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High-performance liquid chromatographic assay of 5-aminosalicylic acid (5-ASA) and its metabolites N-β-D-glucopyranosyl-5-ASA, N-acetyl-5-ASA, N-formyl-5-ASA and N-butyryl-5-ASA in biological fluids

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ABSTRACT

A fast, highly sensitive high-performance liquid chromatographic method for the simultaneous determination of 5-aminosalicylic acid (5-ASA) and its metabolites N-β-D-glucopyranosyl-5-ASA, N-formyl-5-ASA, N-acetyl-5-ASA and N-butyryl-5-ASA has been developed using a dynamically modified silica approach on a 40 mm × 4.6 mm I.D. column packed with 3-μm Hypersil. Plasma proteins are precipitated with acetonitrile. After extraction of the acetonitrile into 1,1,1-trichloroethane an undiluted aqueous phase containing the analytes is obtained. The detection limits are in the range 0.002–0.05 μg/ml in plasma at a signal-to-noise ratio of 3 using fluorescence detection.

INTRODUCTION

5-Aminosalicylic acid (5-ASA) is a drug used in the treatment of inflammatory bowel diseases [1]. A number of assay procedures for the determination of 5-ASA and its major metabolite N-acetyl-5-ASA (Ac-5-ASA) are described [2–14]. Recently three new metabolites of 5-ASA have been described and characterised, namely N-β-D-glucopyranosyl-5-ASA (Glc-5-ASA) [15], N-formyl-5-ASA (F-5-ASA) [16] and N-butyryl-5-ASA (Bu-5-ASA) [17]. In this paper we present a high-performance liquid chromatographic (HPLC) method using dynamically modified silica for the determination of 5-ASA and all its known metabolites in biological fluids.

EXPERIMENTAL

Chemicals

5-ASA and Ac-5-ASA were kindly supplied by Ferring (Copenhagen, Denmark). F-5-ASA, Bu-5-ASA and N-isobutyryl-5-ASA (iso-Bu-5-ASA) were synthesised according to methods previously described [16,17]. Glc-5-ASA was

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formed in a solution of 0.83 *M* glucose and 1.6 m*M* 5-ASA in 0.2 *M* potassium phosphate buffer (pH 7.4)–methanol (1:1) stored at ambient temperature for 24 h. All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

Apparatus

A Kontron (Tegimenta, Switzerland) liquid chromatograph was used. It consisted of an Anacomp 220 HPLC controller, a Kontron 420 HPLC pump, a Kontron MSI 660 HPLC autosampler and a Kontron 480 column oven. For detection a Jasco (Tokyo, Japan) 821-FP intelligent spectrofluorometric detector was used (excitation at 315 nm and emission at 470 nm if no other values are stated).

Sample preparation

A 1.0-ml volume of plasma was mixed with 2.0 ml of acetonitrile for 20 s on a vortex mixer. After 15 min at −20°C and 10 min at ambient temperature the mixture was mixed prior to centrifugation at 2000 *g* for 15 min. The supernatant was decanted into a 10-ml Kimax glass tube with a screw cork, 5.0 ml of 1,1,1-trichloroethane were added and the acetonitrile was extracted into the organic phase by shaking for 2 min. After centrifugation at 2000 *g* for 15 min the supernatant was transferred to the autosampler vials and 20 μl were injected onto the column.

A 1.0-ml aliquot of urine was mixed with 4.0 ml of methanol. After 15 min at −20°C the mixture was centrifuged for 15 min at 2000 *g*. An aliquot of the supernatant was diluted with distilled water (1:1) and 20 μl were injected onto the column.

Faeces was collected in and extracted by 500 or 1000 ml of methanol depending on the amounts of faeces delivered. The extract was centrifuged for 4 min at 10 000 *g*, an aliquot of the supernatant was diluted with distilled water (1:1) and 20 μl were injected onto the column.

Preparation of standards

External standard quantification was used. Standards of 5-ASA, F-5-ASA, Ac-5-ASA and Bu-5-ASA in water were used for the determination in urine and faeces. Blank plasma spiked with each of the analytes was used as standard solution for the assays of plasma samples.

