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DETERMINATION OF ACETYLSALICYLIC ACID AND METABOLITES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new method has been developed for the determination of acetylsalicylic acid, salicylic acid, salicyluric acid and gentisic acid in plasma, urine and tissue homogenates by simple extraction with ethyl acetate, evaporation and redissolution and measuring by high-performance liquid chromatography. Linearity, reproducibility and recovery were determined. Experiments were carried out to investigate the decomposition of acetylsalicylic acid in plasma with fluoride at different temperatures. The method has been used for pharmacokinetic experiments and an example is given.

INTRODUCTION

Acetylsalicylic acid (ASA) is rapidly hydrolysed to salicylic acid (SA) and acetic acid in the body [1]. SA is further metabolized to salicyluric acid (SU) (conjugation with glycine), gentisic acid (GA) (hydroxylation), salicylacylglucuronide (SAG) and salicylphenolglucuronide (SPG) (conjugation with glucuronic acid) (see Fig. 1). The formation of SU and SPG is saturable at therapeutic concentrations [2, 3] and contributes to a mixed zero- and first-order kinetics for salicylic acid.

In pharmacokinetic investigations of ASA an easy and rapid method for analyses of ASA and metabolites in biological fluids is needed. Several methods for this purpose have been developed based on high-performance liquid chromatography (HPLC) [4—11]. Terweij-Groen et al. [4] determined SA in

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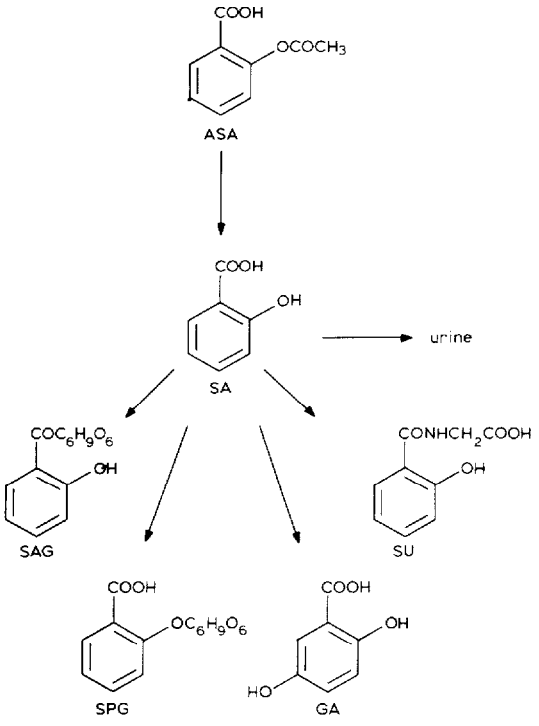

Fig. 1. Diagram showing the metabolism of acetylsalicylic acid (ASA) in humans. The formation of salicyluric acid (SU) and salicylphenolglucuronide (SPG) are saturable at therapeutic concentrations and contribute to a mixed zero- and first-order kinetics of salicylic acid (SA). SAG = salicylacylglucuronide, GA = gentisic acid.

plasma after deproteinization and injection of the supernatant, while Cham et al. [5] improved this method to include SU and GA. Maulding and Young [6] used the same method, but have improved it to include the determination in whole blood, urine and faeces. Bekersky et al. [7] determined SU and SA after deproteinization by trichloroacetic acid and extraction with diethyl ether.

None of these methods determines ASA. Harrison et al. [8] and Lo and Bye [9] determined ASA and SA after extraction with methylene chloride and chloroform, respectively, but none of the metabolites are determined by these methods. Peng et al. [10] determined ASA, SU and SA in plasma after extraction with a mixture of ethyl acetate and benzene, while Amick and Mason [11] have improved the method to include GA and determinations in urine. Rumble et al. [12] determined ASA, SU, SA and GA in plasma by deproteinization with perchloric acid and methanol. Lo and Bye [9], Amick and Mason [11] and Rumble et al. [12] used fluoride to inhibit the acetylsalicylic acid esterase, and only the two last-mentioned methods determine more than three substances.

On this basis an HPLC method was developed, by which GA, SU, ASA and SA can be determined in plasma, urine and tissue homogenates following a simple extraction by ethyl acetate, and at the same time steps have been taken to inhibit the esterase by fluoride and cooling. The method is based on the method of Amick and Mason [11], with some modifications.

EXPERIMENTAL

Reagents

Acetylsalicylic acid, salicyluric acid, gentisic acid and orthophosphoric acid are all of quality for laboratory use; salicylic acid and sodium hydroxide are Ph. Eur. grade and methanol, ethyl acetate and sodium fluoride are all of analytical quality.

Equipment

For separation and detection an HPLC pump Model M-6000 A from Waters Assoc., a Model U6K injector (Waters Assoc.), an LDC UV III Monitor (1203) detector at 280 nm and a recorder (Servo/riter II, Texas Instruments) are used. The column is 15 cm × 4 mm I.D., packed with LiChrosorb RP-18 (5 μm) and thermostatted at 45°C in a waterbath. The working pressure of the pump is 200—275 bars at a flow-rate of 1.5 ml/min.

