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IMPROVED METHOD FOR THE DETERMINATION OF ASPIRIN AND ITS METABOLITES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATIONS TO HUMAN AND ANIMAL STUDIES*

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SUMMARY

An improved method has been developed for the determination of acetylsalicylic acid, salicylic acid, gentisic acid, and salicyluric acid in plasma and urine of rabbits and man. Samples are extracted with dichloromethane containing mephenytoin as an internal standard, the solvent is evaporated under reduced pressure, the residue reconstituted and analyzed by high-performance liquid chromatography. Extraction efficiencies, linearity and assay precision were determined. This method has been applied to human bioavailability studies and the data are presented.

INTRODUCTION

The pharmacokinetics of aspirin (ASA), one of the most widely used analgesics, remains a field of active investigation [1, 2]. In the species studied, ASA is hydrolyzed to salicylic acid (SA), which is further metabolized by conjugation to form salicyluric acid (SU), salicyl phenolic glucuronide and salicyl acyl glucuronide [2]. To a minor extent, SA is hydroxylated to form gentisic acid (GA), some of which is conjugated with glucuronide to form gentisuric acid [3]. Recently, SU has been reported to form a double conjugate with glucuronic acid [4]. The kinetics of ASA metabolite formation in

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man have been extensively studied by Levy and co-workers [5—10] who showed capacity-limited formation of SU and salicyl phenolic glucuronide.

In order to conduct a large pharmacokinetic study of ASA in humans, our laboratory needed a rapid, sensitive, and selective assay for aspirin and its major metabolites in plasma and urine. Colorimetric assays for ASA and its metabolites are neither specific nor sufficiently sensitive [11—13]. The more recent assays using high-performance liquid chromatography (HPLC) are selective, but either fail to quantify ASA or fail to prevent its hydrolysis during sample preparation [14—19]. Other HPLC methods do not quantify metabolites of ASA and frequently do not provide for complete analysis of metabolites in urine [20—25]. While this study was in progress, two additional assays for ASA were published. One method requires a back-extraction step, not required in our procedure, and does not quantify GA [26]. The other method does not quantify urinary conjugates and does not use an internal standard [27].

Consequently, we developed a semi-automated method for analysis of ASA and its major metabolites by HPLC, which has been used successfully for measurement of over 2000 samples in studies of ASA bioavailability in humans, as well as pharmacokinetic investigations in rabbits and rats.

EXPERIMENTAL

Reagents

All reagents and solvents were reagent or HPLC grade and used as received. ASA was a gift from Endo Labs. (Garden City, NY, U.S.A.). SU, β-D-glucuronidase (97,900 I.U./ml, crude from *Helix pomatia*, EC 3.2.1.31), and heparin sodium salt, 140 USP K units/mg were obtained from Sigma (St. Louis, MO, U.S.A.). GA was purchased from Aldrich (Milwaukee, WI, U.S.A.). Mephenytoin (MP) was a gift from Sandoz (East Hanover, NJ, U.S.A.). Sodium fluoride, SA, and HPLC-grade phosphoric acid were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.). HPLC-grade methanol, acetonitrile, and dichloromethane from both Fisher Scientific and Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) were used.

Equipment

A Model 1084B liquid chromatograph equipped with a Model 79875A variable-wavelength detector, a Model 79850B LC terminal, a Model 79841A automatic injector and a Model 79842A autosampler, all from Hewlett-Packard, were used for all analyses. A 25 cm × 4.6 mm I.D. Nucleosil C_{18} column with 5-μm spherical particles was used (Alltech Assoc., Deerfield, IL, U.S.A.).

