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RAPID AND SENSITIVE DETERMINATION OF ACETYLSALICYLIC ACID AND ITS METABOLITES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic technique was developed for the simultaneous determination of gentisic acid, salicyluric acid, acetylsalicylic acid and salicylic acid in plasma and serum. The method involved a single deproteinization step and separation using a reversed-phase column eluted with a buffered methanol (35%) mobile phase. Detection was achieved with a variable-wavelength ultraviolet detector set at 235 nm and a given chromatographic analysis could be completed in less than 10 min. The method was tested in both human and animal (rat) models given a single dose of acetylsalicylic acid.

INTRODUCTION

The analgesic, anti-inflammatory and antipyretic actions of acetylsalicylic acid (ASA) have resulted in the widespread and frequent use of this drug. Most recently, it is being employed as an antithrombotic agent based on its ability to inhibit platelet function [1]. In man, ASA is rapidly hydrolysed to salicylic acid (SA) by non-specific esterases found in many tissues. SA is eliminated from the body by renal excretion and by hepatic biotransformation to salicyluric acid (SU), salicylic phenolic glucuronide, salicylic acyl glucuronide and gentisic acid (GA) [2].

Various methods have been reported for quantifying ASA and its metabolites in different biological media. These include the standard colorimetric [3] and fluorometric [4] methods and the more modern analysis by gas—liquid chromatography [5] and high-performance liquid chromatography (HPLC) [6—13]. Of these analytical methods only HPLC presently offers the required

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level of specificity, sensitivity and simplicity needed to analyse large numbers of samples in a short period of time.

Of many HPLC methods which analyse various combinations of ASA and its metabolites in different biological fluids [6—13], four employ a time-consuming solvent extraction and nitrogen evaporation process [6—10]. Others, which analyse plasma following deproteinization detect GA, SU and SA but not ASA [11, 12]. Only one method has measured ASA and its major metabolites GA, SU, ASA and SA in plasma without solvent extraction [13]. Here we describe a modification of this method for the simultaneous determination of GA, SU, ASA and SA in serum and plasma. The method improves separation and reduces sample analysis time to less than 10 min.

EXPERIMENTAL

Chemicals and solvents

The salicylate standards ASA, SA, GA and SU were obtained from Sigma (St. Louis, MO, U.S.A.). The internal standard 3,4,5-trimethoxybenzaldehyde (TMB) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Chromatographic mobile phases were prepared with double-distilled water in HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The buffering agent was HPLC grade potassium dihydrogen phosphate from Fisher Scientific. All solvents were filtered and degassed prior to use. All other chemicals were analytical grade.

Chromatographic conditions

The chromatographic system consisted of a Model 110A solvent pump (Beckman Instruments, Berkeley, CA, U.S.A.), a Model 7120 injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop, a guard column, 40 mm × 2 mm I.D. packed with Co:Pell ODS, particle size 30—38 μm (Whatman, Clifton, NJ, U.S.A.) and a reversed-phase C_8 column (octyl siloxane bonded phase on silica; 250 mm × 4.6 mm I.D.), particle size 10 μm (Alltech Assoc., Deerfield, IL, U.S.A.) at ambient temperature. The effluent was monitored using a variable-wavelength detector UV-50 (Varian Instruments, Palo Alto, CA, U.S.A.). Quantitation was accomplished with a Model 3380A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Conditions for optimal separation of the compounds of interest were studied using a mobile phase consisting of methanol and 0.1% potassium dihydrogen phosphate buffer in which the methanol and final pH of the mobile phase were systematically modified. These studies indicated an optimal mobile phase concentration of 35% methanol, pH 3.9 ± 0.1. The flow-rate was 2 ml/min and the effluent was monitored at 235 nm. Standard curves were constructed based on peak area ratios obtained by internal standardization. The line of best fit was measured using a least-squares linear regression method.

Preparation of plasma and serum samples

Blood samples (500 μl) were collected into chilled test tubes containing 5 mg/ml potassium fluoride (25%), to prevent ASA hydrolysis. For the analysis of plasma, blood samples were mixed with 1.0% disodium ethylenediamine-

tetraacetate (EDTA, 1:10 parts blood), then centrifuged at 750 *g* for 15 min and the plasma was separated. For serum preparation, blood samples were allowed to coagulate, then centrifuged at 9000 *g* for 4 min and the serum was separated.

