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Direct analysis of salicylic acid, salicyl acyl glucuronide, salicyluric acid and gentisic acid in human plasma and urine by high-performance liquid chromatography

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Abstract

A method for the simultaneous direct determination of salicylate (SA), its labile, reactive metabolite, salicyl acyl glucuronide (SAG), and two other major metabolites, salicyluric acid and gentisic acid in plasma and urine is described. Isocratic reversed-phase high performance liquid chromatography (HPLC) employed a 15-cm C_{18} column using methanol–acetonitrile–25 m*M* acetic acid as the mobile phase, resulting in HPLC analysis time of less than 20 min. Ultraviolet detection at 310 nm permitted analysis of SAG in plasma, but did not provide sensitivity for measurement of salicyl phenol glucuronide. Plasma or urine samples are stabilized immediately upon collection by adjustment of pH to 3–4 to prevent degradation of the labile acyl glucuronide metabolite. Plasma is then deproteinated with acetonitrile, dried and reconstituted for injection, whereas urine samples are simply diluted prior to injection on HPLC. *m*-Hydroxybenzoic acid served as the internal standard. Recoveries from plasma were greater than 85% for all four compounds over a range of 0.2–20 μg/ml and linearity was observed from 0.1–200 μg/ml and 5–2000 μg/ml for SA in plasma and urine, respectively. The method was validated to 0.2 μg/ml, thus allowing accurate measurement of SA, and three major metabolites in plasma and urine of subjects and small animals administered salicylates. The method is unique by allowing quantitation of reactive SAG in plasma at levels well below 1% that of the parent compound, SA, as is observed in patients administered salicylates.

Keywords: Salicyl acyl glucuronide; Salicyluric acid; Gentisic acid; Salicylate

1. Introduction

Salicylate is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) on the market today. The major metabolic and elimination pathways of salicylic acid (SA) are now well characterized [1,2]. The major metabolites of SA are salicyluric acid (SU), salicyl acyl glucuronide (SAG), gentisic acid (GA) and salicyl phenolic glucuronide (SPG). Small amounts of other metabolites of SA, such as salicyluric acid phenolic glucuronide (SUPG), gentisuric acid (GU) and 2,3-dihydroxybenzoic

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acid have also been identified in urine and plasma following the ingestion of aspirin [3–5]. It is now well established that acyl glucuronides of NSAIDs are capable of undergoing hydrolysis, intra-molecular rearrangement and inter-molecular covalent binding reactions with proteins [6–8]. It has also been postulated that acyl glucuronides which form adducts with proteins may cause immune response that could be associated with some of the observed toxicity of these drugs [9]. In order to characterize the pharmacokinetics of SAG and the relationship between SAG and SA protein adducts, a sensitive, specific, accurate and reproducible analytical method is necessary for the measurements of SA, SAG and other metabolites in plasma and urine samples.

A number of analytical methods have been published for the determination of SA and its metabolites in plasma or urine [10–20]. However, only one method permitted the determination of SAG in plasma directly. In very early studies of salicylate, Schachter et al. [17,18] employed a fluorimetric method to measure the concentration of SAG in plasma by converting SAG to its stable hydroxamate derivative at room temperature and neutral pH. Such chemical estimation is complex, time consuming and requires large sample of 5 ml plasma. Mallikaarjun et al. [16] more recently used the reaction of hydroxylamine with SAG to form the stable hydroxamate prior to the analysis of SA and its metabolites in urine by HPLC. Shen et al. [19] also developed a suitable isocratic HPLC method to determine the concentration of SA and its metabolites including SAG in human urine. Then Vree et al. [20] employed a gradient HPLC method for the determination of SA and its metabolites in human plasma and urine, but was unable to quantify or detect SAG in human plasma samples of subjects administered salicylates.

We report here the development and validation of a simple, direct, sensitive and reproducible HPLC analytical method which can be applied for determination of GA, SAG, SU and SA in pharmacokinetics studies in humans and animals.

