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Short communication

# Simultaneous determination of acetylsalicylic acid and salicylic acid in human plasma by high-performance liquid chromatography

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**Abstract**

A high-performance liquid chromatographic (HPLC) method is described for the simultaneous determination of acetylsalicylic acid (ASA) and its main metabolite salicylic acid (SA) in human plasma. Acidified plasma is deproteinized with acetonitrile which is separated from the aqueous layer by adding sodium chloride. ASA and SA are extracted into the acetonitrile layer with high yield, and determined by reversed-phase HPLC (column: Novapak $C_{18}$ 4 $\mu$m silica, 150×4 mm I.D.; eluent: 740 ml water, 900 $\mu$l 85% orthophosphoric acid, 180 ml acetonitrile) and photometric detection (237 nm). 2-Methylbenzoic acid is used as internal standard. The method allows the determination of ASA and SA in human plasma as low as 100 ng/ml with good precision (better than 10%). The assay was used to determine the pharmacokinetic parameters of ASA and SA following oral administration of 100–500 mg ASA in healthy volunteers.

*Keywords:* Acetylsalicylic acid; Salicylic acid

## 1. Introduction

Acetylsalicylic acid (ASA) is widely used as an analgesic, antiinflammatory and antipyretic drug. In addition, low-dose ASA is employed as an antithrombotic agent. ASA is rapidly hydrolysed in vivo to salicylic acid (SA) which is also active. SA is further metabolized by hydroxylation to gentisic acid (GA), and by conjugation to salicyluric acid (SUA) and other conjugates [1]. Colorimetric, fluorimetric and chromatographic methods have been described for the determination of ASA, degradation products and metabolites in pharmaceutical preparations and biological fluids [2]. Currently, reversed-phase HPLC techniques with photometric detection are the methods of choice [3].

Two problems are associated with the HPLC analysis of ASA and SA in biological fluids. Firstly, ASA hydrolyses to SA in protic solvents such as water or methanol, and also in plasma. The half-life of ASA, when incubated in plasma at 37°C, is about one hour [4]. In water the rate of hydrolysis is dependent on pH with best stability of ASA at pH 2–3. At pH 7 about 20% of ASA are degradated to SA within one day [5]. Therefore, partial degradation of ASA may occur after sampling and before injection into the HPLC system [6]. Secondly, part of SA may be lost by sublimation during sample treatment when evaporation steps are included [3,7–9]. The aim of the present investigations was to develop an HPLC method for the simultaneous

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determination of ASA and SA in human plasma which circumvents these analytical problems.

## 2. Experimental

### 2.1. Reagents

ASA, SA, GA, and SUA were obtained from Sigma Chemie (Deisenhofen, Germany), the internal standard 2-methylbenzoic acid from Bayer (Leverkusen, Germany), acetonitrile (LiChrosolv gradient grade), sodium chloride (p.a.), orthophosphoric acid (85%, p.a.) and hydrochloric acid (37%, p.a.) from E. Merck (Darmstadt, Germany). Water was purified through a Milli-Q water purification system (Millipore, Eschborn, Germany).

The stock solutions of ASA and SA (1000 μg/ml) were prepared in acetonitrile, that of the internal standard, 2-methylbenzoic acid (MBA, 100 μg/ml), in water. All solutions were stored at 4°C. At this temperature, decomposition of ASA to SA was less than 2% within one month, SA and MBA were stable for at least two months.

### 2.2. Collection and storage of samples

Venous blood samples from healthy volunteers were collected into heparinized plastic tubes (LiHeparin Monovette, Sarstedt, Nümbrecht, Germany), immediately chilled, and the plasma was separated by centrifugation at 4°C within 15 min. The plasma was shock frozen with liquid nitrogen and stored at −70°C until the assay.

### 2.3. Sample preparation

Frozen plasma samples were thawed in an ice-water bath. An amount of 200 μl plasma was added to 200 μl internal standard solution (5 μg/ml MBA in a 50:50 mixture of 0.2 *M* hydrochloric acid and 0.2 *M* orthophosphoric acid) and vortex-mixed for 1–2 s (Reax 2000, Heidolph, Kelheim, Germany). The resulting pH of the mixture was about 2.7. An amount of 400 μl acetonitrile was added and the solution was mixed again. After 15 min at 4°C the mixture was centrifuged (1 min; 10 500 g; Biofuge B, Heraeus Sepatech, Osterode, Germany) to separate precipitated proteins. The supernatant was transferred into 1.5-ml tubes containing 100–120 mg sodium chloride. The suspension was vortex-mixed briefly and incubated at 4°C for 10 min. After vortex-mixing and centrifuging once again (1 min, 10 500 *g*), 200 μl of the upper organic layer was transferred into autosampler vials, and 10 μl was injected into the chromatograph. For quantification spiked plasma samples of 2.5 μg/ml ASA and 5.0 μg/ml SA were assayed with each run. The procedure was also applied to whole blood [3] using 200 μl lysed blood, 400 μl internal standard solution, 600 μl acetonitrile and about 200 mg sodium chloride per sample. In this case, the volume of internal standard solution had to be doubled in order to keep the mixture at pH<3, because at higher pH the extraction of ASA and SA into the acetonitrile layer is poor.

