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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF SALICYLIC ACID AND ITS METABOLITES IN URINE BY DIRECT INJECTION

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SUMMARY

A direct injection method has been developed for the determination of salicylic acid and its metabolites in urine. Urine samples are treated with hydroxylamine to convert salicyl acyl glucuronide to salicylhydroxamic acid, which can be accurately quantitated by direct injection into a high-performance liquid chromatographic system along with salicylic acid, gentisic acid and salicyluric acid. Salicyl phenolic glucuronide is quantitated by difference after hydrochloric acid hydrolysis at 65°C with no loss of salicylic acid by sublimation or hydrolytic loss of salicyluric acid. This method has been applied to urine samples from human subjects and the results are discussed.

INTRODUCTION

Salicylates were first introduced in medicine more than a hundred years ago and are still widely used for their antiinflammatory and analgesic activities and more recently recommended as a prophylactic agent in the treatment of angina pectoris [1]. Aspirin, the most commonly used salicylate, is hydrolyzed in the gut and the plasma to salicylic acid (SA) which is subsequently metabolized by conjugation to salicyluric acid (SU), salicyl acyl glucuronide (SAG) and salicyl phenolic glucuronide (SPG) [2]. A small fraction of SA is hydroxylated to form gentisic acid (GA). There also have been reports of conjugation products of GA [3] and SU [4,5] excreted in the urine. The pharmacokinetics of SA have been studied extensively [6,7] and it has been shown that the formation of SU and SPG from SA occur by capacity-limited processes [8,9].

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A study was done to compare the excretion of SA and its metabolites in four ethnic groups and for this a rapid and robust method was required to quantitate SA, GA, SU, SAG and SPG in urine. There are numerous methods for the estimation of SA, GA and SU in urine and plasma by gas chromatography (GC) [10] and high-performance liquid chromatography (HPLC) [11–16]. Methods for the estimation of SAG and SPG are few as reference samples of the glucuronides are not easily available and the quantitation is generally indirect [17,18]. The acyl glucuronide is labile and is susceptible to intramolecular rearrangements [19,20] with the acyl group migrating to different positions on the glucuronide moiety. The rearranged glucuronide isomers are resistant to hydrolysis by β-glucuronidase and SAG determined by this method is underestimated. The hydroxylamine treatment described by Schacter [17] overcomes this problem as both the rearranged and unrearranged SAG react with hydroxylamine giving the correct estimate of SAG. The only HPLC method in the literature for the determination of SAG using the hydroxylamine treatment is by Hutt et al. [4], but their method was complex, included an extraction step and had a long run time. The only methods available for the estimation of SPG are hydrochloric acid hydrolysis which includes a tedious sealing step [6] and a complex fluorimetric method also involving an extraction step [21]. Consequently, we developed a method for the analysis of SA, GA, SU, SAG in urine samples by direct injection in an HPLC system and SPG by hydrolysis circumventing the sealing step.

EXPERIMENTAL

Reagents

All reagents were reagent or HPLC grade and were used as received. GA, SU, salicylhydroxamic acid (SHA) and hydroxylamine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade phosphoric acid and methanol, SA, dipotassium phosphate, concentrated hydrochloric acid and sodium hydroxide pellets were purchased from Fisher Scientific (Springfield, NJ, U.S.A.).

Equipment

A Waters Model 6000A HPLC pump with an LDC Spectromonitor II variable-wavelength UV detector set at 310 nm, attached to a Model C-R1A Shimadzu integrator, was used. The column was a Vydac ODS 10 μm, 25 cm $\times$ 4.6 mm I.D. with a pre-column packed with 32–50 μm ODS particles and a Rheodyne Model 7125 injector with a 100-μl loop.

Determination of GA, SA, SU, SAG and SPG in urine

Determination of the optimal pH of the mobile phase. A series of 0.05 *M* potassium dihydrogenphosphate solutions in 6% methanol was prepared at different pH values. A standard mixture of SA, GA, SU and SHA was prepared and used for determining the different retention times at the various pH ranges. The pH values studied were 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0. Retention times were plotted against pH and from the plot (Fig. 1) the optimum pH value for the mobile phase was determined to be 4.0.

