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Novel direct high-performance liquid chromatographic method for determination of salicylate glucuronide conjugates in human urine

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ABSTRACT

A novel direct high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of three salicylate glucuronide conjugates and other salicylate metabolites in human urine has been developed. Salicylate glucuronide conjugates were purified by HPLC from the urine of a volunteer after oral administration of aspirin and identified by selective hydrolysis with β-glucuronidase and with sodium hydroxide. This method gave high reproducibility with coefficients of variation less than 10%. The total urinary recovery of salicylic acid after a single 1.2-g dose of soluble aspirin was greater than 90%. This assay has been successfully used to re-evaluate the capacity-limited pharmacokinetics of salicylic acid in humans.

INTRODUCTION

Salicylic acid (SA), usually administered as its precursor aspirin, is widely used for its analgesic–antipyretic effects and in the chronic management of rheumatoid arthritis and osteoarthritis [1,2]. Aspirin is rapidly hydrolysed to SA on absorption [3]. SA is metabolised to form various metabolites (Fig. 1). Three important metabolites are salicyluric acid (SU), salicyl phenolic glucuronide (SPG) and salicyl acyl glucuronide (SAG) [3,4]. Kinetic characteristics of their formation in man has been extensively described by Levy and co-workers [4–9] who emphasized the role of capacity-limited formation for SU and SPG.

A reliable analytical method for glucuronides is necessary to study the pharmacokinetics of SA. Spectrophotometric [10] and fluorimetric [11] methods have been reported earlier for the estimation of salicylate and its glucuronide conjugates in urine. The indirect determination of SPG, by hydrolysis of all salicylate glucuronide metabolites and subtraction of the estimated amounts of SU, SAG etc., fails to note the contribution of salicyluric phenolic glucuronide (SUPG) [8]. As a consequence pharmacokinetic parameters estimated for SPG may be inaccurate. Only one direct HPLC assay for SPG in human urine has been reported [12] but this method did not quantitate SAG directly.

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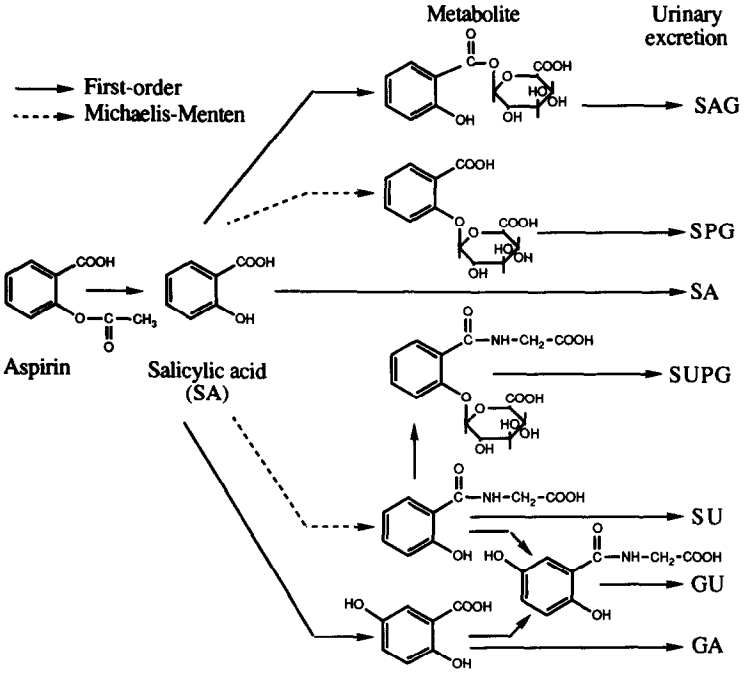

Fig. 1. Metabolic pathways of salicylic acid (SA) in human. Pathways indicated with solid lines follow first-order kinetics whereas those indicated with dashed lines are saturable pathways. SAG = salicyl acyl glucuronide; SPG = salicyl phenolic glucuronide; SU = salicyluric acid; SUPG = salicyluric phenolic glucuronide; GU = gentisuric acid; GA = gentisic acid.

