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SIMPLE AND RAPID METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE EIGHT MAIN METABOLITES AND CONJUGATES OF SULPHASALAZINE IN HUMAN PLASMA, URINE AND FAECES USING DYNAMICALLY MODIFIED SILICA

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SUMMARY

A simple and rapid method for the simultaneous determination of sulphapyridine, N-acetylsulphapyridine, 5-aminosalicylic acid, N-acetyl-5-aminosalicylic acid, hydroxysulphapyridine, N-acetylhydroxysulphapyridine, sulphapyridine O-glucuronide and N-acetylsulphapyridine O-glucuronide in plasma, urine and faeces is presented. After precipitation of plasma proteins by addition of methanol the samples are injected directly into the liquid chromatographic system. The limit of detection is 1 μg/ml or less at a signal-to-noise ratio of 5 for all compounds using ultraviolet, fluorescence or electrochemical detection. The advantages of the dynamically modified silica approach in this reversed-phase high-performance liquid chromatographic method are demonstrated with respect to regulation and reproducibility of the selectivity.

INTRODUCTION

Sulphasalazine (salicylazosulphapyridine, SASP) has been used in the treatment of Crohn's disease and ulcerative colitis for more than four decades [1,2]. Investigation has shown that the drug might be active in the treatment of rheumatoid arthritis [3]. The SASP molecule consists of 5-aminosalicylic acid (5-ASA) joined to sulphapyridine (SP) by an azo linkage (Fig. 1). When the drug is given orally it is partly absorbed unchanged and partly cleaved to 5-ASA and SP by bacterial azo reductase in the large intestine [4]. 5-ASA and SP are further metabolized (Fig. 1).

5-ASA and N-acetyl-5-aminosalicylic acid (Ac-5-ASA) have been deter-

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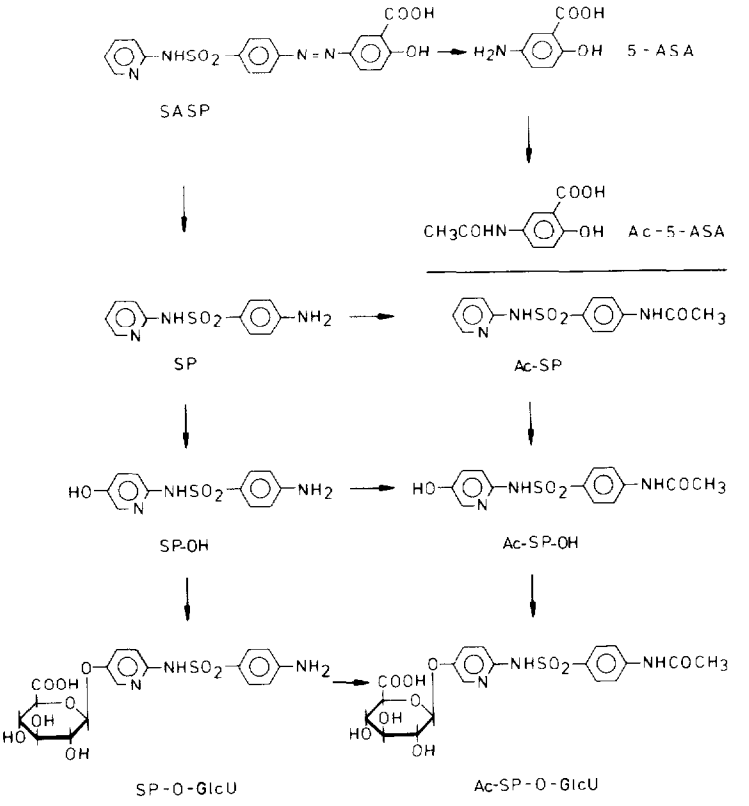

Fig. 1. Metabolism of sulphasalazine (SASP).

mined by fluorimetry [5], colorimetry [6] and by high-performance liquid chromatography (HPLC) using fluorescence detection [7–13] and electrochemical detection [13].

SP and acetylsulphapyridine (Ac-SP) and the hydroxylated metabolites 4-hydroxysulphapyridine (SP-OH) and 4-hydroxyacetylsulphapyridine (Ac-SP-OH) have been determined by spectrophotometry [15] as well as by HPLC [10,16–18].

The determination of the intact glucuronides of the hydroxylated metabolites of SP has not been demonstrated in the above methods, and neither has the simultaneous determination of 5-ASA, Ac-5-ASA, SP, Ac-SP and the hydroxylated metabolites. The method described in this paper demonstrates the simultaneous separation of all eight metabolites and conjugates as well as the advantage of the simultaneous use of three different methods of detection – UV absorption at 254 nm, fluorescence detection with excitation at 315 nm and emission at 430 nm, and electrochemical detection at a potential difference of +600 mV vs. Ag/AgCl.

EXPERIMENTAL

Apparatus

A Waters liquid chromatograph (Milford, MA, U.S.A.) consisting of a Model 6000 A pump, a 710 B WISP autoinjector, a Model 730 data module and a Model 720 system controller was used. The columns were thermostated in a Shimadzu CTO-6A oven. For detection a Shimadzu SPD-6A (Kyoto, Japan) UV detector (254 nm), a Kontron SFM 23 (Zürich, Switzerland) LC spectrophotofluorimeter (excitation at 315 nm, emission at 430 nm) and a Shimadzu L-ECD-6A electrochemical detector operated at +600 mV were used in series, in that order. For peak current measurements in the electrochemical detector a glassy carbon vs. an Ag/AgCl reference electrode was used.