2. Experimental

2.1. Chemicals and materials

GA, SU, SA, 2,3-dihydroxybenzoic acid, *m*-hydroxybenzoic acid and β-glucuronidase, type B-10 from bovine liver, were obtained from Sigma (St. Louis, MO, SUA). HPLC-grade methanol and all other analytical grade reagents were purchased from Fisher (Norcross, GA, USA). SAG, SPG and SUPG were isolated from human urine after ingestion of 3 g SA (see below) and these studies in patients were approved by the University's Institutional Review Board. Pooled expired blank human plasma was obtained from the blood bank of the University of North Carolina Hospital. Blank human urine was from a healthy volunteer after an overnight fast.

2.2. Equipment

The preparative HPLC system consisted of a Pharmacia LKB 2150 pump (Bromma, Sweden), a manual injector connected to a 1-ml loop, a Hewlett-Packard series 1050 detector set at 225 nm, an Econosil ODS reversed-phase column (250 mm × 10 mm I.D. with 10-μm particles) (Deerfield, IL, USA) and methanol–acetonitrile–25 m*M* acetic acid (12:12:76, v/v/v) at 5 ml/min as the mobile phase. Chromatograms were recorded on a Hewlett-Packard 3396 Series II integrator (Palo Alto, CA, USA). The analytical HPLC system consisted of a Bio-Rad Model AS-100 HPLC automatic injector, a Hewlett-Packard Series 1050 UV detector set at 310 nm with an absorbance range setting of 0.2 AU, an Axxiom ODS reversed-phase column (150 mm ×4.6 mm I.D. with 5-μm particles) (Springfield, VA, USA) coupled with a RP-18 guard column (15 mm × 3.2 mm I.D., with 7-μm particles) (Brownlee Labs, San Jose, CA, USA) and methanol–acetonitrile–25 m*M* acetic acid (8.5:8.5:83, v/v/v) at 1 ml/min was employed as the mobile phase.

2.3. Isolation and characterization of SAG

Urine from a normal healthy volunteer was collected for 12 hours after taking 2.9 g of aspirin orally over a four hour period. The pH of the urine was adjusted to 3–4 with 1 ml 43% H_3PO_4 immediately after collection. By using an infusion pump at a rate of 1 ml/min, 60 ml of urine was passed through a Sep-Pak (C_{18} cartridge size 360 mg, Waters, Milford, MA, USA) to remove lipophilic material. The Sep-Pak cartridges were eluted with 3 ml of 10% acetonitrile–water. The effluent collected during passage of urine through the Sep-Pak cartridge and the first eluent were combined and lyophilized. The residue of lyophilization was reconstituted with 50 ml 70% acetonitrile–water, then lyophilized again after filtration with 45-μm nylon membrane filter (Millipore, Bedford, MA, USA). The residue was dissolved in 30 ml of methanol–25 m*M* acetic acid solvent (30:70, v/v) and injected onto the preparative HPLC column. Aliquots of 1 ml were injected and fractions were collected every min for 20 min. The collected fractions containing SAG (see below) were pooled, evaporated with a rotary evaporator at 37°C to remove most of the methanol, then frozen and lyophilized to dryness.

The identity of SAG was initially confirmed by release of SA after hydrolysis with strong base. Aliquots of the SAG fractions from preparative chromatography were adjusted to pH 10 with 0.2 *M* sodium hydroxide, then incubated at 37°C for 1 h. SA was determined to be stable to this brief treatment in strongly basic solution. The samples were neutralized with acetic acid to pH 3 after incubation, then the solution was injected onto the analytical HPLC system. Standardization of SAG content after preparative HPLC was done by comparing the UV response on HPLC of a series dilution of SAG stock solution before and after hydrolysis with 0.2 *M* NaOH at 37°C for 1 h. The areas obtained from these samples were compared with standard solutions of SA. To confirm that the conjugate was a β-1-glucuronide, SAG was also hydrolyzed with β-glucuronidase as described previously [21]. The identity of the metabolites (SAG, SPG and SUPG) were also confirmed by fast-atom bombardment (FAB)-MS using a thiolglycerol matrix.

In order to evaluate whether there was any interference within plasma that may overlap with SAG, we also used HPLC followed by lyophilization to collect SAG from human plasma samples from a subject taking oral SA. These SAG samples were reconstituted and incubated at 37°C for 30 min in either 0.2 ml 150 m*M* sodium phosphate buffer (pH 5) containing 2000-unit β-glucuronidase, or in 0.2 ml 150 m*M* sodium phosphate buffer (pH 10), then reassayed to assess the presence of material with the same retention as SAG and to determine SA content.