The present investigation reports a direct high-performance liquid chromatographic (HPLC) method for a simultaneous analysis of gentisic acid (GA), SU, SA and three glucuronide metabolites namely SUPG, SAG and SPG. This method does not require chemical or enzymatic hydrolysis of salicyl glucuronide conjugates to SA.

EXPERIMENTAL

Materials

All chemicals and reagents used are of analytical grade. Aspirin, SA, GA, SU and β-glucuronidase (type VII from *Escherichia coli*) were purchased from Sigma (St. Louis, MO, U.S.A.). Phenoxyacetic acid (PAA), potassium dihydrogenphosphate (KH_2PO_4), triethylamine (TEA), orthophosphoric acid, HPLC-grade acetonitrile and methanol were obtained from BDH (Poole, U.K.). SPG was a gift from Professor F. Bochner and Ms. D. Imhoff (University of Adelaide, Adelaide, Australia). Soluble aspirin (Aspro Clear tablets) were obtained from Nicholas Kiwi (NZ) (Otahuhu, Auckland, New Zcaland).

Chromatographic conditions

Separation and quantification of analytes was performed on a reversed-phase μBondapak phenyl column (300 mm × 4.6 mm I.D., 10 μm average particle size, Waters Assoc., Milford, MA, U.S.A.). A Shimadzu Model LC-6A solvent pump (Tokyo, Japan) and a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl injector loop were used. The absorbance of the eluent was determined using a Shimadzu Model SPD-6AV UV–VIS spectrophotometric detector.

Conditions for optimal separation of the compounds of interest were studied using a mobile phase consisting of methanol–acetonitrile–TEA– 10 m*M* KH_2PO_4 (18:110:1:871, v/v, pH 2.8 ± 0.05) in which the final pH of the mobile phase was adjusted with concentrated orthophosphoric acid. After adjustment, the mobile phase was degassed and filtered using a membrane filter (0.45 μm, Millipore, Bedford, MA, U.S.A.). The flow-rate was 1 ml/min and the effluent was monitored at 237 nm.

Hydrolysis procedures

Urine samples collected from a volunteer over a 36-h period following 1.2-g soluble aspirin were used in the hydrolysis procedures as follows.

Alkaline hydrolysis. A 1.0-ml urine sample was diluted with 0.8 ml of 0.1 *M* phosphate buffer (pH 6.8) containing 0.5 mg/ml PAA (internal standard), mixed and incubated with 100 μl of 0.1 *M* NaOH at 37°C for 15 min. A second 1.0-ml aliquot of the above urine sample was incubated with 100 μl of water under the same conditions as above. After incubation, 100 μl of 0.1 *M* HCl were added to the first sample and 100 μl water to the second sample. The tubes were centrifuged at 2000 *g* for 15 min and a 20-μl sample of the clear supernatant was injected onto the HPLC column.

β-Glucuronidase hydrolysis. A 1.0-ml urine sample was diluted with 0.1 *M* phosphate buffer (pH 6.8) containing 0.5 mg/ml PAA, mixed and incubated with 2000 U/ml β-glucuronidase at 37°C for 2 h in a water bath. Another 1.0-ml aliquot of urine was incubated under the same conditions without β-glucuronidase. After incubation, the samples were centrifuged at 2000 *g* for 15 min and the clear supernatant (20 μl) injected onto the HPLC column.

Isolation and characterization of salicylate glucuronides

Urine samples were collected from a volunteer during a 36-h period prior to (control) and after the ingestion of 1.2 g of soluble aspirin (glucuronide-containing urine). Samples were analysed using the chromatographic conditions previously described. This chromatographic analysis was carried out for both control and glucuronide-containing urine, prior to and after treatment with NaOH and β-glucuronidase, as described for salicylate glucuronide hydrolysis.