### 2.4. Chromatography

The HPLC system (Shimadzu, Duisburg, Germany) consisted of a solvent delivery system LC-10AD, a degaser DGU 3A, an autosampler SCL-10A (equipped with a sample cooler set to 7°C to minimize evaporation of acetonitrile), a variable wavelength monitor SPD-10A (237 nm), and Class-LC10 integration software (quantitation was based on peak heights). Separation was performed using a prepacked stainless-steel column (150×3.9 mm I.D.) filled with Novapak $C_{18}$ 4-μm silica (Waters, Eschborn, Germany). The temperature was kept at 30°C (column oven ERC-125; ERC, Alteglofsheim, Germany). The mobile phase consisted of 740 ml water, 900 μl orthophosphoric acid (85%) and 180 ml acetonitrile. The resulting pH was about 2.5. The retention times (flow-rate 1 ml/min; back-pressure 93 bar) were: 2.1 min (GA), 2.9 min (SUA), 4.2 min (ASA), 6.8 min (SA) and 8.9 min (MBA). Before starting, the analytical column had to be primed with di-*n*-butylamine to avoid strong tailing of the SA peak. For this purpose, 400 μl di-*n*-butylamine was added to 200 ml eluent, and the mobile phase was recycled overnight at a flow-rate of 0.3 ml/min.

### 2.5. Validation

All dilutions in plasma were prepared keeping the tubes in an ice-water bath. Standard samples and samples for quality control were prepared by appro-

priate dilution of the stock solutions with blank plasma, and stored frozen at −70°C until assay where they were stable for at least three months. Aqueous dilutions of ASA were prepared in 0.01 *M* HCl, where ASA is sufficiently stable for at least 24 h at room temperature [5]. To determine linearity plasma standards were prepared by spiking drug-free human plasma with known amounts of each analyte (ASA, SA) to obtain concentrations of 0.2, 0.4, 0.8, 2.0, 4.0, 8.0, 20.0 μg/ml ASA and 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 μg/ml SA. To determine the limit of quantification dilutions of 0.0, 0.1, 0.15, 0.2, 0.4 μg/ml ASA and SA in plasma were prepared using a solution of 2.5 μg/ml ASA and SA in plasma for calibration. To determine the limit of detection dilutions of 100, 50, 25, 12.5 ng/ml in 0.01 *M* HCl were prepared.

## 3. Results

A simple and specific HPLC method has been developed for the simultaneous determination of ASA and SA in plasma using methyl benzoic acid (MBA) as internal standard. Gentisic acid (GA) and salicyluric acid (SUA) are also extracted into the acetonitrile layer with a similar recovery as for ASA. Fig. 1 shows typical chromatograms. SUA, ASA and SA are well separated from endogenous plasma components, and can be determined quantitatively. The assay was validated only for ASA and SA. Five calibration curves with six different concentrations (range 0.2–20 μg/ml for ASA and 0.5–50 μg/ml for SA) forced through the origin resulted in correlation coefficients with a linearity better than 0.9997 both for ASA and SA (Table 1). The mean recovery from plasma, calculated from these calibration curves, was 106.8±8.4% for ASA and 121.7±4.8% for SA, and 129.0±2.1% for MBA (conc. 5 μg/ml). The limit of detection (signal-to-noise ratio 3:1) was 75 pg on column for ASA and 100 pg for SA, respectively. The limit of quantification was 100 ng/ml for both compounds. Precision and accuracy at this level was acceptable (ASA: 99±9 ng/ml; SA: 117±9 ng/ml; $n=5$). Better sensitivity could not be achieved, because of considerable peak broadening when injecting larger volumes of acetonitrile. Even at a 10-μl injection volume the peak of ASA was broadened by about 20% versus the injection of 0.01 *M* HCl (see Fig. 1).