Aliquots of plasma or serum (200 μl) were mixed with 20 μl of a 30% perchloric acid solution containing the internal standard TMB (0.02%) in 1.5-ml polypropylene Eppendorf micro test tubes (Brinkmann Instruments, Westbury, NY, U.S.A.) followed by 200 μl of methanol (spiked with standards or drug-free). The mixture was then vortexed for 2 min and centrifuged at 9000 *g* for 4 min. A 20-μl sample of the clear supernatant was injected into the chromatograph.

For standardization, freshly prepared methanolic solutions of GA, SU, ASA and SA ranging in concentration from 1.0 to 480 μg/ml were added to drug-free rat or human sera.

Preparation of urine samples

Equal volumes of urine and hydrochloric acid (10 *M*) were mixed in a 1.5-ml polypropylene test tube and deep frozen (−80°C) until analysis. After thawing, 400-μl samples were transferred to 2-ml glass ampoules, flushed with nitrogen and immediately sealed. The ampoules were heated at 120°C for 3 h to hydrolyse the conjugates. On cooling, the contents of the ampoules were vortexed and 20 μl of the hydrolysate mixed with 20 μl of internal standard (0.02%, TMB), 180 μl of double-distilled water and 200 μl of methanol (spiked with standards or drug-free). The mixture was clarified by centrifugation at 9000 *g* for 2 min. A 20-μl sample of the supernatant was injected into the chromatograph. For analysis of urine the wavelength was changed from 235 to 313 nm as preliminary studies revealed that an endogenous peak co-chromatographed with SA at the lower wavelength. Freshly prepared standard solutions were made from SA (1—100 μg/ml) dissolved in methanol and added to drug-free hydrolysed urine.

RESULTS AND DISCUSSION

The method reported here improves resolution of the four compounds (GA, SU, ASA, SA) over the original technique of Rumble et al. [13] and the analysis time was reduced to less than 10 min following preparation of the supernatant from the deproteinized sample.

Optimal conditions for the separation of GA, SU, ASA and SA were studied in preliminary experiments. Based on these results (Figs. 1 and 2) we chose as mobile phase methanol—0.1% potassium dihydrogen phosphate buffer pH 3.9 (35:65).

TMB was chosen as the internal standard in the present assay as it eluted closely after, and was completely resolved from, the compounds of interest. Blank serum, plasma and urine showed no peaks that interfered with TMB.

All four salicylate compounds and the internal standard were monitored at various wavelengths ranging from 205 to 240 nm. A wavelength of 235 nm was found to be most suitable for monitoring all the compounds simultaneously.

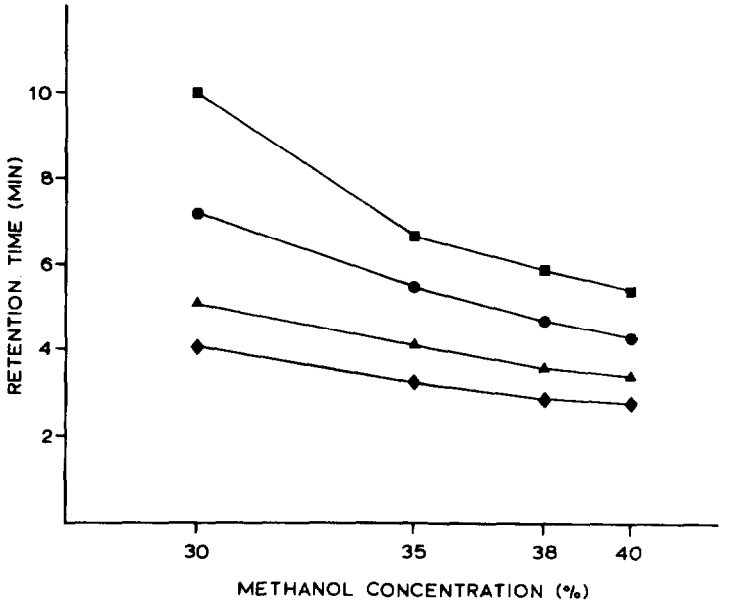

Fig. 1. Effect of methanol concentration on the retention times of GA (♦), SU (▲), ASA (●) and SA (■) at a constant pH (3.9 ± 0.2).