Determination of the optimal methanol concentration. Four mobile phases were tested with methanol concentrations at 2.5, 5.0, 7.5 and 10.5%. The pH was kept constant at 4.0 and the buffer concentration at 0.05 *M*. The standard solution used was the same mixture of SA and its metabolites. The optimal methanol concentration was determined to be 6% from the plot of methanol concentration against retention times (Fig. 2).

The final mobile phase used for the assay was a 0.05 *M* dipotassium phosphate buffer, adjusted to pH 4.0 with phosphoric acid, with 6% (v/v) methanol.

Determination of the time for reaction of hydroxylamine with SAG. Since an authentic sample of SAG could not be obtained, the cumulative urine sample of subject No. 1 presumed to contain SAG was used. A 1-ml volume of the sample was treated with 1.0 ml of 2.0 *M* hydroxylamine solution. The sample mixture was injected into the HPLC system described below at 0, 0.25, 1.0, 1.5, 2.0, 3.1, 5.22 and 7.78 h. The SHA peak at 6.10 min was characterized by using

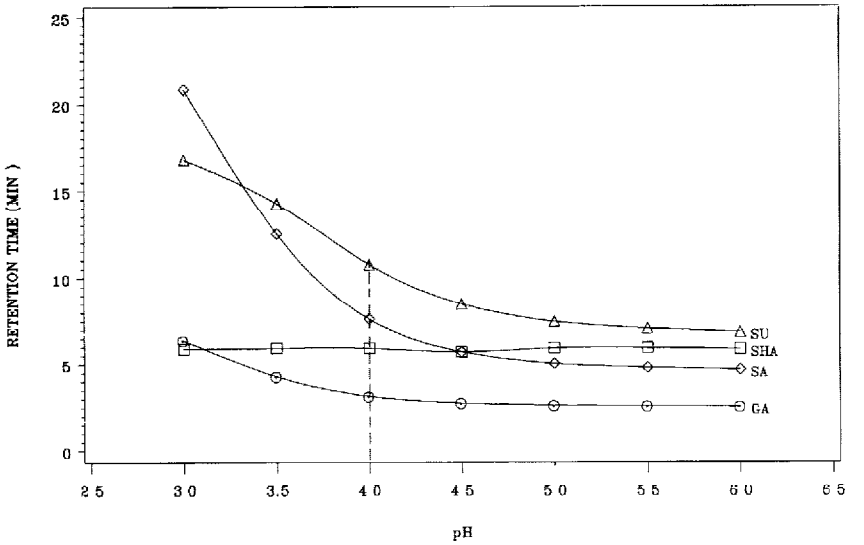

Fig. 1. Plot of retention time of GA, SHA, SA and SU against pH of the phosphate buffer in the mobile phase. The dotted line indicates the optimal pH determined from the plot.

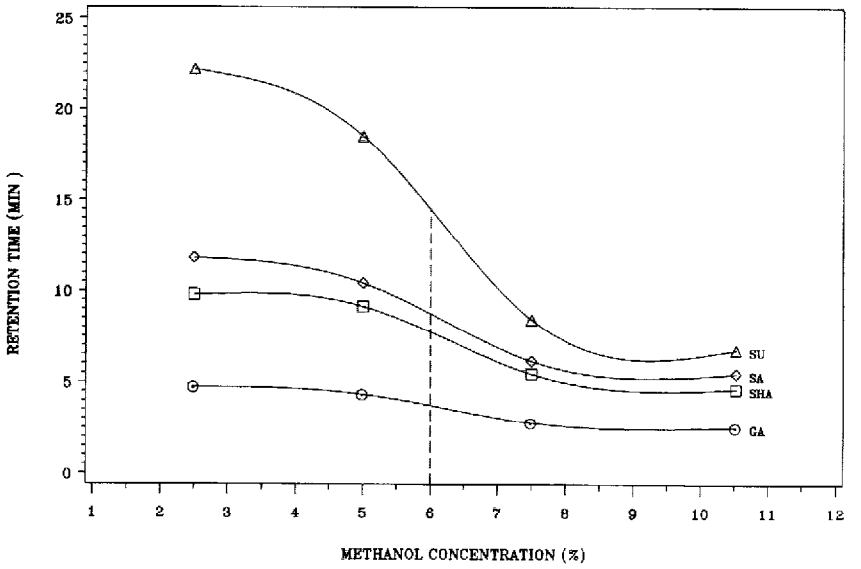

Fig. 2. Plot of methanol concentration of the mobile phase against the retention times of GA, SHA, SA and SU. The optimal methanol concentration is shown by the dotted line.