On the basis of the above treatments, chromatographic peaks were identified which corresponded to SAG, SPG and SUPG, respectively. The column effluent corresponding to these peaks was collected following injections of samples. The collected fractions were freeze-dried by lyophilization.

Standards for calibration curves

Calibration curves were constructed by introducing SPG, SA, GA and SU into drug-free urine. Dilutions of the stock standards were made to cover the concentration range 0.5–200 μg/ml for SA and SU and 1.25–200 mg/ml for GA and SPG. Concentrations of SAG and SUPG were the differences of SA and SU concentrations measured from the hydrolysed fractions of different dilutions and the pre-hydrolysed fractions of the corresponding dilutions. The differences were multiplied by the molecular weight factors 2.28 (for SAG) and 1.90 (for SUPG).

Quantitation of SAG, SPG, SUPG and salicylate in urine

SPG and salicylate concentrations were calculated from the chromatograms generated from the pre-hydrolysed urine aliquot. Peak-height ratios were measured and the concentrations were intrapolated from the above standard curves. Concentrations of SAG and SUPG were calculated from their peak-height ratios, SA and SU standard curves and their molecular weight factors.

Stability studies

Three urine samples were mixed with equivolumes of different 0.1 *M* phosphate buffers (pH 4.0, 7.0 and 9.0, respectively) and allowed to stand at ambient temperature over three days. The samples were monitored by HPLC at different times.

Optimal conditions for enzymatic hydrolysis

β-Glucuronidase (2000 U/ml) was added to a 1.0-ml aliquot of urine sample buffered with 1.0 ml of 0.1 *M* phosphate buffer (pH 6.8). Duration of incubation was varied from 15 min to 4 h and the incubation temperature varied from 25 to 70°C.

RESULTS AND DICUSSION

Initially, a μBondapak C_{18} column (300 mm × 4.6 mm, 10 μm average particle size, Waters Assoc.) and a C_8 column (150 mm × 4.6 mm, 10 μm average particle size, Alltech/Applied Science, Deerfield, IL, U.S.A.) were tested using the chromatographic conditions described in Experimental. It was found that the more polar metabolites (*i.e.* the SA glucuronides) were inadequately retained. Subsequently testing with a μBondapak phenyl column yielded the smallest range of k' values for all solutes studied, *i.e.* it offered the best compromise between the small k' values of the glucuronides and the large k' value of SA (Table I). The more hydrophilic compounds, salicylates and SA glucuronides, required a less hydrophilic stationary phase. As a consequence, the μBondapak phenyl column was chosen as the most appropriate for the analysis rather than C_8 and C_{18} column. The addition of TEA to the mobile phase gave a good resolution of the glucuronides and improved peak shape, and by adjusting the pH of the mobile

TABLE I

RETENTION ON REVERSED-PHASE HPLC COLUMNS FROM DIFFERENT SUPPLIES

Mobile phase consisting of methanol–acetonitrile–TEA–10 m*M* KH_2PO_4, pH 2.8 (18:110:1:871) was used at a flow-rate of 1 ml/min. Other chromatographic conditions, see Experimental.

Compound[a]	Capacity factor (k')		
	Phenyl	C_{18}	C_8
SUPG	0.9	1.4	0.5
SPG	1.1	1.6	1.2
GA	1.4	2.0	1.6
SAG	2.2	2.1	1.6
SU	3.2	5.2	3.5
SA	4.2	7.8	8.4

[a] Refer to Fig. 1 for abbreviations.

phase to 2.8 a separation of the glucuronides and urine interfering peaks could be obtained.

