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Note

Specific and sensitive method for the determination of aspirin and salicylic acid in plasma using reversed-phase high-performance liquid chromatography

LING YEE LO and ALAN BYE*

Department of Clinical Pharmacology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS (Great Britain)

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Assays of aspirin (ASA) and salicylic acid (SA) by gas—liquid chromatography [1, 2] have been reported. However, time consuming chemical derivatizations, such as silylation, were necessary. The compounds have also been analysed by high-performance liquid chromatography (HPLC) [3—6]. One method [3] dealt with analyses of ASA and SA in pharmaceutical formulations and two other methods [4, 5] measured just SA and salicyluric acid (SU) in biological fluids. Only one method [6] determined simultaneously ASA, SA and SU in body fluids, but it required a mixture of solvents for extraction including benzene which is toxic.

This paper describes a rapid determination of ASA and SA in human plasma. One simple, non-toxic extraction solvent is used and a small plasma sample is needed.

EXPERIMENTAL

Materials

Methanol (HPLC grade) and chloroform (Distal Reagent) were obtained from Fisons (Loughborough, Great Britain); aspirin and salicylic acid from Sigma (London, Great Britain); and the internal standard, 3,4-dimethylbenzoic acid from Aldrich (Gillingham, Great Britain). Deionised water was further purified for HPLC by passing through two mini-filters (Whatman, Maidstone, Great Britain), firstly grade 80 (8 μm) and secondly grade 10 (0.9 μm).

A 0.072% acid solution (w/v, pH 2) was prepared by diluting 14.4 ml of a 5% orthophosphoric acid (w/v, pH 1) to 1 l with filtered deionized water.

*To whom correspondence should be addressed.

Instrumentation

Chromatography was performed on a Model SP8000 microprocessor-controlled high-performance liquid chromatograph with in-built data system (Spectra-Physics, St. Albans, Great Britain) equipped with the Model SP770 variable-wavelength detector (Spectra-Physics) set at 234 nm. An injection loop of 10 μl was used. The chromatographic column was a stainless-steel tube (250 × 4.6 mm I.D.) pre-packed with LiChrosorb RP-18 10 μm (Owens Polyscience, Macclesfield, Great Britain). The mobile phase consisted of methanol—0.072% orthophosphoric acid (55:45, v/v) and the flow-rate was 1.5 ml/min at 40°. The working pump pressure was approximately 94 bars (9.4 MN/m^2).

The ratios of the areas relating to the peaks of the drug or metabolite standard to that of the added internal standard peak were plotted against the known concentrations to provide standard curves. The best straight line was calculated using the least square linear regression method (Texas Instruments SR-51-II calculator).

Collection and storage of samples

The blood samples were collected into lithium heparin plastic tubes (Searle Diagnostic, High Wycombe, Great Britain) containing aqueous potassium fluoride solution (50 μl, 50% w/v) and kept in an ice bath. The samples were then centrifuged at 2°. The plasma was separated and analysed immediately or deep frozen (−20°) until analysis.

Sample preparation

A suitable range of concentrations for ASA (0.5—20 μg/ml) and for SA (0.5—100 μg/ml) were prepared by adding to human plasma known amounts of the two compounds dissolved in acetonitrile (1 mg/ml).

Aliquots of plasma (200 μl) were pipetted into 20-ml (16 mm O.D.) extraction tubes (Sovirel, Levalloix-Perret, France) and followed by the addition of 300 μl of a 10 μg/ml solution of 3,4-dimethylbenzoic acid in acetonitrile, 1 ml of 5% orthophosphoric acid (w/v, pH 1) and 10 ml of chloroform. After screw-capping, the tube contents were mixed on a reciprocating table (25 oscillations per min) at room temperature for 30 min and then centrifuged at 1000 *g* for 10 min. The lower organic layer (9 ml) was transferred into a 10 ml BC24/C14T conical centrifuge tube (Quickfit & Quartz, Corning, Stone, Great Britain) and evaporated to dryness under nitrogen in an ice water bath. The dry residues were dissolved in 200 μl of the mobile phase used and 10 μl of this was injected into the chromatograph.

Validation

Reproducibility of the assay was determined by carrying out six replicate analyses of each plasma standard with ASA concentrations at 1 and 20 μg/ml and SA concentrations at 2 and 100 μg/ml.

RESULTS

Typical chromatograms are shown in Fig. 1. The retention times of ASA, SA and 3,4-dimethylbenzoic acid were 165, 246 and 433 sec respectively. No other interfering peaks were observed.

The calibration curves for ASA and SA were linear ($y = 0.0360x - 0.0057$, and $y = 0.4307x + 0.0108$, respectively).

The coefficients of variation determined from peak area ratios of the compound to the added internal standard in plasma were less than 7% at all concentrations investigated. The results for the method precision and reproducibility are summarised in Table I.

The recovery of ASA and SA was determined by comparing the peak areas from extracted and non-extracted standards and found to be > 95% for ASA and 70% for SA.

A plasma profile from a healthy female volunteer who had taken 600 mg soluble ASA orally is given in Fig. 2. ASA was rapidly absorbed and eliminated, and its concentration at 2 h was below 0.5 μg/ml. However, the metabolite (SA) did not reach a peak level until 70—90 min after ASA dosing and was detectable at 24 h.