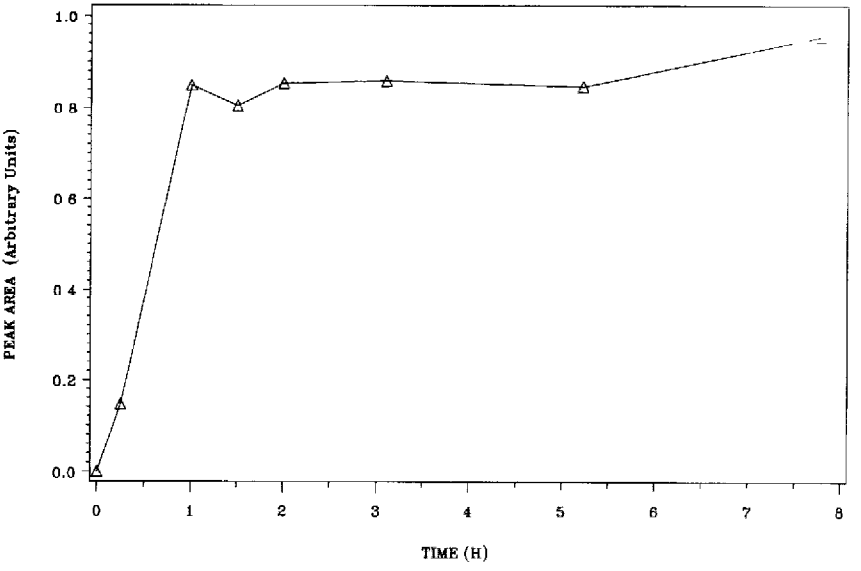

Fig. 3. Peak area of SHA is plotted as a function of the incubation time of the urine sample and hydroxylamine solution. The formation of SHA is essentially complete at 1.0 h.

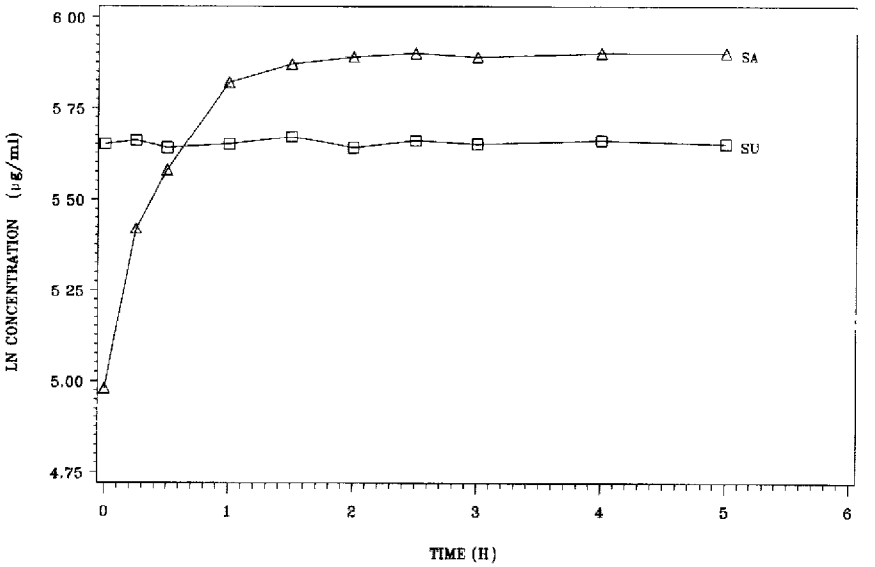

Fig. 4. Concentration of SA and SU in a urine sample after hydrochloric acid hydrolysis at 65 °C. Hydrolysis of SA glucuronides is complete after 1.5 h without any degradation of SU.

a solution of pure SHA. A plot of the peak area at 6.10 min against time revealed that by 1.0 h the acyl glucuronide had completely reacted with hydroxylamine (Fig. 3).