Following a 1.2-g single-dose administration of soluble aspirin to a healthy volunteer, three glucuronide conjugates of salicylate were present in urine (Fig. 2). Incubation of these urine samples with 0.1 *M* NaOH and β-glucuronidase (Fig. 3) showed that peaks F_1, F_2 and F_3 are SUPG, SPG and SAG, respectively. It has been reported that alcohols and phenols form ethereal glucuronides, which are resistant to hydrolysis by mild alkali. Carboxylic acids form ester glucuro-

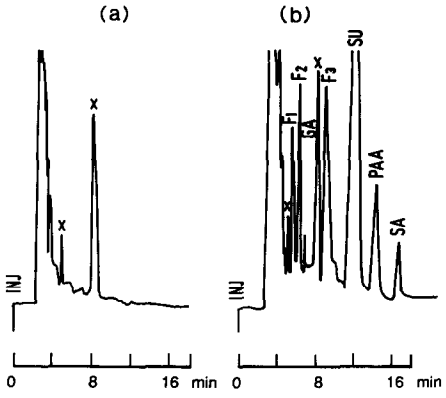

Fig. 2. Urinary chromatograms before (a) and after (b) a single 1.2-g dose of soluble aspirin. Blank urine (a) contains two endogenous peaks, X. F_1 (5.1 μg/ml), F_2 (17.0 μg/ml) and F_3 (18.2 μg/ml) are SA glucuronide conjugates in the urine sample after dosing (b). Other peaks are: GA = gentisic acid (3.8 μg/ml); SU = salicyluric acid (59.6 μg/ml); PAA = phenoxyacetic acid (internal standard); SA = salicylic acid (4.3 μg/ml). Refer to Fig. 1 for abbreviations.

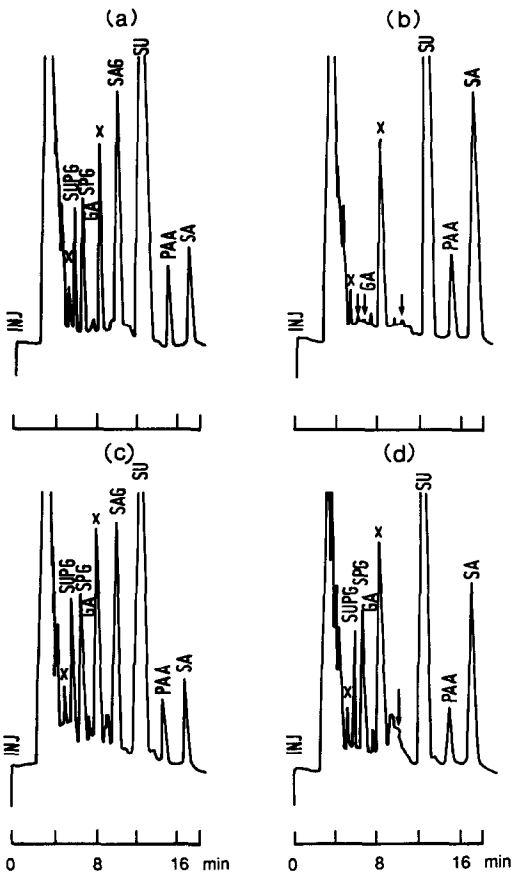

Fig. 3. Chromatograms of human urine samples after a single dose of 1.2 g soluble aspirin. (a) Before hydrolysis with β-glucuronidase, *i.e.* the sample was incubated under the same conditions without the enzyme. Concentrations were: SA (12 μg/ml), SU (65 μg/ml), GA (2.8 μg/ml), SAG (21.5 μg/ml), SPG (12.2 μg/ml) and SUPG (8.5 μg/ml). (b) After hydrolysis with β-glucuronidase (2000 U/ml) at 37°C for 2 h. Concentrations were: SA (26 μg/ml), SU (69.5 μg/ml), GA (2.1 μg/ml) and SAG, SPG and SUPG (undetectable). (c) Urine sample incubated at 37°C for 15 min without the treatment of NaOH. Concentrations of salicylate and its glucuronide conjugates are the same as (a).(d) Urine sample after hydrolysis with 0.1 *M* NaOH at 37°C for 15 min. Only SA concentration was increased from 12 to 21 μg/ml and SAG was not detected. The other salicylate concentrations were not significantly changed.