Procedures

The blood samples are collected in vials prepared with sodium fluoride and heparin (4 mg of NaF and about 50 I.U. of heparin per 1.5 ml of blood). The vials are kept on ice for no longer than 0.5 h until further processing. The addition of sodium fluoride and keeping on ice is to minimize the hydrolysis of ASA [13]. The blood samples are centrifuged (1500 *g* for 10 min at room temperature) and the plasma is immediately extracted as follows. In a glass vial are added 50 μl of concentrated phosphoric acid, 200 μl of plasma and 600 μl of ethyl acetate. The contents are shaken for 30 sec on a whirlimixer and centrifuged at 600 *g* for 10 min at 10°C; 400 μl of the supernatant are transferred to a plastic vial and stored at −26°C until analysis. Immediately before measuring by HPLC, the 400 μl of supernatant are evaporated to dryness under a gentle stream of air (7 min) on an icebath. The residue is redissolved in 200 μl of mobile phase (methanol—water, 40:60; adjusted to pH 3.00 with 0.005 *M* phosphoric acid and sodium hydroxide), and 100 μl are injected into the liquid chromatograph. External standards are injected for every six samples and the concentration is calculated by measuring relative peak heights and correcting for dilution and recovery.

The tissue homogenates are made as follows: 500 mg of tissue and 2 ml of distilled water are homogenized on a Virtis homogenizer Model 60 K for 30 sec at 40,000 rpm. The homogenate is treated as described for plasma. For determinations in urine, the urine is diluted tenfold with water and processed as described for plasma.

RESULTS AND DISCUSSION

Chromatograms

Fig. 2a shows a chromatogram after analysing rabbit plasma without any materials added. Fig. 2b shows a chromatogram of rabbit plasma 15 min after intravenous administration of 50 mg/kg ASA. The chromatogram shows in addition to ASA, SU and SA in the rabbit plasma. No GA was measured after ASA administration, but it could be also separated. The retention times are: GA, 2 min; SU, 2.7 min; ASA, 3.7 min; and SA, 5.5 min.

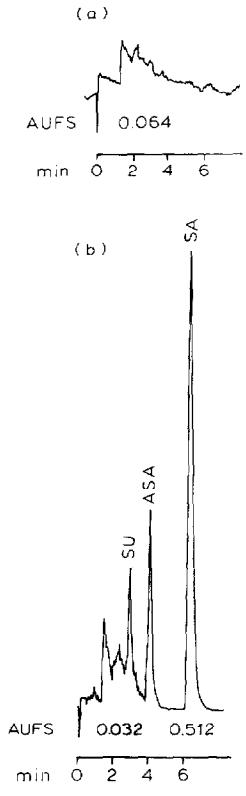

Fig. 2. Chromatograms of (a) blank samples from rabbit plasma and (b) plasma analysis 15 min after intravenous administration of 50 mg/kg ASA to a rabbit.

Linearity

Standard solutions of ASA, SA, SU and GA at different concentrations in the mobile phase (0.5, 2, 5, 10, 50, 100, 200, 300, and 500 μg/ml) were measured and the correlation between the corrected peak heights (peak height × a.u.f.s.) and the concentrations were determined. GA and SU show linearity up to 100 μg/ml (r = 0.993 and r = 0.997, respectively), while ASA and SA are linear up to 250 μg/ml (r = 0.994 and r = 0.997, respectively). Determination of higher concentrations of SA can be performed by injecting a smaller volume: 50 μl or 25 μl.

Standard solutions in plasma were measured as well, and the overall coefficient of variation for GA, SU and ASA was 4% and for SA it was 6%.

Reproducibility

To both rabbit and human plasma were added GA, SU, ASA and SA, and eight samples of each were extracted and measured. Table I shows the added and measured concentrations, demonstrating a good recovery with small variation.

Recovery

Plasma samples with different concentrations of GA, SU, ASA and SA added were extracted and measured, and the recovery was determined. Fig. 3 shows

TABLE I

REPRODUCIBILITY OF EIGHT DETERMINATIONS OF STANDARD SOLUTIONS OF GA, SU, ASA AND SA IN HUMAN AND RABBIT PLASMA

	GA	SU	ASA	SA
Human plasma				
Added (μg/ml)	24.7	45.1	63.3	80.5
Determined $\bar{x}$ (μg/ml)	24.9	42	62	72
S.D.	0.6	1	2	4
C.V. (%)	2.4	2.4	3.3	5.6
Recovery (%)	101 ± 2	94 ± 3	98 ± 3	89 ± 5
Rabbit plasma				
Added (μg/ml)	29.0	39.1	67.0	82.7
Determined $\bar{x}$ (μg/ml)	29.4	40	62	79
S.D.	0.95	1	3	3
C.V. (%)	3.2	2.9	4.7	4.3
Recovery (%)	101 ± 3	102 ± 3	93 ± 4	95 ± 4