Determination of temperature and time for hydrochloric acid hydrolysis of SPG. A modification of Levy's method [6] was utilized for the determination of SPG. Levy's procedure required the sample to be treated with an equal volume of concentrated hydrochloric acid (10 *M*) and heated for 3 h at 100 °C in sealed ampules. Erratic results were observed when this method was used for the samples with loosely closed screw-capped tubes because SA sublimes at 74 °C and a substantial amount is lost when heated at 100 °C. Decreasing the temperature to 65 °C was effective as there was no perceptible loss of SA (Fig. 4). It is apparent from Fig. 4, that by 1.5 h the hydrolysis is essentially complete. Also, from the figure, it can be seen that SU is unaffected by the hydrolysis and so the SA obtained from the hydrolysis is the sum of free SA and SA glucuronides.

A suitable internal standard was not found due to interference with the peaks of interest. The samples were weighed before and after hydrolysis and the difference was made up by the addition of water to restore the original weight. This difference was generally very minimal and was never more than 0.003 mg.

Standards and standard curves

Standard solutions for the assay were mixtures of the metabolites from stock solutions made up in urine. The concentrations of the standard solutions for

the hydroxylamine treatment were 2.0–28.57 μg/ml for GA, 5.0–95.24 μg/ml for SHA, 20.0–190.48 μg/ml for SA and 50.0–638.10 μg/ml for SU. Standards for the hydrolysis treatment were prepared in the same manner as for the hydroxylamine treatment except for the concentrations which ranged from 25.0 to 476.19 μg/ml for SA and from 50.0 to 476.19 μg/ml for SU. GA and SHA were not added to the standards for hydrolysis.

Six standard solutions were prepared for both the hydroxylamine and hydrolysis treatments and were injected into the HPLC system at the beginning and at the end of the analysis. The standards were treated in exactly the same manner as the samples for both the treatments.

Analytical procedure

SA, SAG, GA and SU in the urine samples were estimated by treatment with hydroxylamine and SPG by hydrochloric acid hydrolysis.

Hydroxylamine treatment. The urine sample tubes were thawed at room temperature. They were then vortexed using a Vortex-Genie for 1 min. This step is extremely important as otherwise anomalous results are obtained. After vortexing, 500 μl of the pooled urine sample were pipetted into a tube and 500 μl of 2.0 *M* hydroxylamine solution (pH 7.0) were added. The mixture was allowed to stand at room temperature for 2 h. After 2 h the samples and the standards were centrifuged at 1750 *g* for 10 min and 100 μl of each injected into the HPLC system.

Hydrolysis treatment. A 300-μl volume of concentrated hydrochloric acid (37%) was added to 300 μl of the urine sample in a tube, and after capping incubated in a constant-temperature water-bath at 65 °C for 2.0 h. The samples were weighed before and after hydrolysis and the difference in weights was made up with distilled water. After the hydrolysis, the samples were allowed to cool and 300 μl of 9 *M* sodium hydroxide were added to neutralize the hydrochloric acid. The samples were again allowed to cool, centrifuged at 1750 *g* for 10 min and 100 μl were injected into the HPLC system. The standards were treated in exactly the same manner as the samples including the hydrolysis and the addition of distilled water when necessary.

RESULTS AND DISCUSSION

Chromatography

The chromatogram for the hydroxylamine treatment is shown in Fig. 5. The retention times were 3.3, 6.3, 7.7 and 11.1 min for GA, SHA, SA and SU, respectively. Fig. 6 shows the chromatogram obtained for the hydrolysis treatment. SA eluted at 7.6 and SU at 11.0 min. Both SA and SU were well separated but GA could not be quantitated due to interference from the solvent front peaks.