Determination of ASA, SA, and SU in plasma

Blood was collected in sterile 7-ml tubes containing 30 mg of sodium fluoride per tube (Becton-Dickinson, Rutherford, NJ, U.S.A.). The tubes were inverted gently several times to dissolve the sodium fluoride and immediately placed on ice. The blood samples were centrifuged at 0° C in a Beckman TR-6 centrifuge at 1500 *g* for 10 min. Plasma (1 ml) was pipetted into a 50-ml centrifuge tube containing 150 μl of 3 *M* orthophosphoric acid, 400 mg of

sodium chloride, and 12 ml of dichloromethane, which contained 3 μg/ml MP as the internal standard. The time which elapsed from the collection of blood to the pipetting of plasma was 30 min or less and the samples were kept on ice at all times. The tubes were shaken for at least 10 min at 300 oscillations per min on an Eberbach shaker, centrifuged and the upper layer was removed by aspiration and discarded. From the organic (lower) layer 8 ml were transferred to a 15-ml conical centrifuge tube and the solvent evaporated under reduced pressure of a water aspirator at room temperature using a Buchler Evapomix. Care was taken to remove the tubes immediately upon evaporation of the solvent. The residue was dissolved in 200 μl of mobile phase. The mixture was vortexed for 1 min, placed in a septum-capped vial, and a 10-μl portion injected on column. The mobile phase was pH 2.5 5 m*M* phosphate buffer—methanol—acetonitrile (68:16:16) and the flow-rate was 1.3 ml/min. The ultraviolet (UV) absorbance was measured at 237 nm. The retention times of GA, SU, ASA, SA and MP were 4.5, 5.7, 7.4, 10.0, and 11.6 min, respectively. Sample chromatograms are shown in Fig. 1.

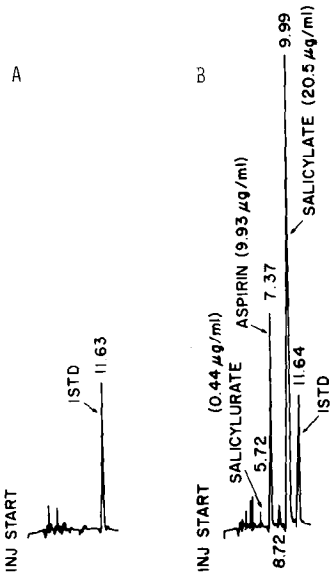

Fig. 1. HPLC chromatograms of an extract of human plasma (A) prior to dosing; (B) 20 min after oral administration of 975 mg of aspirin.

Rabbit plasma was analyzed in a manner similar to human plasma except that ca. 1 ml of blood was collected in a 5-ml polypropylene tube containing 50 μl of sodium fluoride solution (160 mg/ml). A 0.5-ml aliquot of plasma was assayed, and the quantities of salt and phosphoric acid reduced by half.

Determination of SA, SU, and GA in urine

ASA metabolites were analyzed by mixing 1 ml of urine with 1 ml of pH 5.0 0.2 *M* acetate buffer, a drop of chloroform and 20 μl of β-D-glucuronidase, and incubating at 37°C for 20 h in a Dubnoff metabolic shaking incubator. The solution was transferred quantitatively to a 10-ml volumetric flask, diluted

to volume, mixed well, and a 1-ml portion pipetted into a 50-ml conical centrifuge tube containing 12 ml of dichloromethane (9 μg/ml internal standard) and the same amounts of salt and phosphoric acid as above. The samples were shaken, centrifuged and evaporated as for plasma. The residue from evaporation of dichloromethane was reconstituted in 1 ml of mobile phase, of which 10 μl were injected on column and the UV absorbance measured at 237 nm.

When quantification of GA was desired, the residue from evaporation of the organic phase was reconstituted in 0.5 ml of mobile phase of which 50 μl were injected on column. The UV absorbance was measured at 330 nm for 4.8 min to optimize detection of GA acid and then at 237 nm for the remainder of the run. Determinations of free SA and SU in urine were essentially the same except that the addition of β-D-glucuronidase was omitted. Representative chromatograms of extracts of human urine are shown in Fig. 2.

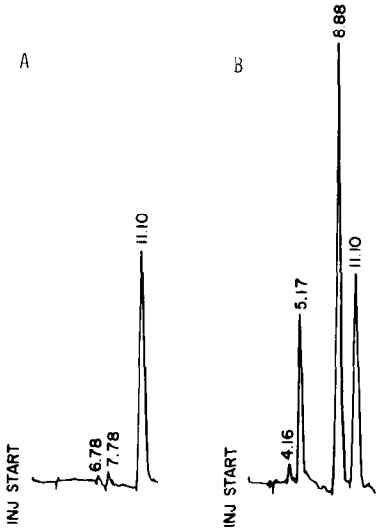

Fig. 2. HPLC chromatograms of an extract of blank human urine (A) prior to dosing with aspirin; (B) after a single oral dose of 975 mg of ASA. GA, SU, SA, and MP (internal standard) appear at retention times of 4.16, 5.17, 8.88, and 11.10 min, respectively.