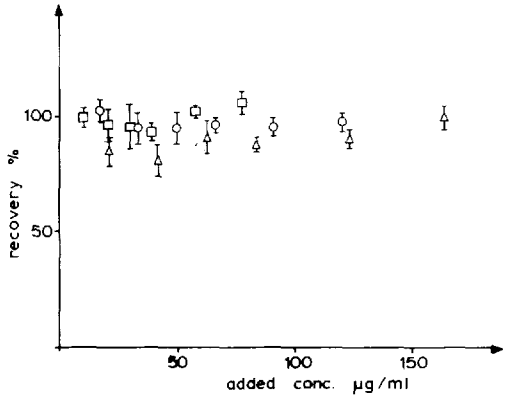

Fig. 3. Mean percentage recovery as a function of added concentration (μg/ml) to plasma of ASA (○), SA (△) and SU (□). The bars indicate the standard deviations. The percentage recovery is independent of the concentration. (SU: r = 0.646, α = 0.1204, variance ratio (VR) = 0.18. ASA: r = 0.421, α = −0.0343, VR = 0.054. SA: r = 0.883, α = 0.0832, VR = 0.88. $F_{1,4}^{0.90}$ = 4.54. F-test of dependence.)

the percentage recovery of SU, ASA and SA at different concentrations, and it is seen that the recovery does not depend on the concentration (F-test of dependence [14]).

Selectivity

In order to detect any interference, the following compounds as pure materials or tablets in solution were analysed as described for plasma: ascorbic acid, codeine, caffeine, dextropropoxyphene, inulin, paracetamol and tea. Only paracetamol interferes with SA, which should be considered when using this method.

Sensitivity

In plasma the lowest detectable concentrations were: GA, 2.5 μg/ml; SU and SA, 0.2 μg/ml; and ASA, 0.5 μg/ml. In urine: SU and SA, 10 μg/ml. In mobile phase: GA, SU and SA, 0.1 μg/ml; ASA, 0.5 μg/ml.

Storing of samples

The effect of storage of the plasma samples with added ASA and fluoride was studied after a storage time of 27 days at temperatures of 5°C, −26°C, and −80°C. Fig. 4 shows the concentration of SA formed by hydrolysis. It is seen that the samples can be stored at −80°C with only a little hydrolysis, possibly due to the thawing phase. Because of this finding the plasma samples were not stored, but were extracted within 0.5 h after blood sampling.

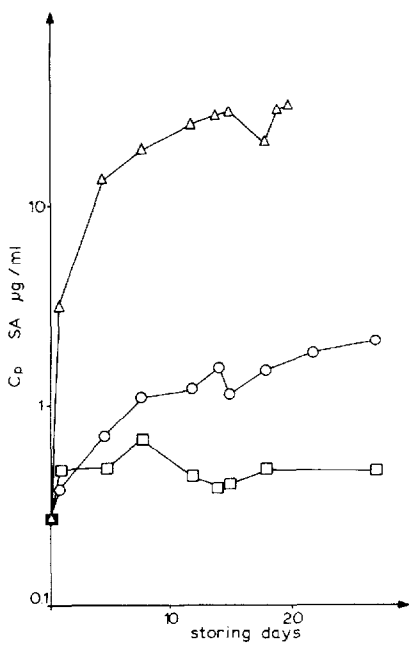

Fig. 4. SA plasma concentrations measured at different times after adding ASA to plasma with fluoride and storing at different temperatures for up to 27 days. (□), −80°C; (○), −26°C; (△), +5°C.

Use of the method

The method has been used for the determination of plasma and tissue concentration after intravenous administration of ASA or SA to rabbits and after oral administration of ASA to human volunteers. Fig. 5 shows the plasma concentration curve after administration of 650 mg of ASA per os as effervescent tablets to a human volunteer. ASA, SA and SU were measured, but no GA could be detected after ASA administration.

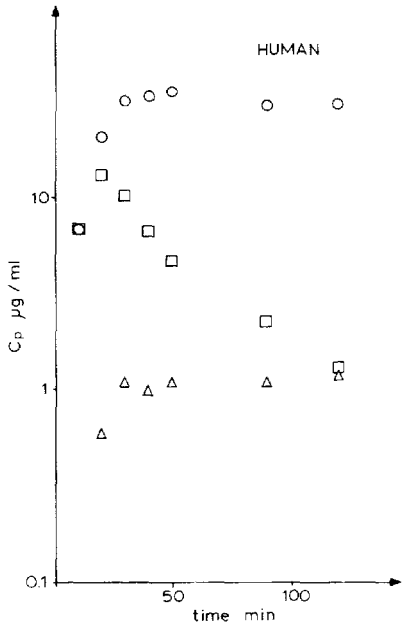

Fig. 5. Plasma concentrations of ASA (□), SA (○) and SU (△) after oral administration of 650 mg of ASA as effervescent tablets to a male human volunteer weighing 76 kg.

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