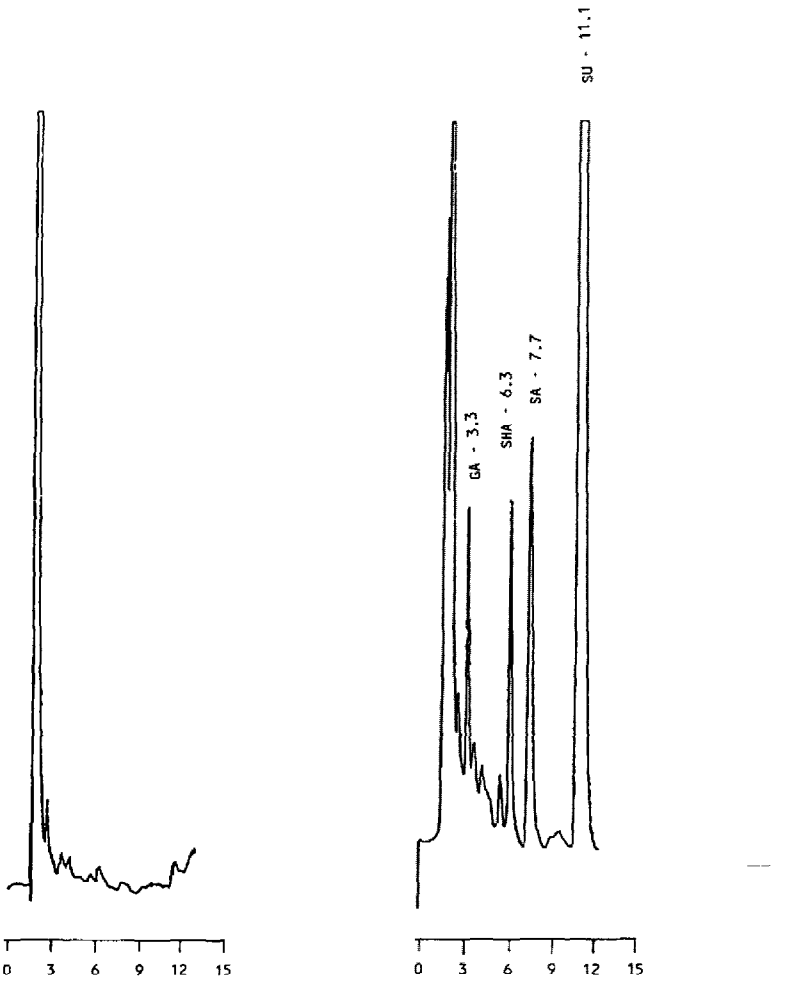

Fig. 5. Chromatogram of (left) blank urine and (right) urine sample from a subject after hydroxylamine treatment. The numbers indicate retention time in minutes. Estimated concentrations in $\mu g/ml$ are 6.60 for GA, 19.29 for SHA, 20.21 for SA and 192.77 for SU.

The pH and methanol concentration of the mobile phase were tightly controlled as the retention times changed significantly if they were not.

Quantitation

Using a linear regression program, concentrations were regressed against peak areas and the slope and intercept determined (Table I). These were then used to calculate concentrations of the metabolites in the urine samples. The concentrations were multiplied by the volume of urine voided and the appropriate factor to convert them to SA equivalents.

