5.31 ± 0.32	pH 8.32 ± 0.19
Body weight (kg)	90	90
Dose (mg acid)	1183	1263
Dose (mg sodium salt)	1382	1465
Salicylic acid		
C_{max} (μg/ml)	94	114
t_{max} (h)	1	2
$t_{1/2}$ (h)	3.0	2.6
Volume of distribution (l/kg)	0.18	0.19
Percentage of the dose excreted (%)		
Salicylic acid	0.6	22.2
Salicylic acid acyl glucuronide	6.0	2.3
Salicylic acid phenolic glucuronide	4.9	4.7
Salicyluric acid	68.7	58.3
Salicyluric acid phenolic glucuronide	5.2	3.9
total	85.4	91.4
Renal clearance (mean ± S.D.)(ml/min)		
Salicylic acid ($n = 5$)[a]	0.16 ± 0.05	9.0 ± 2.9
Salicyluric acid ($n = 15$)[a]	422. ± 42	554. ± 51
Maximal renal excretion rate (mean ± S.D.)(mg/h)		
Salicyluric acid ($n = 9$)[b]	63.6 ± 1.9	71.4 ± 9.0
Salicylic acid phenolic glucuronide ($n = 6$)[b]	8.4 ± 0.3	10.0 ± 1.0
Protein binding (mean ± S.D.)(%)		
Salicylic acid (*in vivo*, $n = 6$)	93.8 ± 0.95	n.d.[c]
Salicyluric acid (*in vivo*, $n = 6$)	89.7 ± 2.2	n.d.

[a] Renal clearance (ml/min) = renal excretion rate (μg/min)/plasma concentration (μg/ml).
[b] Based on n urine samples.
[c] n.d. = not detected.

SAAG. This hydrolysis process was rapid in phosphate buffer of pH 7.4 (4 h reaction time, Figs. 4,5). With alkaline urine pH, SAAG was not present due to the hydrolysis, while the isomerisation process apparently did not take place as the isoglucuronides were not visible (sample taken from the second experiment in which the urine of the same volunteer was kept alkaline; pH > 7).

However, the urine collection time and storage in the bladder was 3.3 h, which must have been long enough to allow a visible isomerisation. An explanation may be that the SAAG concentration in the urine samples was too low to generate a visible concentration of the isoglucuronides, like those for probenecid acyl glucuronide [8] and naproxen acyl glucuronide [9]. The increase in the percentage of the dose of salicylic acid excreted under alkaline urine conditions compared to that under acidic conditions (21.6%) is much higher than the decrease of SAAG (3.7%) available for alkaline hydrolysis.

To avoid large fluctuations in the renal excretion of salicylic acid a volunteer study must be carried out with controlled and preferably acidic urine (pH 5–5.5) to prevent hydrolysis during the transfer time of the glucuronides in the kidney and storage in the bladder.

5. Conclusion

The HPLC method enables the measurement of salicylic acid and its metabolites. The pilot pharmacokinetic experiment is in agreement with earlier studies. A full pharmacokinetic study of salicylic acid and its conjugates will be published elsewhere.

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