nides, which are easily split by mild alkali to liberate free glucuronic acid [13]. Fig. 4 shows the chromatograms of the purified fractions of individual glucuronide peaks prior to and after treatment with β-glucuronidase. All glucuronide peaks disappeared following these treatments, and there was a corresponding increase in the SU and SA peaks. No peaks other than those due to SU and SA resulted from these treatments. F_1 is considered to be SUPG since the hydrolysis of the F_1 fraction yielded a peak corresponding to SU (Fig. 4, a1 and a2). SUPG was the

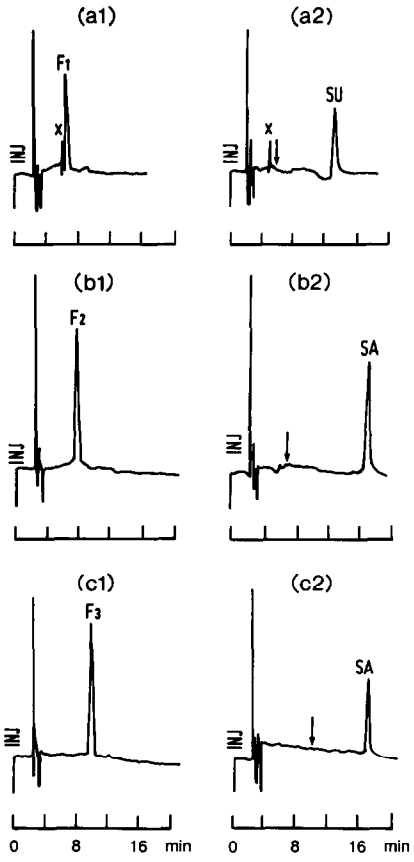

Fig. 4. Chromatograms of the reconstituted lyophilised SA glucuronide fractions before and after hydrolysis with 2000 U/ml β-glucuronidase at 37°C for 2 h. (a1) and (a2) show the chromatograms before and after the hydrolysis of fraction F_1 and (b1) and (b2) for fraction F_2 before and after the hydrolysis, respectively. (c1) and (c2) show the chromatograms before and after the hydrolysis of fraction F_3. Other abbreviations for peaks, refer to Fig. 2. Amount injected was 17, 25 and 23 μg/ml for F_1, F_2 and F_3, respectively.

first isolated from urine of a volunteer after oral ingestion of aspirin. This metabolite has been reported to be found in the plasma from a uremic patient on intermittent hemodialysis [14]. The fractions corresponding to the SUPG peak were collected for further characterization by enzymatic, chromatographic and UV absorbance criteria. F_2 was identified as SPG by comparison with the authentic compound. The evidence from the β-glucuronidase hydrolysis of the F_2 fraction also supports this conclusion. The F_2 peak completely disappeared and yielded a corresponding peak of SA after the hydrolysis (Fig. 4, b1 and b2). F_3 was characterized by its different response to NaOH and β-glucuronidase hydrolysis. The alkaline hydrolysis of the F_3 fraction abolished the F_3 peak and produced a peak corresponding to SA (Fig. 3c and d). After the hydrolysis of the F_3

fraction with β-glucuronidase, the F_3 peak disappeared and yielded a peak corresponding to SA (Fig. 4, c1 and c2). Thus these results sugggest that F_3 was SAG.

It has been reported that the hydrolytic conditions of phenolic glucuronides are optimized by incubation at 60°C for 30 min [15–17]. In this study, we found that the collected glucuronide fractions form HPLC could be totally hydrolysed by incubation with 2000 U/ml β-glucuronidase at 37°C (pH 6.8) for 2 h (Fig. 5).

