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Determination of the theophylline solubilizer salicylamide-*O*-acetic acid in serum and urine using high-performance liquid chromatography

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Abstract: A high-performance liquid chromatographic method for the determination of the theophylline solubilizer salicylamide-*O*-acetic acid has been developed in the range 0.5 to 10 μg/ml for human serum and 5 to 400 μg/ml for urine. Reversed-phase ion-pair chromatography was employed with tetrabutylammonium hydrogen sulphate as counter-ion and 2-nitrophenylacetic acid as internal standard. Preliminary pharmacokinetic single-dose studies show that the sensitivity and the selectivity of the assay are adequate to measure lower concentrations in the late β-phase.

Keywords: *Salicylamide-O-acetic acid; theophylline solubilizer; reversed-phase ion-pair liquid chromatography.*

Introduction

In view of the enhanced efforts undertaken to improve the safety of drugs there is an increasing need to investigate the excipients used in formulations with respect to their effects on the human body, the potency of interaction with the parent drug, and their elimination pathways. This is certainly the case for the anti-asthmatic drug Solosin®, an injection that contains salicylamide-*O*-acetic acid (SAA) in comparatively high amounts as a solubility-increasing excipient for theophylline.

In the present paper a sensitive and specific HPLC method for the determination of SAA in biological fluids is described. No corresponding analytical procedure has previously been described in the literature.

Experimental

Chemicals

Water was prepared using a Milli-Q-system (Millipore Corp., Bedford, USA), supplied with deionized water. Acetonitrile and methanol (HPLC grade) were products of Baker Chem. Corp. (Gross-Gerau, FRG). The internal standard (I.S.), 2-nitrophenyl-

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acetic acid ($C_8H_7NO_4$; NPA), was received from Fluka AG (Buch, Switzerland). Salicylamide-*O*-acetic acid ($C_9H_9NO_4$; SAA) and Solosin® ampoules (containing 208 mg theophylline and 416 mg SAA in 5 ml solvent) were gifts from Cassella–Riedel Pharma GmbH (Frankfurt, FRG). Other chemicals were of analytical grade and were used without further purification.

Reagents

Standard solutions of SAA and the internal standard NPA were prepared by dissolution of the substances in ethanol in concentrations of 1 g/l for urine analysis and 50 mg/l for serum analysis, respectively.

Buffer A. Sodium dihydrogen phosphate monohydrate (0.3 mol/l) was dissolved in water and the solution adjusted to pH 8.5 by the addition of sodium hydroxide solution (6 mol/l).

Buffer B. Potassium dihydrogen phosphate (50 mmol/l) and tetrabutylammonium hydrogen sulphate (9 mmol/l) were dissolved in water and the solution adjusted to pH 7.0 by titration with potassium hydroxide solution (7 mol/l).

Buffer C. Sodium dihydrogen phosphate (0.1 mol/l) was dissolved in water and the solution adjusted to pH 8.0 by the addition of sodium hydroxide solution (6 mol/l).

Mobile phases. For the analysis of serum samples 770 ml of buffer B was mixed with 230 ml methanol whilst for urine samples 860 ml of buffer B was added to 140 ml acetonitrile. Prior to use the mobile phases were filtered (0.5 μm) and degassed by ultrasonic treatment for 30 min.

Collection of specimens

Healthy adult volunteers (male, non-smokers, free of concomitant drug treatment) were subjected to a single i.v. injection of the content of a Solosin® ampoule containing 208 mg theophylline and 416 mg SAA. Blood samples (2 ml) were collected by venupuncture at intervals after injection (0.166, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0 and 5.0 h). After centrifugation the serum samples were processed as described below. Urine was collected every 2 h over an 8-h period after injection. Serum and urine samples were found to be stable after storage at −20°C for at least two months.

Sample preparation

Serum samples (0.25 ml) were mixed in stoppered glass tubes with buffer A (0.25 ml) and with 20 μl of internal standard solution (50 mg/l NPA). After the addition of ethyl acetate (2 ml pre-saturated with water) the tubes were shaken thoroughly for 1 min followed by centrifugation (5 min; 2000 ***g***). The upper organic phase was then aspirated and discarded. This pre-extraction procedure was repeated with a further volume of 2 ml water-saturated ethyl acetate. Hydrochloric acid (0.25 ml, 1 mol/l) and ethyl acetate (7 ml) were then added to the aqueous phase followed by mechanical shaking for 30 min. After centrifugation the upper organic phase was pipetted off into glass tubes and evaporated under a stream of nitrogen at 30°C. The dry residue was dissolved in 0.2 ml of mobile phase and 0.1 ml of the resulting solution was subjected to HPLC analysis. For the analysis of urine samples 0.1 ml of internal standard solution (1 g/l NPA) was