A pure standard of Glc-5-ASA was not obtainable. Thus the concentration of Glc-5-ASA was determined indirectly. Glc-5-ASA degrades to 5-ASA at weakly acidic pH [15]. Therefore the amount of Glc-5-ASA in the standard preparation was determined as the difference of the 5-ASA content in two diluted standards of Glc-5-ASA, one at pH 7.4 and one at pH 3.0.

Chromatography

An analytical column, 40 mm × 4.6 mm I.D., from Knauer (Berlin, Germany) was packed with Hypersil Shandon (Cheshire, UK) 3-μm particles using the dilute slurry technique [15]. The saturation column, 150 mm × 4.6 mm I.D., situated between the pump and the autoinjector, was dry-packed with LiChroprep Si 60 (15–25 μm particles) (Merck). Both columns were operated at 34°C. The mobile phase consisted of methanol–0.2 *M* potassium phosphate buffer (pH 6.5)–water (40:20:40) with 4 m*M* hexadecyltrimethylammonium bromide (CTMA) added. The flow-rate was 1.7 ml/min.

RESULTS AND DISCUSSION

The dynamically modified silica approach has previously been used for the separation of 5-ASA and Ac-5-ASA in biological fluids [6,14]. In order to obtain a fast and highly sensitive method a short column (40 mm) was packed with silica with a relatively small surface area. This provided short retention times [18]. Particles of 3 μm were chosen in order to obtain a high efficiency of the system. The chromatographic system was investigated with respect to ionic strength, concentration of CTMA, pH of the buffer, percentage of the organic modifier, solvent selectivity and temperature. Dilution of the plasma samples was prevented by precipitating proteins with acetonitrile and subsequent removal of the acetonitrile by extraction with a halogenated hydrocarbon. A similar approach has previously been described for the analysis of cefotetan epimers in human plasma [19].

Solvent selectivity

No difference in the solvent selectivity was observed when using 20% acetonitrile, 18% tetrahydrofuran or 40% methanol in the eluent.

Percentage of organic modifier

The retention of Glc-5-ASA, 5-ASA, F-5-ASA, Ac-5-ASA and Bu-5-ASA decreased as the percentage of methanol in the mobile phase increased.

pH of the buffer

In Fig. 1, the retention of 5-ASA and its metabolites is shown as a function of the pH of the buffer. The retention (k' values) is expected to increase as the pH of the buffer increases due to an increased adsorption of CTMA ions to the surface of the silica. However, the retention of 5-ASA and its metabolites decreased as the pH of the buffer increased. This behaviour of anions in dynamically modified silica systems has previously been described [6].