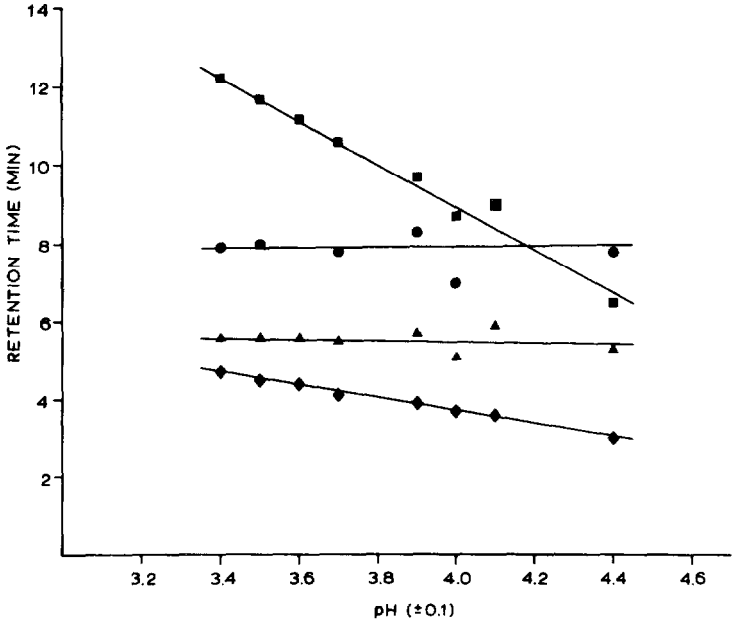

Fig. 2. Effect of pH on the retention times of GA (♦), SU (▲), ASA (●) and SA (■) at a constant methanol concentration (35%).

Sample preparation followed the simple perchlorate deproteinization procedure of Rumble et al. [13] but other agents were tested for their ability to precipitate protein. These included acetonitrile, trichloroacetic acid and formic acid. We elected to use perchloric acid in the present assay although the

other agents were also effective. However, the use of perchloric acid requires a high-speed centrifuge. If unavailable, acetonitrile may be a better choice as the deproteinizating agent [14]. The extraction of salicylate compounds from the precipitated protein gave consistent recoveries (80—86%).

Fig. 3 depicts a chromatogram of GA, SU, ASA, SA and TMB (10 μg/ml) and compares chromatograms of blank serum and spiked serum. Retention times of GA, SU, ASA, SA and TMB were 3.3, 4.1, 5.5, 6.7 and 8.7 min, respectively. The blank serum and plasma (Figs. 3 and 7) gave a baseline that was virtually free of extraneous interfering peaks after the initial solvent front. Only one endogenous peak that could interfere with low levels of SU (< 2 μg/ml) was sometimes observed at 3.9 min.

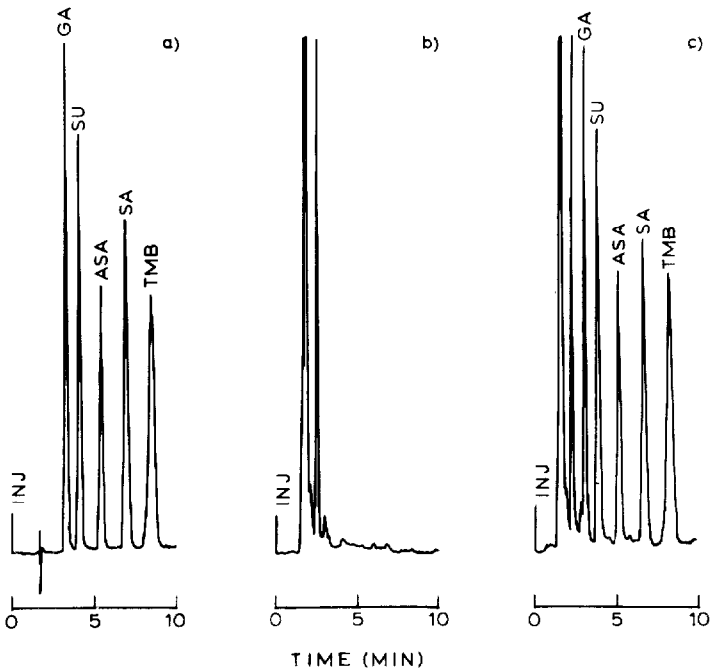

Fig. 3. Chromatograms of (a) methanol spiked with 10 μg/ml each of GA, SU, ASA, SA and TMB, (b) blank serum and (c) serum spiked with 10 μg/ml each of GA, SU, ASA, SA and TMB.