Stored plasma samples were stable for at least three months at −70°C; at −30°C ASA decomposed to SA: within three weeks to 5% and within seven weeks to 13%. At 2–4°C (ice-water bath) the degra-

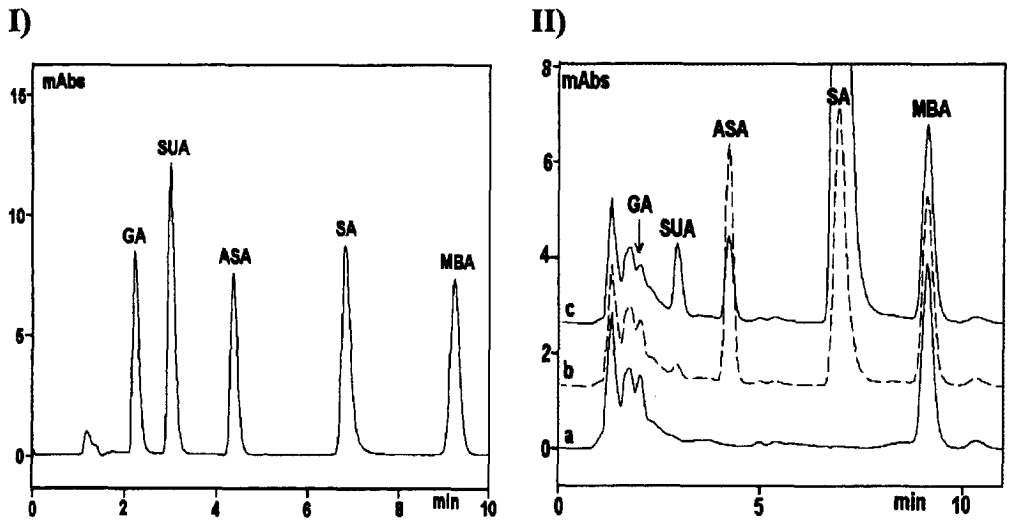


Fig. 1. Chromatograms of: (I) a standard mixture of 50 ng each of GA, SUA, ASA, SA and MBA in 0.01 *M* HCl; (II) plasma of a volunteer before (a), 10 min after (b), and 1 h after (c) oral administration of 500 mg ASA. Injection volume: 10 μl. Concentrations: b, ASA=7.5 μg/ml; SA=7.0 μg/ml; c, ASA=2.5 μg/ml; SA=32.2 μg/ml. GA=gentisic acid; SUA=salicyluric acid; ASA=acetyl salicylic acid; SA=salicylic acid; MBA=2-methyl benzoic acid (internal standard).

Table 1
Linearity of the determination of ASA and SA in human plasma

| ASA | | | SA | | |
|---|---|---|---|---|---|
| Added (μg/ml) | Found (μg/ml) | R.S.D. (%) | Added (μg/ml) | Found (μg/ml) | R.S.D. (%) |
| 0.20 | 0.201 | 7.7 | 0.50 | 0.481 | 4.2 |
| 0.40 | 0.402 | 6.0 | 1.00 | 0.9951 | 5.9 |
| 0.80 | 0.815 | 4.4 | 2.00 | 1.86 | 2.7 |
| 2.00 | 2.00 | 2.9 | 5.00 | 4.81 | 1.6 |
| 4.00 | 3.90 | 2.1 | 10.00 | 9.61 | 1.9 |
| 8.00 | 7.89 | 1.3 | 20.00 | 19.6 | 1.2 |
| 20.00 | 20.1 | 0.3 | 50.00 | 50.2 | 0.2 |
| *r* | 0.99993 | | *r* | 0.9998 | |

The concentrations were calculated as "unknown" against the linear regression lines (forced through the origin). The data are from $n=5$ standard curves (mean and relative standard deviation).

dation of ASA was 5% within one hour; the addition of potassium fluoride [2] had no influence.

The method has been applied for the analysis of about 3000 plasma samples from four bioequivalence studies in healthy volunteers following oral doses of 100–500 mg ASA. Between 34 and 68 plasma samples of the volunteers, 4 plasma controls, and 3–4 plasma standards (conc. ASA 2.5 μg/ml, SA 5 μg/ml) were processed in one run. The results of the quality control samples are listed in Table 2. In Fig. 2 the mean plasma concentration–time curve of ASA and SA is depicted following single oral administration of 500 mg ASA to twelve volunteers.

## 4. Discussion

Simple deproteinization of serum by perchloric acid–methanol mixtures or acetonitrile preceding HPLC analysis of ASA and SA resulted in only moderately precleaned samples and only moderate sensitivity [10–12]. Therefore, a common pretreat-

Table 2
Assay precision and accuracy of the determination of ASA and SA in spiked human plasma (means of *n* assays)