pipetted into stoppered glass tubes and evaporated. The urine sample (0.25 ml), buffer C (0.75 ml) and 1,2-dichloroethane (6 ml) were then added to the tube, the mixture was shaken mechanically for 15 min and then centrifuged for 10 min. A portion (0.5 ml) of the upper urine phase was pipetted off and passed through a micro filter (0.45 μm; Millex-HV_4; Millipore Corp., Bedford, USA). Aliquots (10–100 μl) of the clear filtrate were injected into the HPLC valve. Urine samples with concentrations of SAA exceeding 400 μg/l were diluted appropriately with aqueous NaCl solution (0.15 mol/l) prior to analysis.

High-performance liquid chromatography

The liquid chromatograph consisted of an HPLC pump (Model 6000 A, Waters Assoc., Milford, USA), an injection valve with a 100 μl-loop (Model 7125, Rheodyne Corp., Berkeley, USA) and a variable wavelength UV-detector (Model 1203, Milton Roy GmbH, Hasselroth, FRG), which was adjusted to either 290 or 300 nm and linked to a 10 mV-recorder or to a computing integrator (Model C-R1B, Shimadzu Corp., Tokyo, Japan). The separation column (125 × 4.6 mm i.d.) was coupled directly with a guard column (20 × 4.6 mm i.d.). Both columns were filled with ODS-Hypersil (5-μm, Shandon Southern Products, Cheshire, UK), and were commercially packed (Bischoff GmbH, Leonberg, FRG). The flow rates used were 1.2 or 1.3 ml/min. Micro syringes were products of Hamilton KG (Bonaduz, Switzerland).

Calibration

Standards for calibration were prepared by spiking samples of drug-free human serum or urine with SAA in the range of 0.5–10.0 μg/ml (= 2.56 – 51.2 μmol/l) for serum analysis and 10.0–400 μg/ml (= 51.2 – 2049 μmol/l) for urine analysis, respectively. These standard samples were processed as described above in parallel to samples from volunteers.

Recovery

The efficiency of the ethyl acetate extraction of SAA and NPA from serum was estimated by comparing their peak areas in HPLC chromatograms of serum extracts with those of pure standards injected directly into the same HPLC system.

Calculations

Calibration plots were calculated on a programmed desk computer (Model HP 87, Hewlett–Packard GmbH, Frankfurt, FRG) by the method of linear regression analysis employing the ratio of peak areas (SAA to I.S.).

Results and Discussion

Sample preparation

The procedure described above for the extraction of SAA from human serum has been experimentally optimized with respect to extraction recovery and removal of undesired materials disturbing the chromatographic evaluation. Neutral and basic substances are removed through pre-extraction with ethyl acetate at pH 8.5. Under these conditions SAA and the internal standard (NPA) remain in the aqueous phase as carboxyl anions. After acidification, both substances are extracted as uncharged carboxylic acids into ethyl acetate with an average recovery of 84% for SAA and 86% for NPA, respectively.

For determinations of SAA in urine, the work-up procedure was simplified due to the relatively high concentrations of SAA observed in human urine specimens obtained after intravenous application of Solosin®. A single pre-extraction with an organic solvent (1,2-dichloroethane) at pH 7.5 removed undesired materials, whereas SAA and NPA remained in the upper aqueous phase, aliquots of which were filtered (0.45 μm) and injected directly onto the HPLC column.

High-performance liquid chromatography

Initially pure standards of SAA were chromatographed using reversed-phase columns of the ODS-type. Narrow peaks were obtained when the ionization of the SAA carboxyl group was suppressed using acidic phosphate buffers (pH 2.8) as part of the mobile phase. This method of hydrophobic chromatography on reversed-phase columns with control of solute ionization has found wide acceptance for the analysis of various carboxylic acids in biological materials [1].

However, due to its relatively high polarity SAA could not be separated satisfactorily from some rapidly eluting blank peaks when this mode of chromatography was applied to the analysis of serum or urine specimens. An increase in the relative retention time of SAA with adequate separation from the blank peaks was achieved on the same ODS-column by using reversed-phase ion-pair chromatography employing tetrabutyl-ammonium as described for other substituted organic acids [2–5].

The presence of the rapidly eluting front peaks made it necessary to find an internal standard eluting later than SAA under these chromatographic conditions. Among several substituted phenyl- and phenoxyacetic acids structurally related to SAA, 2-nitrophenylacetic acid (NPA) proved to be most suitable in view of its chemical stability and chromatographic behavior.