The calibration curves for serum ASA and SA were linear in the range 1—500 μg/ml while those of GA and SU were linear from 1 to 60 μg/ml. The regression equations when peak area ratios were taken were: GA, $Y = 0.054X + 0.007$, $r = 0.9932$; SU, $Y = 0.065X - 0.028$, $r = 0.9933$; ASA, $Y = 0.054X + 0.007$, $r = 0.9984$; SA, $Y = 0.063X + 0.012$, $r = 0.9979$. The limit of detection, defined as a peak at least three times the height of the baseline noise measured over a 2-min interval, for GA, SU, ASA and SA were 0.3, 0.8, 0.3 and 0.2 μg/ml, respectively.

The reproducibility of the method was investigated by analysing six serum replicates of each compound at a concentration of 45 μg/ml. The coefficient of variation for the normalized peak area ratios for GA, SU, ASA and SA were 5.2, 3.2, 3.9, and 4.2, respectively.

TABLE I

RELATIVE RETENTION TIMES OF BENZOIC ACID DERIVATIVES

Relative retention time*	Compound
0.08	Sulphosalicylic acid
0.25	3,4-Dihydroxybenzoic acid
0.27	4-Aminobenzoic acid (PABA)
0.32	Benzoylaminoacetic acid (hippuric acid)
0.34	Gentisic acid
0.34	4-Aminosalicylic acid
0.40	2,3-Dihydroxybenzoic acid
0.40	2,6-Dihydroxybenzoic acid
0.41	4-Hydroxybenzoic acid
0.46	3-Hydroxybenzoic acid
0.47	2,4-Dihydroxybenzoic acid
0.47	2-Acetylbenzoic acid
0.48	Salicyluric acid
0.68	Acetylsalicylic acid
0.83	Salicylic acid
1.00	3,4,5-Trimethoxybenzaldehyde (ISTD)
> 1.4	4-Methylbezoic acid (*p*-toluic acid)
≫ 1.4	4-Methylsalicylic acid

*Based on retention time of ISTD.

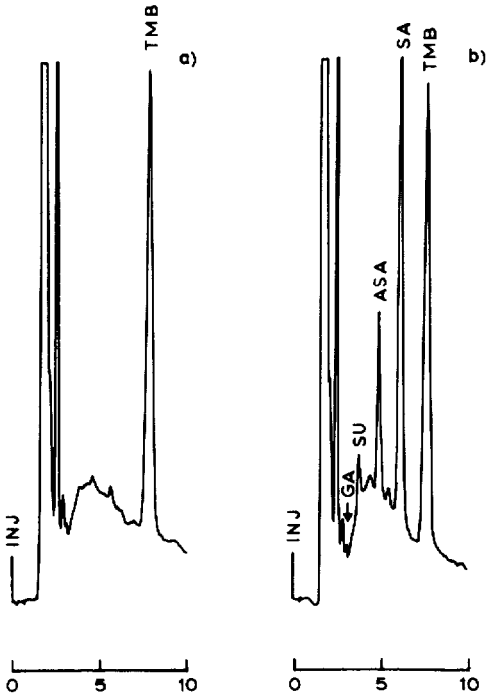

Fig. 4. Chromatograms of (a) blank human plasma containing TMB and (b) human plasma 30 min after administration of 1.3 g aspirin orally.

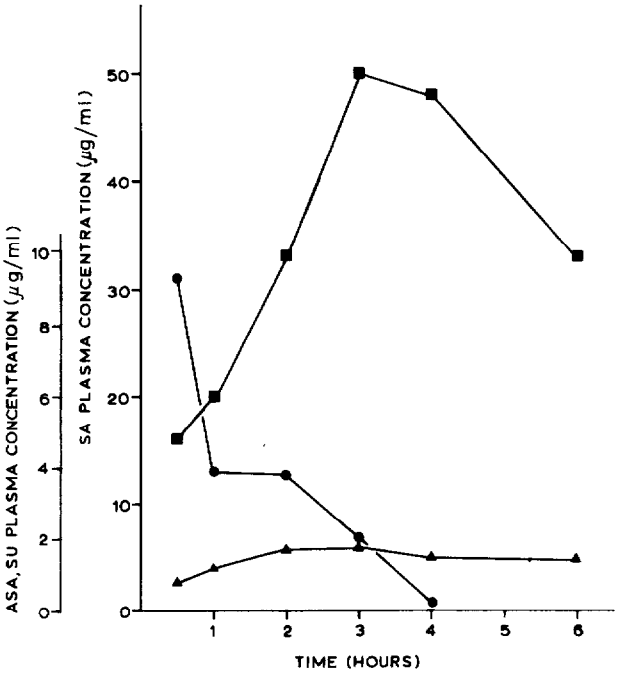

Fig. 5. Concentrations of SA (■), ASA (●) and SU (▲) in human plasma following an oral dose of four 325-mg tablets of ASA. GA was quantifiable at 0.5 and 4 h (0.4 and 0.5 μg/ml, respectively) but values fell below the limit of detection (0.3 μg/ml) at the other sampling times.