| Study | Analyte | Added (μg/ml) | *n* | Found (μg/ml) | Accuracy (%) | Coefficient of variation (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Intra-assay | Inter-assay |
| I | ASA | 10.00 | 13 | 10.4 | 4.4 | 1.7 | 3.2 |
| | | 1.00 | 13 | 0.990 | 5.5 | 2.9 | 6.8 |
| | SA | 20.00 | 13 | 21.0 | 5.0 | 1.4 | 2.5 |
| | | 2.00 | 13 | 1.91 | 6.2 | 4.8 | 9.0 |
| II | ASA | 5.00 | 10 | 5.02 | 2.4 | 2.1 | 3.0 |
| | | 0.50 | 10 | 0.513 | 3.3 | 4.3 | 3.7 |
| | SA | 10.00 | 10 | 10.2 | 1.8 | 1.8 | 2.1 |
| | | 1.00 | 10 | 0.975 | 6.6 | 2.9 | 3.4 |
| III | ASA | 10.00 | 10 | 10.1 | 2.9 | 2.3 | 3.8 |
| | | 1.00 | 10 | 0.944 | 6.3 | 2.1 | 4.2 |
| | SA | 40.00 | 10 | 41.6 | 4.8 | 2.1 | 4.3 |
| | | 4.00 | 10 | 3.80 | 6.2 | 2.1 | 5.0 |
| IV | ASA | 10.00 | 10 | 10.1 | 4.4 | 1.7 | 2.4 |
| | | 1.00 | 10 | 1.02 | 2.1 | 3.9 | 1.7 |
| | SA | 40.00 | 10 | 41.7 | 4.8 | 1.3 | 2.8 |
| | | 4.00 | 10 | 3.87 | 3.3 | 1.3 | 0.8 |

Control samples were processed in duplicate with each run.

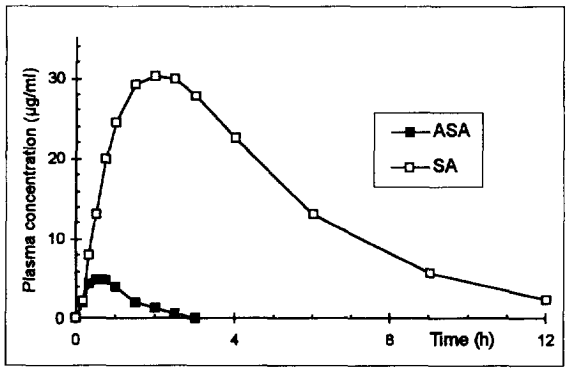


Fig. 2. Mean plasma concentration–time course of ASA and SA in twelve healthy volunteers following oral administration of one tablet containing 500 mg ASA.

ment of biological fluids is as follows: ASA and SA are extracted into organic solvents such as ethyl acetate, dichloromethane or chloroform at low pH. The organic solvent is evaporated, and the residue is dissolved in eluent preferable of low pH, where ASA is sufficiently stable [1–3,7,8,13–16]. Back extraction of ASA and SA into aqueous buffer of neutral pH is also possible [12,17], but not recommended, because ASA hydrolyses to SA in neutral aqueous solution or other protic solvents [1,6,18]. On the other hand, partial loss of SA by sublimation was observed during evaporation of the organic solvent [1,7–9,13,14].

The method described above does not differ from published HPLC procedures with respect to sensitivity, accuracy and precision, but it is outstanding with respect to simplicity in sample treatment combined with stability of ASA in the samples waiting for injection. Specimens could be stored for two days at room temperature and at least one week at 4°C before analysis without decomposition, e.g. hydrolysis of ASA to SA). The recovery was 20% higher for SA and MBA compared to ASA, and apparently more than quantitative, when referred to the extraction volume of 400 $\mu$l acetonitrile. This can be explained, because part of the acetonitrile is dissolved in the aqueous sodium chloride solution, and another part may evaporate during sample treatment. Therefore, the analytes are concentrated in acetonitrile to some extent. In an earlier investigation we also used acetonitrile successfully as final solvent for specimens waiting for injection into the HPLC apparatus to keep stable water- and methanol-sensitive compounds, namely *o*-phtaldialdehyde adducts of aliphatic amines [19].

The chromatographic system is based on published procedures [10] (see also [2]), and is has been demonstrated that ASA, SA, SUA and GA are clearly separated from each other (see Fig. 1). In man these compounds are further metabolized to glucuronides. But these conjugates are more hydrophilic than the respective parent compounds, detectable only in urine, and under reversed-phase HPLC conditions they elute before SUA [20]. Therefore, it is unlikely that these conjugates may interfere in the determination of ASA and SA in plasma.

We did not add enzyme inhibitors (e.g. potassium fluoride or physostigmine) to plasma in order to prevent enzymatic hydrolysis of ASA to SA. According to earlier investigations the preservatives are not very efficient, because enzymatic hydrolysis in plasma overlaps with chemical hydrolysis [2,16]. Therefore, immediate acidifying [16] and/or cooling and storage at −70°C after sampling are the best steps to prevent degradation of ASA in plasma. Thawed plasma samples should be handled in an ice-water bath, where they are sufficiently stable for one hour (degradation <5%).

In conclusion, the HPLC method presented here is simple and has the necessary precision, sensitivity and accuracy to allow the simultaneous determination of ASA and SA in plasma. It has been proven very useful to determine the plasma concentration–time course of ASA and SA after oral administration of 100–500 mg ASA to human volunteers, and to calculate the pharmacokinetic parameters.

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