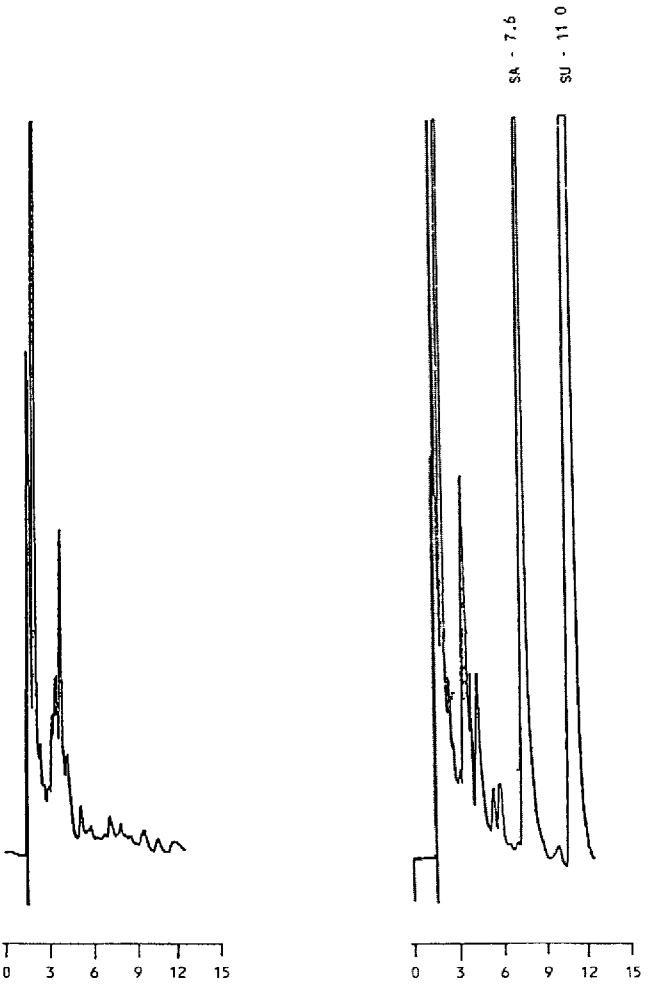

Fig. 6. Chromatogram of (left) blank urine and (right) urine sample from a subject after hydrolysis treatment. The numbers indicate retention time in minutes. Estimated concentrations in μg/ml are 93.39 for SA and 319.17 for SU.

The SA and SU amounts from the hydrolysis were determined in the manner described above. Since free SA and SAG were known from the hydroxylamine treatment, SPG was calculated by subtracting SA and SHA from the SA obtained by hydrolysis.

Linearity and precision

All the standard mixtures were treated in the appropriate manner and injected into the HPLC system. The intra-day and inter-day variability were

TABLE I

TYPICAL REGRESSION PARAMETERS FOR STANDARD CURVES OF GA, SHA, SA AND SU FOR HYDROXYLAMINE AND HYDROLYSIS TREATMENTS

Compound	Slope	Intercept	Correlation coefficient
Hydroxylamine treatment			
GA	2.58	0.14	0.999
SHA	1.45	−0.57	0.999
SA	2.06	1.80	0.999
SU	1.60	−7.41	0.999
Hydrolysis treatment			
SA	1.30	−2.86	0.999
SU	1.01	−7.64	0.999

TABLE II

INTER-DAY AND INTRA-DAY VARIABILITY OF GA, SHA, SA AND SU STANDARDS FOR THE HYDROXYLAMINE TREATMENT

Values in parentheses are concentrations in μg/ml.

Standard No.	Coefficient of variation (%)			
	GA	SHA	SA	SU
Inter-day variability (n = 10)				
1	–	6.84 (5.0)	8.90 (20.0)	6.12 (50.0)
2	7.23 (2.0)	4.82 (10.0)	5.16 (40.0)	3.22 (100.0)
3	3.14 (5.0)	6.75 (20.0)	5.75 (60.0)	5.84 (200.0)
4	10.98 (10.0)	4.12 (30.0)	5.63 (80.0)	6.23 (300.0)
5	5.97 (15.0)	3.64 (50.0)	3.96 (100.0)	4.61 (500.0)
6	5.77 (28.57)	5.83 (95.24)	5.93 (190.48)	6.21 (638.10)
Intra-day variability (n = 10)				
1	–	4.88 (5.0)	1.32 (20.0)	3.35 (50.0)
2	3.81 (2.0)	3.42 (10.0)	0.82 (40.0)	0.98 (100.0)
3	0.87 (5.0)	3.87 (20.0)	2.78 (60.0)	3.07 (200.0)
4	3.55 (10.0)	1.43 (30.0)	1.02 (80.0)	1.69 (300.0)
5	1.84 (15.0)	1.92 (50.0)	1.95 (100.0)	1.12 (500.0)
6	0.95 (28.57)	3.05 (95.24)	1.69 (190.48)	1.08 (638.10)

calculated by determining the percentage coefficient of variation for each metabolite at each standard concentration.

The within-day coefficient of variation for all the metabolites was below 5% for all the concentrations (Table II). Inter-day variability was less than 10% for all the metabolites at all concentrations.

The coefficient of variation was less than 3% for both inter-day and intra-day analysis (Table III) for the hydrolysis treatment. The correlation coefficients were typically $\geqslant 0.999$ for both the treatments.