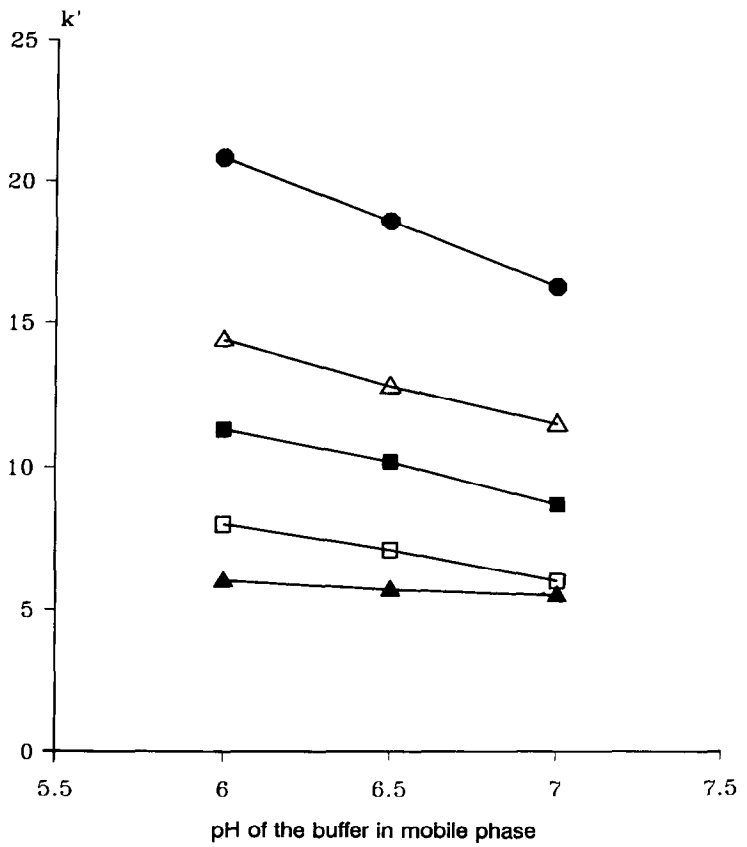

Fig. 1. Relationship between the pH of the buffer added to the eluent and the capacity factor (k') for 5-ASA and its four metabolites. Column: Hypersil 3 μm, 40 mm × 4.6 mm I.D.; eluent: methanol–0.2 *M* potassium phosphate buffer (pH 6.0–7.0)–water (40:20:40), operated at 34°C. (▲) Glc-5-ASA; (□) 5-ASA; (■) Ac-5-ASA; (△) F-5-ASA; (●) Bu-5-ASA.

Ionic strength

An increase in ionic strength decreased the retention of all solutes.

Concentration of CTMA

In Fig. 2 k' values of 5-ASA and its metabolites are shown as a function of the concentration of CTMA in the mobile phase. The retention of all five compounds exhibit a maximum at a distinct concentration of CTMA in the mobile phase, when 30% methanol is present (Fig. 2A). The concentration at which the maximum is reached has previously been shown to correspond to the critical micelle concentration of CTMA in the mobile phase [20]. The observed maximum at 1.5 m*M* CTMA for a mobile phase containing 30% methanol corresponds to previous observations concerning similar compositions of the mobile phase [21]. The

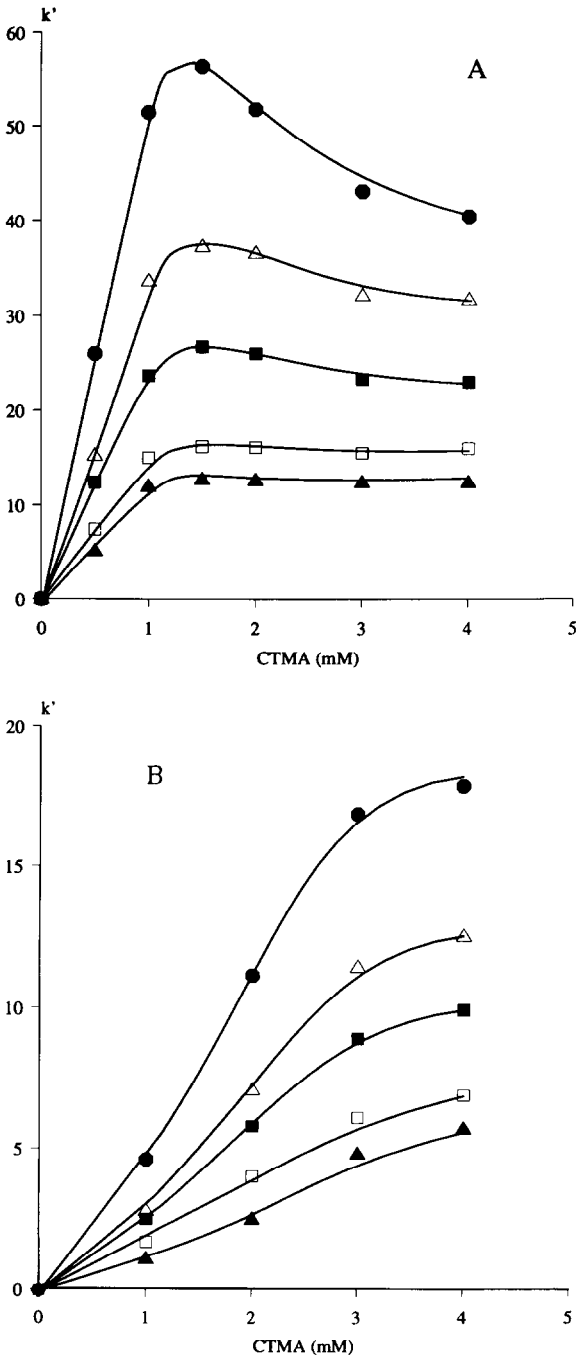

Fig. 2. Relationship between the concentration of CTMA in the eluent and the capacity factor (k') for 5-ASA and its four metabolites. Column as in Fig. 1; eluent: methanol–0.2 *M* potassium phosphate buffer (pH 6.5)–water [30:20:50 (A)] and [40:20:40 (B)]. Temperature as in Fig. 1. (▲) Glc-5-ASA; (□) 5-ASA; (■) Ac-5-ASA; (△) F-5-ASA; (●) Bu-5-ASA.

retention of all five compounds increased as the concentration of CTMA increased when 40% methanol is present in the mobile phase (Fig. 2B).