Standard curves

Standard curves were prepared daily by addition of the analytes to the appropriate fluid (either plasma or urine, human or rabbit) using a 100-μl gas-tight Hamilton syringe. The concentration ranges for standards in plasma were: ASA, 0.2—100 μg/ml; SA, 0.2—100 μg/ml; SU, 0.2—4 μg/ml; and in urine were: GA, 2—20 μg/ml; SA, 20—300 μg/ml; SU, 200—2000 μg/ml. The standards for plasma (ASA, SA, and SU) were prepared in acetonitrile. The standards for urine (SA, SU, and GA) were prepared in water, with sufficient 1 *M* sodium hydroxide added to effect solution. All standards were kept refrigerated, and showed no decomposition over a period of five months. The standard curves were calculated by plotting the ratios of integrated areas of analyte and internal standard against the concentration of analyte. The line

which best fits the data was determined using a linear least-squares program on a Hewlett-Packard 85 computer. The concentrations of analyte in unknowns were then determined by the computer. Standard curves for all analyses were linear over the concentration ranges studied with correlation coefficients $\geqslant$ 0.999.

RESULTS AND DISCUSSION

Stability of ASA under assay conditions

As others have observed, the hydrolysis of ASA is minimized (less than 5%) for samples collected over fluoride, kept on ice, centrifuged at 0°C, and pipetted immediately into the extraction tubes containing solvent [23, 28]. Samples of plasma spiked with ASA and carried through this extraction procedure showed a loss of ASA of 2.2 ± 1.1%. The mobile phase is buffered to pH 2.5 at which point the rate of ASA hydrolysis is reported to be at a minimum [29]. Extracted samples stored in mobile phase for 24 h at room temperature showed a 4% loss of ASA.

Sublimation of SA during solvent evaporation has been reported by others [18, 23, 30]. We investigated the problem by comparing recoveries of SA from extracts of plasma spiked with SA. We observed no loss of SA during solvent evaporation as long as the solvent contained plasma components. However, when SA was added to pure extraction solvent (i.e. dichloromethane that had not been equilibrated with plasma), significant loss of SA occurred during evaporation. At the concentrations tested (equivalent to 40 and 1.6 μg/ml SA in plasma), 3% and 51%, respectively, of SA was lost in the evaporation step. Results in urine were similar. These findings indicate that plasma and urinary components inhibit sublimation of SA. One of us has observed this for other drugs [31]. Since SA is extracted from plasma or urine, including the standards which are spiked into these fluids, its loss through sublimation does not occur at a significant rate under the conditions of this assay.

Recovery and precision

Overall recoveries of ASA, SA, SU, and GA were determined by extracting the substances from plasma or urine and comparing the chromatographic peak areas to those obtained from unextracted standards dissolved in mobile phase. Recoveries (mean ± S.D.) from plasma for ASA, SA, and SU were 92 ± 1%, 85 ± 4%, and 52 ± 8%, respectively, and from urine for SA, SU, and GA were 98 ± 4%, 93 ± 7%, and 55 ± 3%, respectively. To measure the precision of the assay, a volunteer was given 975 mg of ASA by mouth. Blood was collected at 35 and 70 min, divided into five portions and assayed as above. For urine, a sample obtained from 0—8 h after the same dose of ASA was assayed five times. The results of the precision studies in plasma and urine are shown in Tables I and II, respectively.