Sensitivity

The limit of quantitation was 5 μg/ml for SA, 2 μg/ml for SHA, 2 μg/ml for GA and 10 μg/ml for SU. This compares favorably with sensitivities reported in the literature.

TABLE III

INTRA-DAY AND INTER-DAY VARIABILITY OF SA AND SU STANDARDS FOR THE HYDROLYSIS TREATMENT

Values in parentheses are concentrations in μg/ml.

Standard No.	Coefficient of variation (%)			
	Intra-day ($n=10$)		Inter-day ($n=10$)	
	SA	SU	SA	SU
1	3.81 (25.0)	2.95 (50.0)	5.19 (25.0)	3.88 (50.0)
2	2.27 (50.0)	3.48 (100.0)	5.13 (50.0)	5.97 (100.0)
3	2.03 (100.0)	1.66 (150.0)	5.79 (100.0)	4.14 (150.0)
4	1.02 (200.0)	1.56 (200.0)	4.23 (200.0)	4.22 (200.0)
5	0.76 (300.0)	2.87 (300.0)	4.11 (300.0)	3.67 (300.0)
6	0.87 (476.19)	0.98 (476.19)	3.34 (476.19)	2.45 (476.19)

TABLE IV

METABOLITE AND CUMULATIVE AMOUNTS AND RECOVERY IN EIGHT SUBJECTS AFTER ORAL ADMINISTRATION OF 1 g OF ASPIRIN (EQUIVALENT TO 767 mg SA)

All amounts are SA equivalents.

Subject No.	Amount (mg)							
	GA	SAG	SA	SU	Total SA (hydrolysis)	SPG	Cumulative	Recovery (%)
1	9.94	108.32	103.68	459.02	294.13	82.13	763.09	99.49
2	8.62	81.34	190.37	481.09	316.67	44.96	806.38	105.13
7	3.04	24.30	176.16	440.22	342.50	142.04	785.76	102.45
12	0.74	30.09	147.37	474.12	269.11	91.65	743.97	97.01
18	0.15	38.34	103.18	549.07	209 42	67.90	758.64	98.91
20	2.69	73.08	51.23	528.10	236.47	112.16	767.26	100.03
22	0.23	59.83	47.31	562.67	191.20	84.06	754.10	98.32
23	7.01	61.63	62.14	563.65	168.99	45.22	739.65	96.43

Application: ethnic variability study

Urine samples of subjects from four ethnic groups, Caucasians, Orientals, Asian Indians and Blacks were analyzed and the amounts of SA, GA, SAG, SU and SPG determined. Table IV lists the amounts of metabolites in SA equivalents excreted by the subjects. The recovery in most cases was greater than 95%, which is comparable to recoveries reported earlier for SA and its metabolites [6,7].

DISCUSSION

The HPLC–UV detection assay developed had a number of improvements over the existing methods. The total run time was 12.0 as compared to 28 min by the only other similar method [4]. The pH of the mobile phase, 4.05 in our assay, is well within the manufacturer's suggested range of usage for C_{18} columns which is between 2.0 and 7.0. This contrasts sharply with the pH of the mobile phase of the other method [4] which was 11.86, a pH which can be extremely deleterious to the column. Most other methods have used an extraction step for analysis of urine samples [12–16]. This step is essential for plasma samples which have a high protein content and cannot be easily estimated by direct injection. Urine samples, on the other hand, have very little protein in most cases and can be estimated by direct injection.

Treatment of the urine sample with hydroxylamine is essential for accurate determination of acyl glucuronides [19], since they are susceptible to intramolecular rearrangement. The acyl group of the drug moiety, originally attached to the 1-position on the glucuronic acid ring, migrates to another position forming rearranged glucuronides. These rearranged glucuronides are resistant to hydrolysis by β-glucuronidases and quantitation of the glucuronide by this method would underestimate the true amount.

There has been no systematic investigation into the optimum time required for the hydrolysis. The experimental section conclusively establishes that 65 °C and 2 h are the optimum temperature and time, respectively, for complete hydrolysis of both the acyl and the phenolic glucuronides, without any sublimation of SA or hydrolysis of SU.