Temperature

It was observed that an increase in column temperature leads to increasing deformation of the chromatographic peaks, and that a subsequent decrease in the column temperature restored normal peak shapes. The observed deformation of the peaks was more pronounced at lower percentage of organic modifier in the mobile phase. The deformation was observed when using methanol and tetrahydrofuran as the organic modifier, but was absent when using acetonitrile. Column length had no influence on this phenomenon.

In order to investigate whether the phenomenon was due to the difference in temperature between the sample and the mobile phase at the column inlet, a column oven Shimadzu (Tokyo, Japan) CTO 6A equipped with a preheating unit was used. Preheating the mobile phase and the sample before it reaches the column inlet completely restored the peak shape (Fig. 3).

No selectivity changes due to temperature were seen.

Linearity

The calibration curves for Glc-5-ASA, 5-ASA, F-5-ASA, Ac-5-ASA and Bu-5-ASA passed through the origin and were linear in the range 0.005–10 μg/ml ($r = 0.998$).

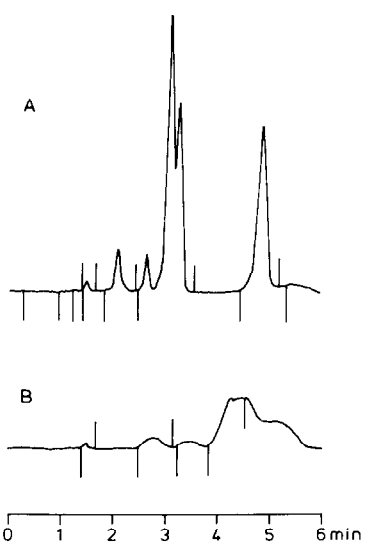

Fig. 3. Chromatograms of standards in water (A) with and (B) without preheating the sample before injection onto the column. Column: Hypersil 5 μm, 120 mm × 4.6 mm I.D.; eluent: methanol–0.2 *M* potassium phosphate buffer (pH 6.5)–water (40:20:40) with 4.0 m*M* CTMA added. Temperature 60°C.

TABLE I

MINIMUM DETECTABLE CONCENTRATIONS OF Glc-5-ASA, 5-ASA, F-5-ASA, Ac-5-ASA AND Bu-5-ASA AT A SIGNAL-TO-NOISE RATIO OF 3 USING FLUORESCENCE DETECTION AT DIFFERENT WAVELENGTHS

Analyte	Minimum detectable concentration (μg/ml)		
	315 nm/430 nm	315 nm/470 nm	335 nm/500 nm
Glc-5-ASA	0.03	0.008	0.005
5-ASA	0.02	0.004	0.002
F-5-ASA	0.002	0.005	0.01
Ac-5-ASA	0.002	0.003	0.008
Bu-5-ASA	0.002	0.005	0.01

Detection limit

The detection limit was chosen at a signal-to-noise ratio of 3. The minimum detectable concentrations of 5-ASA and its metabolites are shown in Table I.

Sample preparation and recovery studies

The recovery of 5-ASA, F-5-ASA, Ac-5-ASA and Bu-5-ASA was studied with respect to the volume of the solvent used for extraction (4.0–8.0 ml) and the volume of acetonitrile (1.0–3.0 ml) used for precipitation of plasma proteins in 1.0 ml of plasma compared to standard solutions in water. There were no major differences in the recoveries of the solutes regardless of the volumes used for extraction and precipitation.

1,1,1,-Trichloroethane and methylene chloride were compared as extractants. Uncorrected data showed that with methylene chloride approximately 30% higher concentrations were obtained for all analytes than with 1,1,1,-trichloroethane. These differences are probably due to differences in the distribution of acetonitrile between the organic and the aqueous phase. Taking the internal standard (iso-Bu-5-ASA) into consideration the recoveries were similar when using the two solvents.