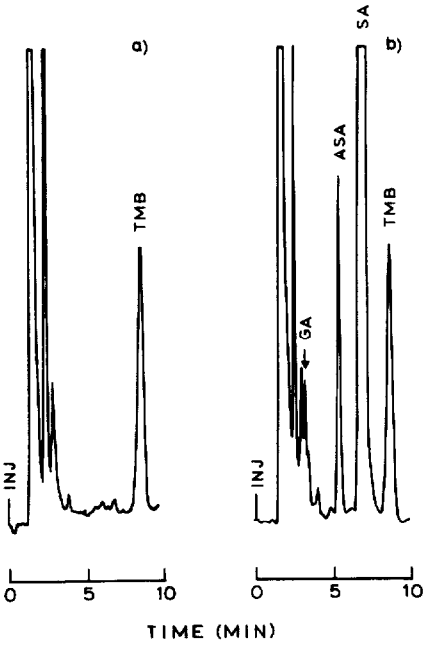

Fig. 6. Chromatograms of rat serum before (a) and 30 min after (b) the administration of 200 mg/kg ASA intravenously. Samples were filtered before being injected onto the chromatograph.

Several benzoic acid derivatives were tested in this system for interference with ASA and its metabolites and the results are shown in Table I.

To accurately determine the concentration of ASA, it was necessary to collect blood into chilled tubes containing an esterase inhibitor such as potassium fluoride to prevent rapid hydrolysis [15]. In addition the samples were analysed as quickly as possible or deep-frozen until analysis to minimize the extent of ASA hydrolysis.

When blood was collected with 30 I.U./ml heparin (Organon, Toronto, Canada) as the anticoagulant, the preservative, benzyl alcohol, was found to co-chromatograph with SU. This could be averted with heparinized blood collection tubes or by using other anticoagulants such as EDTA.

The versatility of the method was tested on human and animal (rat) models. Rats were given ASA by intravenous infusion (200 mg/kg) whereas one human subject was given four 325-mg tablets of ASA by oral administration. Blood samples were obtained from each model at various time periods and analysed for ASA and its metabolites. Urine from rats was also analysed.

Fig. 4 compares chromatograms of human plasma from the same subject before and after the ingestion of a single oral dose of 1.3 g of ASA. The plasma concentration—time profiles of GA, SU, ASA and SA are shown in Fig. 5 from

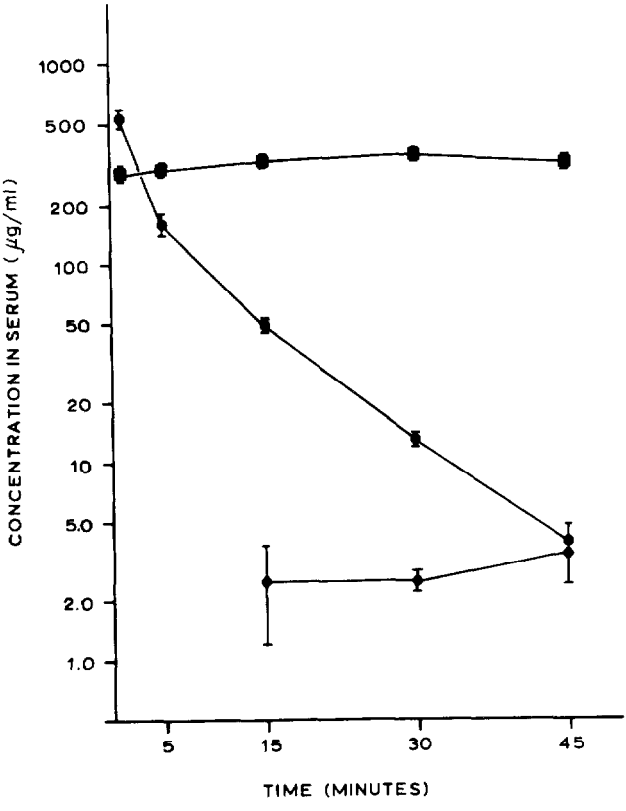

Fig. 7. Time course (mean ± S.D.) of GA (♦), ASA (●) and SA (■) concentrations in rat serum (n = 3) following a 200 mg/kg intravenous dose of ASA. Values of GA were below the limit of detection (0.3 μg/ml) at 1 and 5 min.

samples collected at 0.5, 1, 2, 3, 4 and 6 h. These results are comparable to profiles found by others at similar doses [11, 13, 16, 17]. The maximum concentration of SA (50 μg/ml) was lower than expected but this may be due to the pronounced inter-subject differences in metabolism experienced by normal subjects receiving the same dose of ASA [18].