Hutt et al. [4,5] reported a conjugate of SU, salicyluric phenolic glucuronide in the urine. Any phenolic conjugate present would have revealed itself as an increase in the SU determined by hydrochloric acid hydrolysis. There was an increase in the amount of SU estimated after hydrolysis (Table V), which indicates a strong possibility of the presence of the phenolic conjugate of SU present in the urine. Also, Hutt et al. [4,5] reported that this conjugate comprised about 3% of the dose excreted, which is consistent with our results (Table V).

Wilson et al. [3] reported gentisuric acid as a new metabolite of SA. This report was not confirmed by Hutt et al. [4], who could not detect the metab-

TABLE V

AMOUNTS OF SALICYLURIC ACID ESTIMATED BY HYDROXYLAMINE TREATMENT AND HYDROLYSIS TREATMENT

Subject No.	Amount SA (SA equivalents) (mg)		Difference (%)
	Hydroxylamine treatment	Hydrolysis treatment	
5	431.65	467.57	+7.68
7	440.22	459.40	+4.14
8	422.94	429.85	+1.61
11	596.78	620.71	+3.86
22	562.67	602.63	+6.63

olite. Due to the unavailability of the pure standard, gentisuric acid could not be quantitated by the hydrolysis treatment and the presence of gentisuric acid could neither be confirmed or denied by our method.

In summary, the assay method developed is a definite improvement over existing assays for SA and its metabolites with an economical phosphate buffer–methanol mobile phase, a short run time, no extraction step, accurate quantitation of SAG by hydroxylamine treatment and SPG by acid hydrolysis, without a sealing step.

REFERENCES

1 P.M. Brooks and C.J. Needs, Clin. Pharmacokin., 10 (1985) 164–177.
2 G. Levy, Drug Metab. Rev., 9 (1979) 3–19.
3 J.T. Wilson, H.L. Randall, M.W. Holladay, G.M. Brilis, J. Chrastil, J.T. Watson and D.F. Taber, Clin. Pharmacol. Ther., 23 (1978) 635–643.
4 A.J. Hutt, J. Caldwell and R.L. Smith, Xenobiotica, 12 (1982) 601–610.
5 A.J. Hutt, J. Caldwell and R.L. Smith, Xenobiotica, 16 (1986) 239–249.
6 G. Levy, J. Pharm. Sci., 54 (1965) 959–967.
7 C. Bedford, A.J. Cummings and B.K. Martin, Br. J. Pharmacol., 24 (1965) 418–431.
8 G. Levy, P. Amsel and H.C. Elliott, J. Pharm. Sci., 58 (1969) 827–829.
9 G. Levy, T. Tsuchiya and L.P. Amsel, Clin. Pharmacol. Ther., 13 (1971) 258–268.
10 L.J. Walter, D.F. Biggs and R.T. Coutts, J. Pharm. Sci., 63 (1974) 1754–1757.
11 G.W. Peng, M.A.F. Gadalia, V. Smith, A. Peng and W.L. Chiou, J. Pharm. Sci., 67 (1978) 710–712.
12 B.E. Cham, D. Johns, F. Bochner, D.M. Imhoff and M. Rowland, Clin. Chem., 25 (1979) 1420–1425.
13 D.L. Maulding and J.F. Young, J. Pharm. Sci., 69 (1980) 1224–1225.
14 R.H. Rumble and M.S. Roberts, J. Chromatogr., 225 (1981) 252–260.
15 J.N. Buskin, R.A. Upton and R.L. Williams, Clin. Chem., 28 (1982) 1200–1203.
16 R.J. O'Kruk, M.A. Adams and R.B. Philp, J. Chromatogr., 310 (1984) 343–352.
17 D. Schacter, J. Clin. Invest., 36 (1957) 297–302.
18 D. Schacter and J.G. Manis, J. Clin. Invest., 37 (1958) 800–807.
19 R.G. Dickinson, W.D. Hooper and M.J. Eadie, Drug Metab. Dispos., 12 (1984) 247–252.
20 E. Faed, Drug Metab Rev , 15 (1984) 1213–1249.
21 D.A. Imhoff, P.A. Reece, E. Dimitradis, D. Ward and F. Bochner, Ther. Drug Monit., 8 (1986) 321–325.