Sensitivity

The lowest measurable limits of the assay as described here for plasma are: ASA, 0.2 μg/ml; SA, 0.2 μg/ml; SU, 0.2 μg/ml; and for urine are: SA, 0.2 μg/ml; SU, 0.2 μg/ml; GA, 2 μg/ml. Simple modifications can increase the

TABLE I

REPRODUCIBILITY OF FIVE DETERMINATIONS OF ASA, SA, AND SU IN PLASMA OF A HEALTHY VOLUNTEER FOLLOWING ORAL ADMINISTRATION OF 975 mg OF ASA

	Plasma concentration (μg/ml)					
	35 min post dosing			70 min post dosing		
	ASA	SA	SU	ASA	SA	SU
$\bar{X}$ ± S.D.	9.18 ± 0.20	45.5 ± 0.5	1.17 ± 0.06	3.87 ± 0.12	53.8 ± 1.7	1.43 ± 0.09
C.V. (%)	2.2	1.1	5.1	3.1	3.2	6.2

TABLE II

REPRODUCIBILITY OF SIX DETERMINATIONS OF SA, SU, AND GA IN UNHYDROLYZED URINE OF A HEALTHY VOLUNTEER FOLLOWING ORAL ADMINISTRATION OF 975 mg OF ASA

	Urine concentration (μg/ml), 0—8 h post dosing		
	SA	SU	GA
X ± S.D.	85.0 ± 1.1	1480 ± 20	24.5 ± 0.8
C.V. (%)	1.3	1.6	3.1

sensitivity of the assay if this is desired. With plasma samples, we have routinely injected 10 μl; 50-μl injections increase sensitivity five-fold and enhance the detection limit of all analytes. If urine is diluted less than ten-fold, a corresponding increase in sensitivity can be achieved.

Blanks

Human plasma has no interfering substances for the compounds assayed. Human urine has an SU blank as high as 5 μg/ml in some subjects. SU has been reported as a normal constituent of human urine [32]. Since typical SU concentrations are in the range of 1 mg/ml, this is not a serious drawback. In some subjects, an interfering peak eluted within 0.3 min of GA. This peak appears to be the aglycone of an endogenous glucuronide, since it is not present in unhydrolyzed urine. The blank for SA in urine is zero.

Rabbit plasma has no substances which interfere with either ASA or SU. However, the blank for SA amounts to 0—0.2 μg/ml. In rabbit urine, the blank for SA is 6—25 μg/ml and for SU 6—19 μg/ml. The blank for GA is too large to permit its measurement.

Use of the method

The procedure has been used in a 24-patient crossover study of ASA bioavailability. The mean concentration profiles and the pharmacokinetic parameters calculated from this data will be presented elsewhere [33].

Six other volunteers were given 975 mg of ASA by mouth and timed blood samples were removed through an indwelling venous catheter. Figs. 3 and 4 show the mean plasma curves. With ASA, a peak concentration of 11.7 ± 1.7

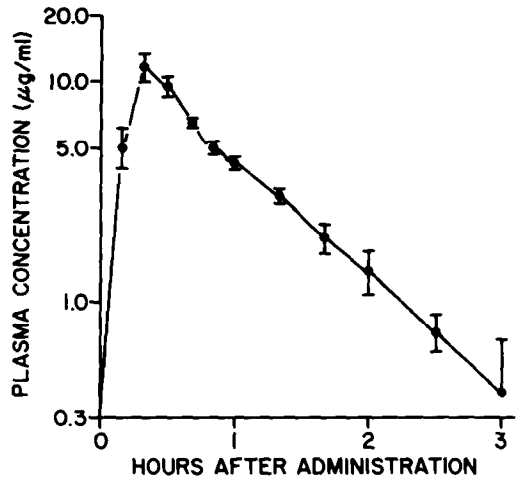

Fig. 3. The concentration of ASA in plasma of humans each given a single oral dose of 975 mg of ASA. Each point represents the mean ± S.E.M. of five different subjects.