2.4. Preparations of stock solutions

Stock solutions containing 0.001, 0.01, 0.1 and 1 mg/ml of SAG (SA equivalents), GA, SU and SA as a mixture and similar stock solutions containing only GA, SU and SA were prepared in 10% acetonitrile–water solution adjusted to pH 3 with phosphoric acid. The concentration of SAG is stated in SA equivalent throughout the text. Blank human plasma or urine were also adjusted to pH 3 with 43% phosphoric acid to prevent pH-dependent degradation of SAG. Both plasma and urine standard curves were prepared daily as needed. No degradation of SA and its metabolites in these stock solutions at all concentrations were noted over 3 months when stored at −80°C. The stability of SA and it metabolites in stock solutions and plasma was also confirmed by inter-day validation studies.

2.5. Standard curves for analysis of plasma

Aliquots of 0.50 ml blank human plasma previously adjusted to pH 3 with 43% phosphoric acid were spiked with 5–25 μl stock solutions to final concentrations of 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 10, 50 and 100 μg/ml for SA, GA, SU and SAG. The solutions were allowed to equilibrate for 10 min at room temperature after vortex-mixing for 30 s. Then 300 μl of acetonitrile

containing 3 μg of *m*-hydroxybenzoic acid as internal standard (I.S.) and an additional 1.2 ml acetonitrile were added to precipitate proteins. Following vortexing for 1–2 min and centrifugation at 2000 *g* for 10 min, the supernatant was removed into a clean test tube and evaporated to dryness under a nitrogen stream at 37°C. After reconstitution with 0.5 ml HPLC mobile phase, the samples were transferred to polypropylene tubes and centrifuged at 15000 *g* for 2 min. An aliquot of 50 μl of the clear supernatant was then injected onto the analytical HPLC.

2.6. Inter-day and intra-day validation for plasma samples

Stock solution of the GA, SAG, SU and SA mixture was added to pooled blank human plasma to final concentrations of 0.20, 0.50, 2.0 and 10 μg/ml. Stock solution of GA, SU and SA mixture was also added to another pooled blank human plasma to final concentration of 5.0, 25, and 50 μg/ml. After 10 min mixing and equilibration, each 0.5 ml of plasma was pippetted into a screw cap test tube and stored at −80°C. Upon analysis, three samples of each concentration were thawed and 300 μl of acetonitrile containing 0.3 μg *m*-hydroxybenzoic acid — as internal standard (I.S.) — and an additional of 1.2 ml acetonitrile were added to precipitate the proteins. The analysis procedure then followed the same as described above for standard curve sample preparations. Inter-day validation was done by repeated analysis of these samples over a period of six months. The intra-day validation was conducted by preparing five separate samples at each concentration and analyzing them in a single day.

2.7. Analysis of plasma samples for in vivo studies

An oral dose of 1300 mg of aspirin was administered according to a patient's normal prescribed regimen as a part of her normal medical care. For the analysis of human clinical samples, blood samples were collected from the patient's forearm vein into tubes containing heparin (50 U/ml) kept on ice. After centrifugation at 4°C for 10 min, plasma was transferred to a screw top vial containing phosphoric acid to adjust the pH to 3–4 in order to minimize acyl migration and hydrolysis of SAG, then stored at −80°C until analyzed. Upon analysis, the samples were thawed and treated the same as described above for preparation of standard curve for plasma.

Recovery values of compounds from plasma samples was evaluated by comparing the compound/*m*-hydroxybenzoic acid ratio from spiked plasma samples relative to unextracted standard samples prepared in sodium phosphate buffer (pH 3–4) solution. *m*-Hydroxybenzoic acid was used here as an external standard to determine the recoveries.

2.8. Standard curve for urine samples

Aliquots of 0.50 ml blank human urine were spiked with 10–50 μl stock solutions to final concentrations of 20, 40, 100, 200, 400, 1000 and 2000 μg/ml for SA, GA, SU and SAG. After vortex-mixing for 30 s, the solutions were equilibrated for 10 min at room temperature. Aliquots of 25 μl of standard solutions were diluted to 0.5 ml with 10% acetonitrile–water containing 20 μg I.S., then 25-μl aliquots were injected on the analytical HPLC.