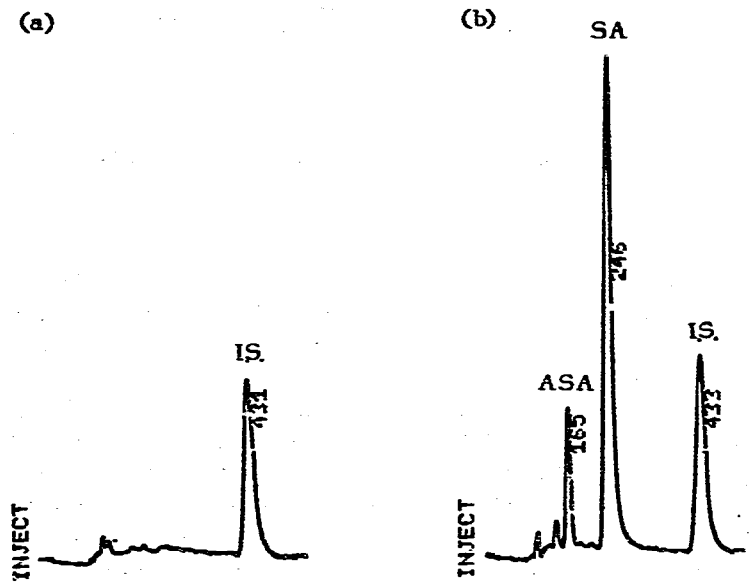

Fig. 1. HPLC determination of aspirin (ASA) and salicylic acid (SA) in plasma. (a) Blank plasma containing internal standard (I.S.) only. (b) Plasma sample containing aspirin (ASA, 9.5 μg/ml), salicylic acid (SA, 36.3 μg/ml) and 3,4-dimethylbenzoic acid (I.S., 10 μg/ml).

TABLE I

METHOD PRECISION AND REPRODUCIBILITY

Compound	Concentration in plasma (μg/ml)	n	Ratio of peak area of compound / peak area of I.S.*			C.V. (%)
			Mean	±S.D.	Range	
Aspirin	1	6	0.033	0.0021	0.030—0.035	6.4
	20	6	0.718	0.0125	0.697—0.730	1.7
Salicylic acid	2	6	0.091	0.0022	0.088—0.094	2.4
	100	3	4.306	0.1684	4.127—4.461	3.9

*I.S. = Internal standard.

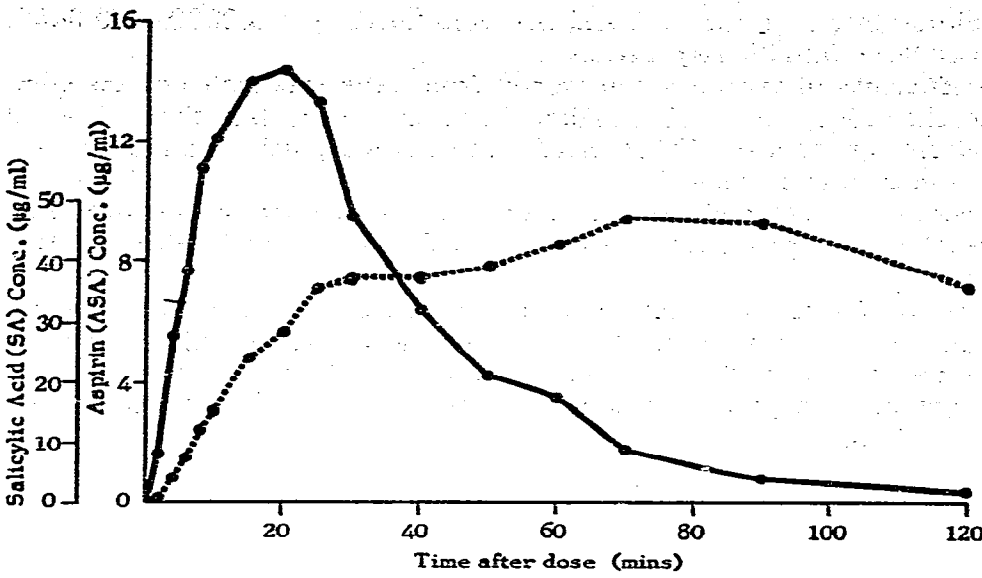

Fig. 2. Typical plasma profile of aspirin (ASA) (—) and salicylic acid (SA) (---) from a healthy fasting female after taking 600 mg of aspirin (2 soluble 300-mg tablets) orally.

DISCUSSION

The maximum concentration of ASA (14.4 μg/ml) and SA (46.8 μg/ml) observed after an oral dose of 600 mg soluble ASA were in the expected range [7, 10]. The plasma half life of ASA (15.5 min) calculated from the results agreed with the value found by Rance et al. [2].

For accurate determinations of ASA and SA, two important precautions, often ignored in some published methods [3, 5] were necessary. Firstly, the blood samples must be collected into fluoride treated tubes on ice to prevent ASA hydrolysis in human blood and plasma [11]. Hydrolysis was demonstrated in two studies [12, 13]. Secondly, the extraction solvent must be evaporated in an ice water bath to prevent underestimations of SA due to the loss through sublimation as has been reported [3].

No method published, to our knowledge, takes account of both precautions together. Only one other HPLC method [6] has been described and the chromatographic conditions are similar to ours. However, no precautions were taken to prevent ASA hydrolysis; the internal standard coeluted with an endogenous peak in dosed human plasma samples and the method was validated for spiked human and dosed rabbit plasma samples only.

The presently described method has adequate sensitivity and precision for monitoring ASA in the presence of a wide range of concentrations of SA. The detection limit for either compound is 0.5 μg/ml. No interference from the other materials present in human plasma was observed. Hence this would be useful in studies concerned with the bioavailability and pharmacokinetics of ASA and its major metabolite, SA, in man.

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