Base-catalysed acyl migration of ester glucuronides of drugs has been reported previously [18–22]. This intramolecular transesterification occurs mainly at mild alkaline pH values higher than pH 7.0 [19], and also when methanol or any nucleophilic solvent is used [23]. This could result from nucleophilic substitution during acyl rearrangement [24,25]. Under mildly alkaline conditions the acyl moiety starts to migrate from the first hydroxy group of the glucuronic acid through the 1,2-*ortho* ester to the second hydroxy group and then to the third and fourth hydroxy group, thereby giving the possibility of several isomers [26]. By contrast, acetonitrile has been shown to enhance the stability of acyl glucuronides [27], especially at low pH. Our experimental conditions, pH of incubation (pH 6.8), immediate acidification of the mixture at the end of the incubation time (about pH 4.0), HPLC solvent (acetonitrile) and pH of the mobile phase (pH 2.8) were chosen to minimize the risk of hydrolysis and acyl shift. The stability of

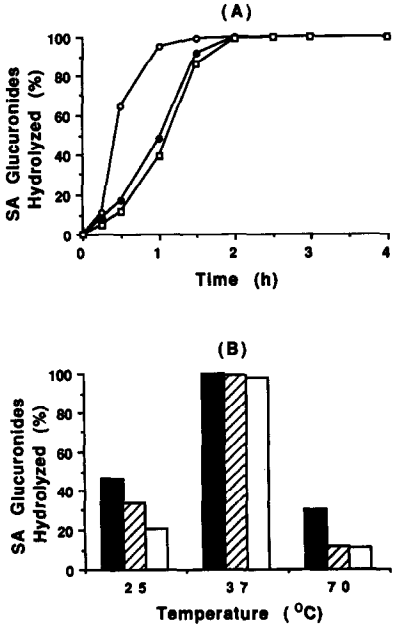

Fig. 5. (A) Effect of incubation time (at 37°C) on the hydrolysis of SA glucuronides with 2000 U/ml β-glucuronidase at pH 6.8. (○) SAG; (●) SPG; (□) SUPG. (B) Effects of incubation temperature on the hydrolysis of SA glucuronides with 2000 U/ml β-glucuronidase at pH 6.8 for 2 h. (■) SAG; (▨)SPG; (□) SUPG.

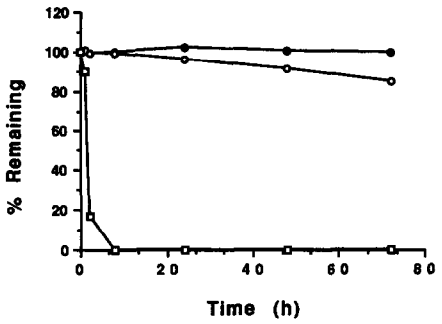

Fig. 6. Effect of pH on the stability of SAG in ambient temperature at different time intervals. (●) pH 4.0; (○) pH 7.0; (□) pH 9.0.

SAG in the 0–72 h urine sample from a volunteer as a function of time at different pH is shown in Fig. 6. The results indicated that hydrolysis of SAG in phosphate buffer (pH less than 7.0), after standing for three days at ambient temperature, was minimal. The half-life of SAG loss at pH 9.0 was 1.34 h. Phenolic glucuronide (SPG) showed very good stability at the different pH values studied.

During the development of the assay for SA glucuronides three approaches for quantitative determination were considered: (a) the use of authentic SPG as a standard; (b) the use of the collected fractions of SAG and SUPG as standards and hydrolyzing them to SA and SU; and (c) calibrating the SAG and SUPG

TABLE II

REPRODUCIBILITY AND PRECISION OF THE ASSAY OF SALICYLATE AND ITS GLUCURONIDES IN HUMAN URINE SAMPLES

Compound[a]	Concentration (μg/ml)	Coefficient of variation (%)	
		Intra-assay ($n=6$)	Inter-assay ($n=4$)
SA	10	1.4	1.0
	100	0.8	1.0
GA	10	1.9	5.6
	100	3.3	1.3
SU	10	0.8	1.0
	100	1.8	0.6
SAG	10	2.0	5.8
	100	3.5	5.4
SPG	10	3.7	1.2
	100	4.1	1.8
SUPG	10	5.0	4.3
	100	6.4	1.3

[a] Refer to Fig. 1 for abbreviations.