2.9. Inter-day and intra-day variation for urine samples

Samples spiked with standards and prepared as per urine standard curves were transferred to screw cap vials and stored at −80°C. At each day of analysis, three samples of each concentration were thawed and prepared as described above for urine samples. Inter-day validation was determined by repeated analysis of these stored samples over a period of 3 months. Intra-day validation was done by preparing three samples at each concentration and analyzing them in one day.

2.10. Analysis of urine samples from in vivo studies

Urine from human subjects administered salicylate was collected sequentially into containers with 1 ml glacial acetic acid, cooled by refrigeration upon voiding and at the end of each collection interval was adjusted to pH 3–4 with phosphoric acid. After measurement of the volume voided, aliquots of urine were stored at −80°C until thawed for analysis.

3. Results

SAG isolated and purified was more than 98% β-1 conjugate with the remainder present as isomeric conjugates formed by intramolecular acyl migration that are resistant to β-glucuronidase cleavage, but susceptible to cleavage by strong base. FAB-MS provided confirmatory identification of SAG with $M_{SAG} + H^+$ (m/z 315) and loss of glucuronic acid to yield SA ($M_{SA} + H^+$, m/z 139).

Fig. 1 shows representative chromatograms obtained with blank plasma, blank plasma spiked with 1.0 μg/ml of SA and its metabolites, and a representative plasma sample at 0.5 h after a patient took 1300 mg oral aspirin. Fig. 2 includes chromatograms of blank urine, blank urine spiked with 40 μg/ml of SA and its metabolites, and the urine sample for a 2–4 h interval after a patient took 1300 mg aspirin. The retention times of GA, SAG, SU, I.S. and SA were 3.8, 6.0, 11.4, 16.5 and 8.2 min, respectively. The retention times of SPG and SUPG that were also isolated from urine and confirmed by FAB-MS are less than 3.0 min, thus these were not detected using this HPLC method, nor did they interfere with the measurement of GA, SAG, SU, or SA. Analysis of ibuprofen, etodolac and ketorolac standard solutions and plasma samples spiked with these drugs demonstrated that they did not interfere with the analysis since there were no interfering signals in the chromatogram.

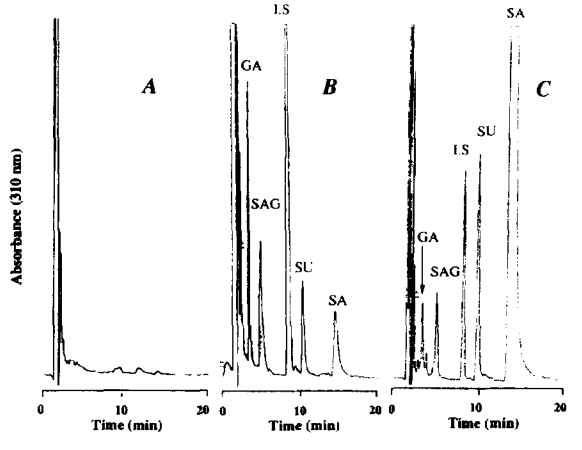

Fig. 1. HPLC chromatogram of SA and its metabolites in human plasma. (A) Blank plasma; (B) blank plasma spiked with SA and its metabolites (1.0 μg/ml); (C) plasma sample at 0.5 h for a subject chronically administered 1300 mg of aspirin four times daily.

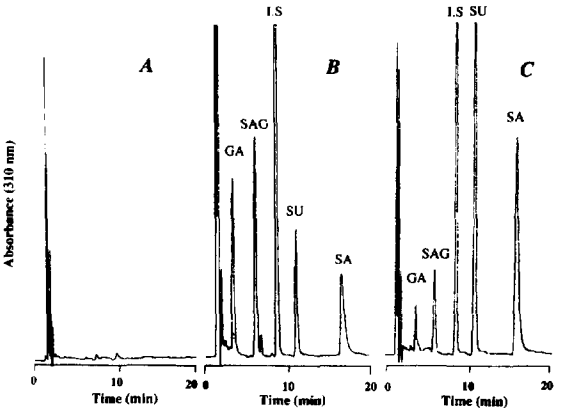

Fig. 2. HPLC chromatogram of SA and its metabolites in human urine. (A) Blank urine; (B) blank urine spiked with SA and its metabolites (40 μg/ml); (C) urine sample at 2–4 h after a subject chronically took 1300 mg of aspirin four times daily.