Fig. 6 shows typical chromatograms of serum before and after a rat was treated with 200 mg/kg ASA intravenously. The time course of serum concentrations (mean ± S.D.) of GA, ASA and SA is shown in Fig. 7. The serum half-life of ASA (7—8 min) determined from these experiments was shorter than the plasma half-life for man (15 min) [19]. Salicyluric acid was not detected in serum in the present study, although SU has been reported in rat urine by Nelson et al. [20]. However, based on an excretion rate of 1 μg/min for SU in the rat [20], serum levels would be close to or below the limits of detection of SU (0.8 μg/ml) in this assay.

Chromatograms of rat urine collected before and after ASA, 200 mg/kg intravenously, are shown in Fig. 8. The retention times of SA and TMB were 6.0 and 7.5 min, respectively. No interfering peaks were seen in blank urine samples. The calibration curve for urine SA was linear in the range 1—100 μg/ml. The regression equation obtained from data based on rat urine samples was $Y = 0.034X - 0.016$, $r = 0.9960$. The coefficient of variation determined from peak area ratios was 2.1% and the detection limit was 1.6 μg/ml.

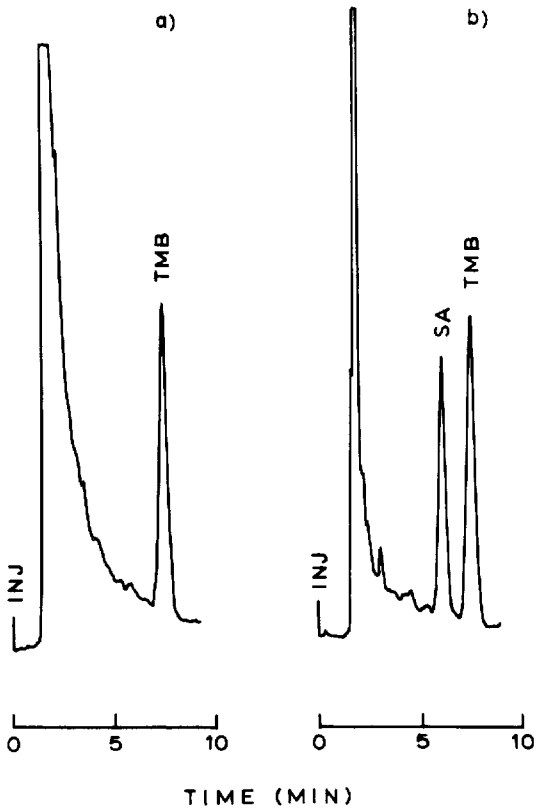

Fig. 8. Chromatograms of (a) blank rat urine containing TMB and (b) rat urine 50 min after administration of 200 mg/kg ASA intravenously. The total salicylate level (mean ± S.D.) was 1.25 ± 0.72 mg/ml ($n = 3$).

ASA is almost completely excreted by the kidneys (99%), as SA or its four major metabolites, GA, SU and the two conjugates salicylic phenolic glucuronide and salicylic acyl glucuronide [21]. Therefore, it is important to completely hydrolyse the glucuronide conjugates in the urine in order to determine total salicylate.

In a previous paper from this laboratory [22], a slightly modified version of this HPLC technique was employed to determine salicylate levels in human serum following ingestion of 325 mg of ASA. This HPLC method is currently being used in a pharmacokinetic study of ASA in doses as low as 1.7 mg/kg, intravenously in an arterial thrombosis rat model.

CONCLUSION

This HPLC technique offers a simple and rapid assay for the determination and quantitation of GA, SU, ASA and SA in biological fluids. The method is highly sensitive and can quantify samples to concentrations as low as 0.2 μg/ml. The assay incorporates a single deproteinization step followed by a rapid chromatographic analysis time of less than 10 min. This method was found suitable for pharmacokinetic studies of ASA and its major metabolites in animals and man.

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