The recovery of 5-ASA, F-5-ASA, Ac-5-ASA and Bu-5-ASA in plasma was investigated in the range 0.05–10 μg/ml. Results are given in Table II. The recovery of Glc-5-ASA could only be estimated indirectly as no standard of Glc-5-ASA was available. A mixture of Glc-5-ASA and 5-ASA was prepared from a known concentration of 5-ASA as described under *Chemicals*, added to plasma in a known concentration and extracted. Glc-5-ASA and 5-ASA were quantified using standard solutions as previously described and the sum of the molar concentrations of 5-ASA and Glc-5-ASA was compared with that of the standard solution used for preparing the mixture. The recovery of Glc-5-ASA was 95% with a coefficient of variation (C.V.) of 4% ($n = 6$).

TABLE II

RECOVERIES OF 5-ASA, F-5-ASA, Ac-5-ASA AND Bu-5-ASA FROM PLASMA AT VARIOUS CONCENTRATIONS

The plasma/acetonitrile ratio was 1:2 and 5.0 ml of 1,1,1-trichoroethane were used for extraction (n = 6).

Concentration (μg/ml)	Recovery (mean ± C.V.) (%)			
	5-ASA	F-5-ASA	Ac-5-ASA	Bu-5-ASA
0.05	99 ± 2	92 ± 3	101 ± 2	81 ± 6
0,1	98 ± 3	97 ± 4	97 ± 2	91 ± 4
0.5	95 ± 6	101 ± 4	108 ± 4	97 ± 4
1.0	94 ± 1	97 ± 2	99 ± 1	95 ± 2
5.0	97 ± 2	99 ± 1	104 ± 1	99 ± 2
10.0	98 ± 1	99 ± 1	103 ± 1	100 ± 0

It was observed that using peak areas gave a higher recovery for 5-ASA than using peak heights. Minor amounts of acetonitrile may be present in the aqueous sample solution injected after preparing plasma samples. Thus the effect of the amount of acetonitrile in the sample solution on the peak height was studied. The

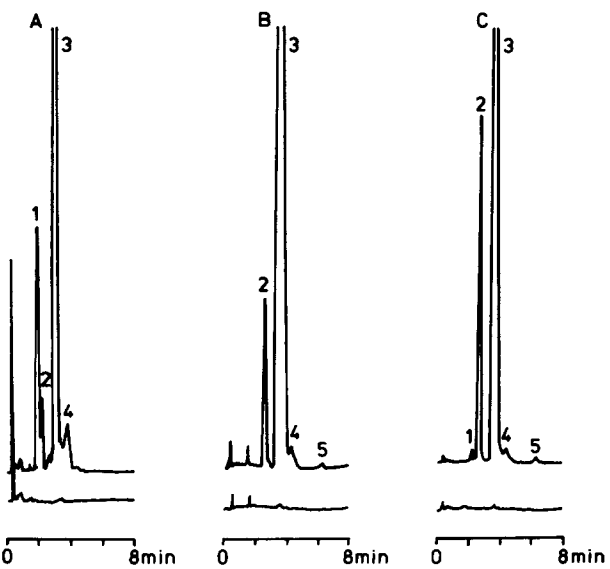

Fig. 4. Representative chromatograms of (A) plasma, (B) urine and (C) faeces samples from a clinical trial. Column as in Fig. 1; eluent: methanol–0.2 M potassium phosphate buffer (pH 6.5)–water (40:20:40) with 4 mM CTMA added. Temperature as in Fig. 1. Peaks: 1 = Glc-5-ASA; 2 = 5-ASA; 3 = Ac-5-ASA; 4 = F-5-ASA; 5 = Bu-5-ASA.

peak height of the analytes decreased concomitantly with decreasing retention as the amounts of acetonitrile in standard solutions analysed increased. Even at low concentrations of acetonitrile (less than 5%) in the sample solution a decrease in peak height compared to that of standard solutions without any acetonitrile added was observed. Peak areas were unaffected. Thus plasma spiked with each of the analytes must be used in order to avoid errors in quantification due to solvent-induced artifacts.

Stability of Glc-5-ASA

The stability of Glc-5-ASA in plasma in different temperatures has previously been investigated [15]. In the worked-up sample Glc-5-ASA was stable for at least 20 h at ambient temperature.

Application

The method described under *Chromatography* was used for analysis of plasma, urine and faeces samples from the clinical trial previously described [22]. Representative chromatograms are shown in Fig. 4.

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