TABLE III

STABILITY OF SA GLUCURONIDE CONJUGATES IN URINE SAMPLE STORED AT −20°C

Storage time (months)	Observed concentration [a] (μg/ml)		
	SAG[b]	SPG [b]	SUPG[b]
0	18.7	16.8	9.8
0.25	18.7	16.8	9.8
0.5	18.7	16.8	9.8
1.0	18.6	16.8	9.8
1.5	18.6	16.8	9.7

[a] Values are an average of duplicates.

[b] Refer to Fig. 1 for abbreviations.

peak-height ratios against SA and SU standard curves. We have recommended a combination of these three approaches which is convenient, economic and simple.

The assay validation was assessed by the inter- and intra-assay reproducibility. The inter- and intra-assay coefficients of variation (C.V.) were below 10% (Table II). Recovery of all salicylate and its glucuronide conjugates from the urine samples at two different concentrations (10 and 100 μg/ml) was estimated by direct injection of the pure standards of the compounds. The mean absolute recoveries for SA and its glucuronide conjugates from human urine were greater than 93%. Under the experimental conditions used, the detection limits of the assay were 2 μg/ml for SAG, SPG and SUPG, 1 μg/ml for GA and 0.5 μg/ml for SA and SU, respectively, using a criterion of a peak-height-to-noise ratio of 4:1. Breakdown of SA glucuronide conjugates when buffered urine (about pH 4.0) was stored at 4°C was estimated by measuring the peak height of SAG, SPG and SUPG at different intervals. These experiments showed negligible breakdown of SA glucu-

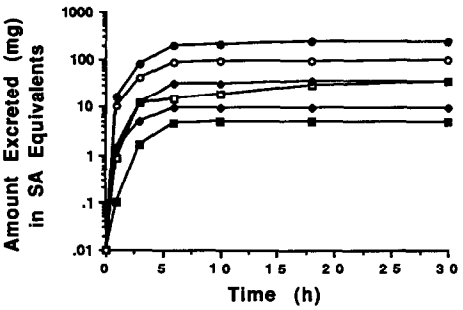

Fig. 7. Cumulative recovery of metabolites of SA in urine over 36 h in one subject after oral administration of 1.2 g of soluble aspirin. (○) SA; (●) SU; (■) GA; (□) SUPG; (◆) SPG; (◇) SAG.

ronide conjugates over 48 h. Urine samples stored at −20°C for up to 1.5 months showed no signs of decomposition and practically the same concentration values were obtained (Table III). This suggests that salicylate glucuronide conjugates are stable under these storage conditions.

A single dose of 1.2 g of aspirin was given to a healthy male volunteer aged 34 years with a body weight of 62 kg. Urine was collected at different intervals. The urine samples were assayed as described under Experimental. The cumulative urinary levels of the different SA metabolites are plotted in Fig. 7. The total dose recovery of SA in urine was greater than 90%. This recovery is similar to our observed total urinary salicylate recovery (free urinary salicylate plus urinary salicyl conjugates after acid hydrolysis at high temperature) [28].

CONCLUSIONS

SA forms two types of glucuronides, a phenolic and an acyl glucuronide (Fig. 1). A sensitive and selective HPLC method was developed for SA metabolites and its glucuronide conjugates in order to further study the dose-dependent aspects of the pharmacokinetics of aspirin or salicylic acid in humans, and to explore the possibility of using this drug as a model compound to investigate factors affecting glucuronide conjugate formation. The usual methods by which glucuronides are measured require an estimate of the aglycone to be made before and after enzymatic or chemical hydrolysis. The difference of these two measurements is then taken as an estimate of the glucuronide present. These approaches lack accuracy and selectivity. The method outlined here overcomes both of the above problems, in that it allows the estimate between low concentration of SA glucuronides in urine with well demonstrated specificity. The SUPG was first detected from the urine of a volunteer after oral administration of aspirin. The present procedure is the first HPLC method for simultaneous determination of SA and its metabolites including three glucuronide conjugates in human urine. This assay has been used to re-evaluate capacity-limited pharmacokinetics of SA in humans following a single-dose administration of salicylic acid to healthy volunteers.

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