Serum analysis

Typical chromatograms are shown in Fig. 1. No interference from endogenous substances with the peaks of SAA or the internal standard was observed during the studies carried out with volunteers. Theophylline and its metabolites were eluted within the polar front peaks.

A linear calibration plot was obtained for peak area ratio (SAA/I.S.) versus concentration of SAA in the range 0.5 to 10 μg/ml, giving $y = 0.130x + 0.010$ with a correlation coefficient of 0.9994. The lower determination limit for the measurement of SAA in human serum, defined as three times the standard deviation (within-assay) of the determination of SAA ($n = 6$) in the lower calibration range, was found to be 0.1 μg/ml. The method described is therefore able to determine the lower serum concentrations of SAA occurring in the late β-elimination phase in pharmacokinetic studies (see Fig. 4).

Urine analysis

Acetonitrile was preferred as the organic modifier instead of methanol due to better resolution of the SAA peak from minor background peaks. For the same reason the wavelength of the UV-detector was shifted to 300 nm, where SAA still exhibits 78% of its maximum absorption at 290 nm.

Under these conditions only negligible blank peaks were observed in the vicinity of the SAA peak; typical chromatographic traces obtained from urine specimens are shown in Fig. 2 and Fig. 3.

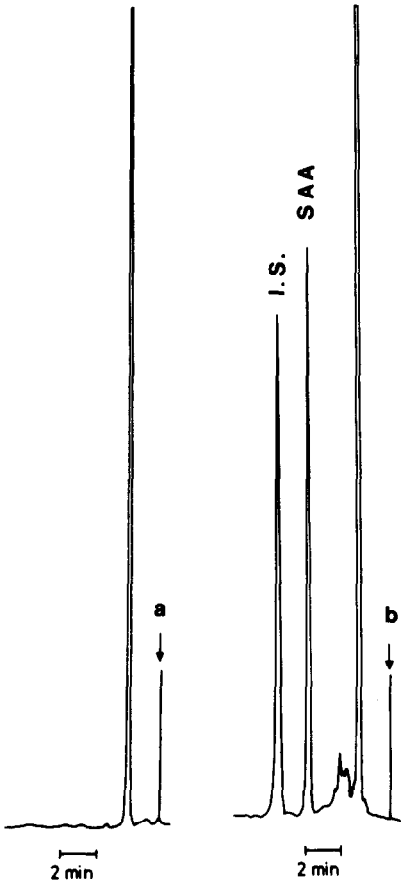

Figure 1
(a) Representative chromatogram of a blank plasma extract. (b) Chromatogram of an extract of volunteer plasma containing SAA at 7.5 μg/ml and the internal standard at 4.0 μg/ml. Chromatographic conditions: see text; detection at 290 nm, 0.10 a.u.f.s.; flow rate: 1.3 ml/min.

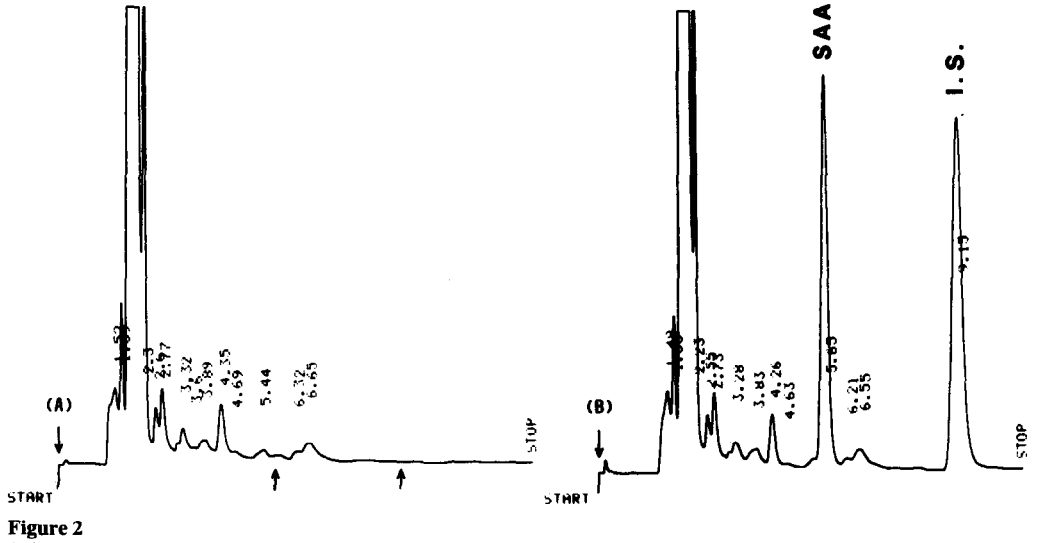

Figure 2
(A) Chromatogram of a human blank urine. (B) Chromatogram of a human blank urine spiked with SAA (333 μg/ml) and with the internal standard (400 μg/ml). Chromatographic conditions: see text; detection at 300 nm; flow rate: 1.2 ml/min.