3.1. Extraction efficiency, linearity and variability

The recoveries of GA, SAG, SU and SA were greater than 85% from plasma over the range of 0.2–20 μg/ml ($n = 3$). The calibration curves were linear over the range 0.1–200 μg/ml for plasma samples and 5–2000 μg/ml for urine with correlation coefficient (r^2) > 0.995 for both plasma and urine when weighted by reciprocal of the concentrations. The intra-day variability for plas-

Table 1
Intra-day variabilities of the assay method for determining the concentration of salicylic acid and its metabolites in plasma

Plasma concentration (μg/ml)	Concentration found (μg/ml) mean ± S.D (n = 5) C.V. (%)			
	GA	SAG[a]	SU	SA
0.2	0.21 ± 0.01	0.20 ± 0.01	0.21 ± 0.02	0.20 ± 0.01
	3.8	5.7	8.8	4.1
0.5	0.44 ± 0.02	0.50 ± 0.01	0.56 ± 0.02	0.59 ± 0.03
	4.7	2.3	3.1	4.8
2.0	2.10 ± 0.04	2.10 ± 0.05	2.03 ± 0.05	2.22 ± 0.07
	1.9	2.3	2.4	3.0
10.0	11.0 ± 0.11	10.5 ± 0.15	9.91 ± 0.12	10.5 ± 0.58
	9.5	5.1	1.2	5.6
50.0	47.4 ± 2.09	53.0 ± 2.49	51.8 ± 3.02	54.8 ± 5.87
	4.4	4.7	5.8	11

[a] SAG concentrations are in SA equivalents.

ma and urine samples are shown in Tables 1 and 2. The intra-day's C.V. of the method ranged from 1.2 to 11 and 0.5 to 6.4% for plasma and urine samples, respectively. These data indicated that the assay method is quite reproducible within the same assay run. The inter-day variability of the assay method as determined for the quality control samples measured over a 12-month period for plasma samples and over a 3-month period for urine samples are presented in Tables 3 and 4. The inter-day variability for the slopes of the standard curves was also calculated. The slopes of the standard curves of GA, SAG, SU and SA did not change significantly over an extended period with C.V.s generally less than 8% for a 3-months period. The minimal concentration tested by the assay was 0.2 μg/ml for SAG and SU with the inter-day and inter-day variation less than 15%. The reproducibility for GA was not as good with the inter-day C.V. exceeding 15% at 0.5 μg/ml, though it was acceptable with a value of 6.3% at 2 μg/ml in

Table 2
Intra-day variabilities of the assay method for determining the concentration of salicylic acid and its metabolites in urine

Urine concentration (μg/ml)	Concentration found (μg/ml) mean ± S.D (n = 3) C.V. (%)			
	GA	SAG[a]	SU	SA
10.0	11.1 ± 0.45	10.7 ± 0.26	10.6 ± 0.68	10.8 ± 0.52
	4.1	2.4	6.4	4.8
100	101 ± 4.5	97.6 ± 2.70	95.7 ± 2.7	99.9 ± 3.0
	4.5	2.8	2.8	3.0
1000	1000 ± 9		972 ± 7	1040 ± 5
	0.9		0.7	0.5

[a] SAG concentrations are in SA equivalents.