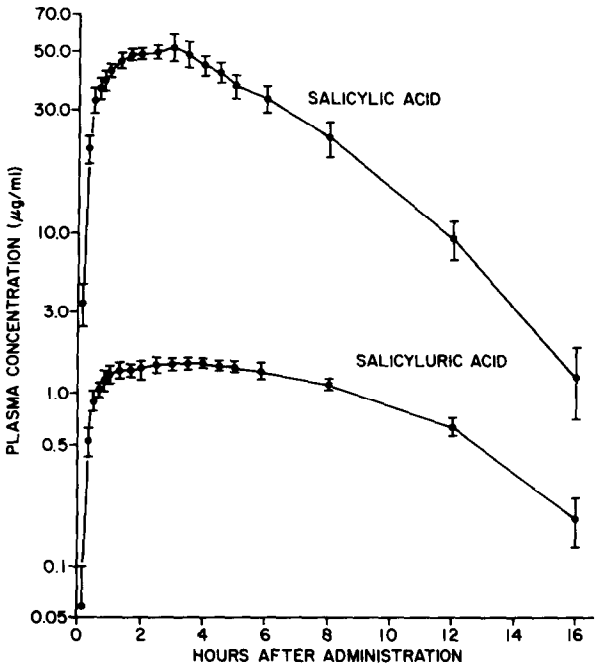

Fig. 4. The concentrations of SA and SU in the plasma of humans each given a single oral dose of 975 mg of ASA. Each point represents the mean ± S.E.M. of five different subjects.

μg/ml was attained 20 min after administration and declined with a half-life of 30.6 min. Half-lives reported for ASA in man following an oral dose of 600—650 mg range from 15 to 20 min [34—36]. Continued absorption of the larger dose of ASA during its exponential decline phase could account for the apparent prolongation of ASA half-life observed in this study. With SA, a peak concentration of 56.7 ± 4.8 μg/ml was attained at 2.37 ± 0.37 h and a terminal half-life of 2.70 h was observed, in good agreement with previously reported

TABLE III

RECOVERY OF URINARY METABOLITES FROM HEALTHY VOLUNTEERS AFTER ORAL ADMINISTRATION OF 975 mg OF ASA

Metabolite	Percent of dose recovered as metabolite						Mean ± S.D.
	KV*	HD	DA	TR	JL	CR	
Free SU	65.6	51.8	69.5	63.2	55.9	61.6	61.3 ± 6.46
Conjugated SU	1.7	2.1	7.2	9.8	16.7	6.8	7.4 ± 5.54
Free SA	17.6	11.7	6.0	7.4	5.0	3.4	8.5 ± 5.27
Conjugated SA	6.6	12.1	6.8	11.3	11.9	8.6	9.6 ± 2.54
GA	1.1	1.2	1.1	1.3	0.9	0.7	1.1 ± 0.22
Total recovery	92.6	78.9	90.6	93.1	90.4	81.1	87.8 ± 6.16

*Subject.

TABLE IV

PHARMACOKINETIC DATA FOR ASA AND SA IN RABBITS FOLLOWING INTRAVENOUS ADMINISTRATION OF 25 mg/kg ASA

Mean ± S.D. (n = 3); k_e = elimination rate constant, $t_{1/2\alpha}$ = distribution half-life, $t_{1/2\beta}$ = elimination half-life, AUC = area under the concentration—time curve, Cl_T = total body clearance.

Parameter	Aspirin	Salicylic acid
k_e (h^{-1})	5.37 ± 0.75	0.305 ± 0.100
$t_{1/2\alpha}$ (h)	0.035*	—
$t_{1/2\beta}$ (h)	0.129*	2.27*
AUC (mg l^{-1} h)	5.88 ± 0.77	135 ± 27
Cl_T (l h^{-1})	15.5 ± 0.3	0.493 ± 0.111

*Harmonic mean.

values [35]. The urinary metabolite and recovery data for these six volunteers were also determined (see Table III). Treatment of urine with β-D-glucuronidase gave significantly higher recoveries (7.4 ± 5.5%) of SU than untreated samples ($p < 0.001$). This indicates that SU, conjugated with glucuronic acid, is present in urine. Chemical evidence for this double conjugate has been observed in rats treated with SU [37] and in humans given ASA [4].

The pharmacokinetics of ASA and its metabolites after intravenous administration of 25 mg/kg ASA to rabbits were determined (Table IV). The data for ASA fitted a two-compartment model with a half-life of 2.1 min and 7.7 min for the α- and β-phases, respectively. The terminal half-life for SA was 2.3 h.

In summary, an improved assay for determination of ASA and its major metabolites in biological fluids was developed. It is rapid, sensitive, and amenable to the analysis of a large number of samples. The automation of the HPLC system allows for the continuous analysis of ASA overnight. Approx.

100 plasma samples can be processed and analyzed within 24 h, thereby eliminating the need to store samples which contributes to the loss of ASA by hydrolysis.

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