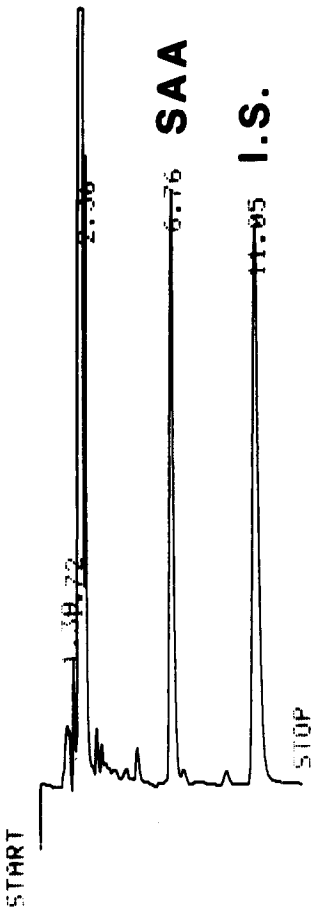

Figure 3
Representative chromatogram of a volunteer urine collected within 2 h after i.v. application of a single Solosin® ampoule. Chromatographic conditions: see Fig. 2.

Table 1
Concentration of unmetabolized SAA in urine fractions collected from a healthy male volunteer after a single i.v. dose of Solosin®

Time period (h post injection)	SAA (μ/ml)	Excretion of SAA (mg)
0–2	411.0	358.4
2–4	24.0	23.1
4–6	5.9	2.3
6–8	5.0	1.0
Total		384.4

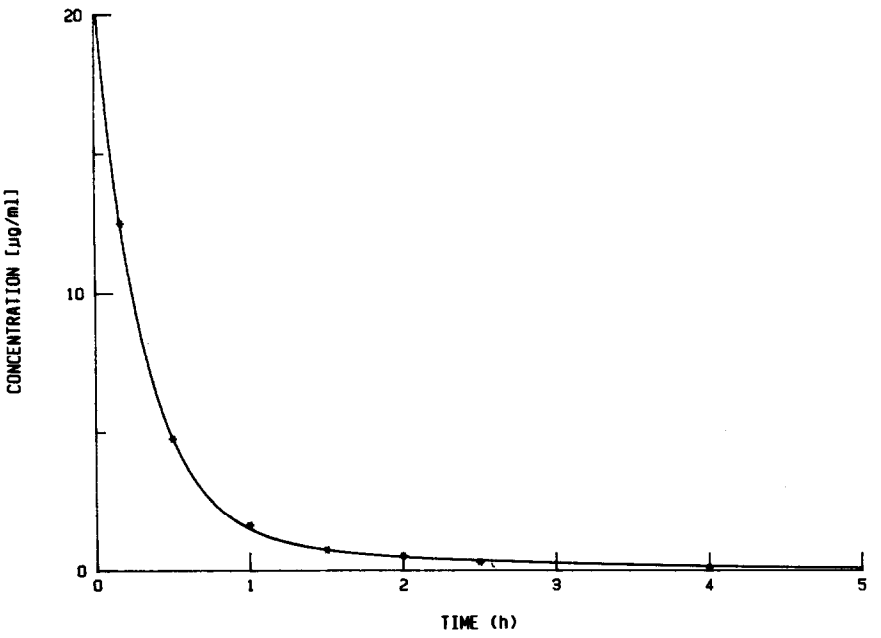

Figure 4
Typical concentration time curve for SAA in human serum after i.v. application of a single Solosin® ampoule.

A linear calibration plot was obtained for peak area ratio (SAA/IS) versus concentration of SAA in the range 5 to 400 µg/ml, giving $y = 2.29 \times 10^{-3}x + 0.02$, with a correlation coefficient of 0.9997. The determination limit (3 × SD) was found to be 5 µg/ml. With this method a precise quantification of SAA in the different urine fractions collected after i.v. application of a Solosin® ampoule was possible (see Table 1).

Conclusion

The described analytical procedure has been successfully applied to the evaluation of the pharmacokinetics of SAA and to the determination of its excretion rate into urine in male volunteers. The results of this study will be published elsewhere in detail.

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