Table 3
Inter-day variabilities of the assay method for determining the concentration of salicylic acid and its metabolites in plasma

Plasma concentration (μg/ml)	Concentration found (μg/ml) mean ± S.D (n = 8) C.V. (%)			
	GA	SAG[a]	SU	SA
0.2	0.19 ± 0.04	0.18 ± 0.02	0.21 ± 0.03	0.18 ± 0.06
	23	10	14	32
0.5	0.55 ± 0.10	0.48 ± 0.03	0.52 ± 0.05	0.50 ± 0.04
	18	5.8	9.5	7.2
2.0	1.94 ± 0.12	1.94 ± 0.13	1.98 ± 0.10	1.80 ± 0.15
	6.3	6.7	5.1	8.4
10.0	10.6 ± 0.61	9.95 ± 0.47	10.5 ± 0.49	9.6 ± 0.56
	5.8	4.7	4.7	5.8
25.0			27.1 ± 0.45[b]	23.3 ± 1.23
			1.7	5.3
50.0			44.7 ± 3.48[b]	46.4 ± 2.96
			7.8	6.4

[a] SAG concentrations are in SA equivalents.
[b] n = 3.

plasma. SA analysis in plasma had an acceptable inter-day precision of C.V. = 7.2% down to the 0.5 μg/ml level.

3.2. Application

Fig. 3 shows the plasma concentration–time profiles of GA, SAG, SU and SA in a patient chronically taking salicylate orally. This patient took 1300 mg per 6 h according to her normal prescribed regimen. The results indicate that plasma levels of SAG are less than 1% of the SAG at every sampling time. The urinary excretion of GA, SAG, SU (all in SA equivalents) was equal to 4.4, 8.1, 45 and 27% of the dose in this same subject for the dosing interval of 6 h. The

Table 4
Inter-day variabilities of the assay method for determining the concentration of salicylic acid and its metabolites in urine

Urine concentration (μg/ml)	Concentration found (μg/ml) mean ± S.D (n = 3) C.V. (%)			
	GA	SAG[a]	SU	SA
10.0	12.0 ± 1.05	11.7 ± 0.58	11.3 ± 0.30	11.0 ± 0.97
	8.7	4.9	2.6	8.8
100	108 ± 6.9	109 ± 10	101 ± 4.7	99.8 ± 6.8
	6.4	9.3	4.7	6.8
1000	1050 ± 58	1080 ± 71	995 ± 25	986 ± 61
	6.3	6.7	5.1	8.4

[a] SAG concentrations are in SA equivalents.

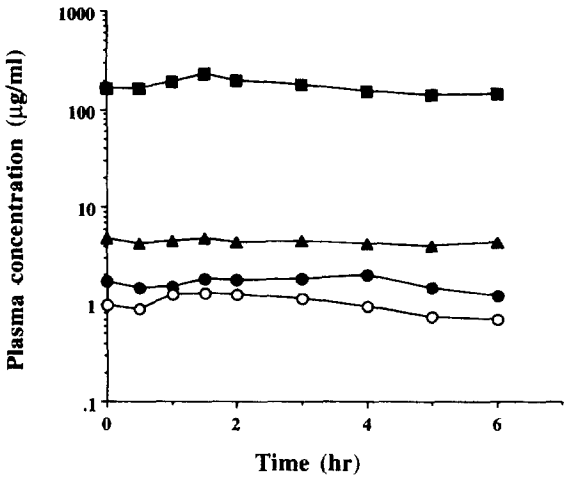

Fig. 3. A representative concentration–time profile of SA and its metabolites in a subject after chronic oral administration of 1300 mg of aspirin four times daily. SAG concentrations are in SA equivalents (●) GA; (○) SAG; (▲) SU and (■) SA.

concentrations for GA, SAG, SU and SA found were in the concentration range validated.

4. Discussion

This method was developed with the specific aim of being able to directly quantify SAG in plasma following administration of salicylates. A number of analytical methods have been published in the literature for the determination of SA and metabolites in plasma or urine [10–20]. However, only one rather dated method was able to determine the concentration of SAG in plasma [18] while several more recent HPLC methods have directly measured SAG in urine or determined it by selective conversion of SAG to its hydroxamate [16,19,20]. Vree et al. [20], in an effort to improve the separation used gradient elution over a 40-cm (150×4.6 mm $C_8 + 250 \times 4.6$ mm C_{18}) column. These more recent analytical HPLC methods were unable to analyze SAG in plasma sample, perhaps because of the low levels of SAG in vivo and the substantial interference seen in plasma. Through the use of lower UV wavelengths than that employed here, the previous methods were able to measure SPG which has little inherent UV absorption at 310 nm. Given the very low levels of SAG relative to SA in plasma, it is evident that indirect methods for measuring SAG which quantifying the increase in SA concentration after enzymic or chemical hydrolysis, as often employed for its analysis in urine or bile, would not be adequate.

The results presented here indicate that the present HPLC method for the determination of GA, SAG, SU and SA is satisfactory for disposition studies of SA and its metabolites in humans and small animals. The validation parameters showed a quantitative recovery, minimal chromatographic background, good intra- and inter-day reproducibility, linearity over 0.1–200 μg/ml for plasma and 5–2000 μg/ml for urine samples. With a change of the guard column at 3–6 months intervals, which represents about 500 plasma samples, good column performance was maintained over a one-year period with frequent use. Though the method does not allow direct quantitation of the more stable glucuronide metabolite, SPG, this method is uniquely applicable to studies of SAG which is labile and potentially reactive. Moreover, at higher doses often used in chronic studies of SA disposition, the urinary excretion of SAG may equal or exceed that of SPG.

This method utilized a higher wavelength of 310 nm for the detection of GA, SAG, SU and SA for both urine and plasma samples than employed by other direct assays. The use of a higher wavelength improved selectivity of the method and reduced the inference from the background and potential interference from other drugs. To ensure that there was no interference from other SA metabolites, SPG and SUPG were isolated from human urine and injected onto the HPLC system. The results indicate that the SPG and SUPG metabolites have very short retention times under the conditions employed and will not interfere with the assay. Collection of SAG from human subject plasma samples by HPLC followed by hydrolysis with β-glucuronidase or by base at pH 10 indicated that there are no interference peaks that overlapped with SAG peak on the HPLC chromatogram.

Because of the huge concentration difference between SA and its metabolites in plasma samples, SA exceeded the detector range if the highest sensitivity was selected to enhance the detection of GA and SAG. For example, increasing the injection volume to 200 μl and using a lower range setting on the detector to 0.1 AU provided an increase in the sensitivity of the assay, with acceptable intra-day C.V.s (<10%) down to plasma concentrations of 0.05 μg/ml. Under such conditions, however, the absorbance of SA often exceeded the detector range. This problem of limited dynamic range can be overcome by either of two approaches. One is to run the samples twice; once at highest sensitivity to measure low levels of metabolites and again at lower sensitivity for the analysis of SA, thus doubling the assay time. An alternative method which we used is to sacrifice some sensitivity of the assay at low concentrations so that all four compounds can be measured at the same time. Although the inter-day C.V.s of some metabolites at very low concentrations are larger than 10% (Table 3), the results of the clinical study in humans shows that such an approach is reasonable because the concentration of GA, SAG and SU are high enough to be measured accurately (Fig. 3). However, when using 0.1-ml plasma samples from single dose studies in rats, GA and SAG concentrations can be measured down to 0.1 μg/ml when the absorbance range of the detector is set at 0.1 AU and one injects 200 μl of the final prepared extract onto HPLC [22].

Many acyl glucuronides are unstable and undergo intramolecular acyl migration under physiological conditions [21–26]. Stability studies conducted in our laboratory show that SAG is unstable and undergo intramolecular acyl migration in 150 m*M* sodium phosphate buffer (pH 7.4) [22]. Dickinson et al. [26] reported that the half-life of SAG was 1.4–1.7 h under pH 7.4 and increased to 70 h under pH 4 at 37°C. Precautions have to be taken to maintain an acidic pH in plasma and urine samples and to cool the samples upon collection in order to minimize the SAG degradation during sample handling and storage. Our validation studies show that no apparent degradation of SAG in urine and plasma were noted over 3–6 months under acidic conditions (pH 3–4).

The applicability of the assay was demonstrated by a preliminary study of the plasma concentration–time profile of SA and its metabolites in a subject who took 1300 mg aspirin every 6 h chronically. The results indicate that the plasma levels of the acyl glucuronide metabolite are less than 1% of those observed for SA and that SA and its metabolites reached steady state levels in this subject. In conclusion, the HPLC method presented is direct, simple, selective, reproducible, sensitive and linear for the simultaneous determination of GA, SAG, SU and SA in human plasma and urine and is adequate for clinical studies.

Acknowledgments

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