Chemicals

5-ASA (+99%) and Ac-5-ASA (+99%) were obtained in pharmaceutical quality from Ferring (Vanløse, Denmark). SP-OH, Ac-SP-OH and Ac-SP were gifts from Pharmacia (Uppsala, Sweden). SP was obtained from Serva (Heidelberg, F.R.G.). β-Glucuronidase from *Escherichia coli* (EC 3.2.1.31) containing more than 200 U/ml was obtained from Boehringer (Mannheim, F.R.G.). All other chemicals were of analytical-reagent grade.

Sample preparation

To 1 ml of urine or plasma, 4 ml of methanol were added and mixed. After 1 h at −20 °C the mixture was centrifuged for 10 min at 5000 *g*. A 1-ml volume of the supernatant was mixed with 1 ml of distilled water and 5 or 20 μl of the final sample solution were injected into the liquid chromatograph. Each urine sample was treated with β-glucuronidase as follows: to 1 ml of urine were added 10 μl of β-glucuronidase; after 4 h at 37 °C the samples was prepared as before.

Faeces was collected for 24 h in 300 ml of methanol. After homogenization, 1 ml was added to 1 ml of distilled water. After centrifugation for 10 min at 5000 *g*, 5 μl were injected into the liquid chromatograph.

Chromatography

The analytical column was a Knauer column (120 mm × 4.6 mm I.D.) packed with LiChrosorb Si 60 (5 μm particle size) or the column packing material stated. 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 particle size). The columns were operated at 35 °C. The final mobile phase was methanol–0.2 *M* potassium phosphate (pH 6.5)–water (45:10:45 v/v) containing 3.75 m*M* cetyltrimethylammonium (CTMA) bromide; the flow-rate was 1.0 ml min^{-1}.

RESULTS AND DISCUSSION

The dynamically modified silica approach has already been shown to be a valuable separation method in the analysis of biological samples [9,19–21]. In this technique, bare silica is used as the column packing material but the addition of CTMA bromide to the eluent results in a reversed-phase system. The presence of CTMA ions also results in an ion-pairing system for anionic substances, because the CTMA cation forms very hydrophobic ion-pairs with anions [22]. In the present chromatographic system some of the solutes are separated by a reversed-phase mechanism and some solutes by an ion-pair reversed-phase mechanism. To obtain a satisfactory separation of the eight metabolites and conjugates of SASP, the chromatographic system has been investigated in detail with respect to the concentration of CTMA bromide in the eluent, buffer pH, buffer ionic strength and column temperature.

As shown previously [23], an increase in the concentration of organic modifier in the eluent has a dramatic effect on the retention. This is due to the fact that the eluotropic effect of the eluent increases concomitantly with a decrease in the amount of stationary phase, thus leading to a decrease in the retention

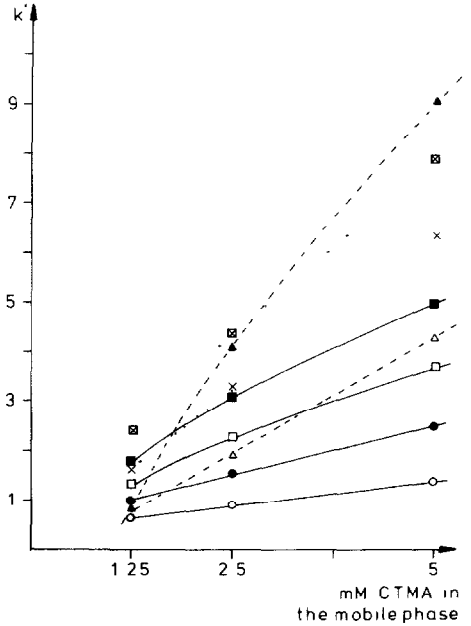

Fig. 2. Relationship between the concentration of CTMA in the eluent and the capacity factor (k') for the eight metabolites and conjugates. Column: LiChrosorb Si 60, 5 μm, 120 mm × 4.6 mm I.D., eluent: methanol–0.2 *M* potassium phosphate (pH 6.5)–water (45:10 45, v/v) with CTMA added in the concentrations indicated. (○) SP; (●) Ac-SP; (□) SP-OH; (■) Ac-SP-OH; (×) 5-ASA; (⊠) Ac-5-ASA; (△) SP-O-GlcU; (▲) Ac-SP-O-GlcU.

of solutes much more pronounced than when chemically bonded phases are used, where the amount of stationary phase remains unchanged.

Concentration of CTMA

When the concentration of CTMA is increased, the amount of CTMA ions adsorbed on the silica surface increases and so does the amount of stationary phase. As expected, the k' values of the solutes chromatographed also increase (Fig. 2). No changes in selectivity are seen between the non-ionic solutes (SP, Ac-SP, SP-OH and Ac-SP-OH). Between the anionic solutes (5-ASA, Ac-5-ASA and the two glucuronides) and between the non-ionic and the anionic solutes, large selectivity changes are seen. A dramatic effect is seen for the two glucuronides (SP-O-GlcU and Ac-SP-O-GlcU) (for formulas see Fig. 1).

pH of the buffer

The pK_a of silica is ca. 6.5–7.0. When the pH of the buffer added to the eluent is decreased, the ionization of the silanol groups decreases, and fewer CTMA ions are adsorbed on the surface. Accordingly, the k' values of the solutes chro-

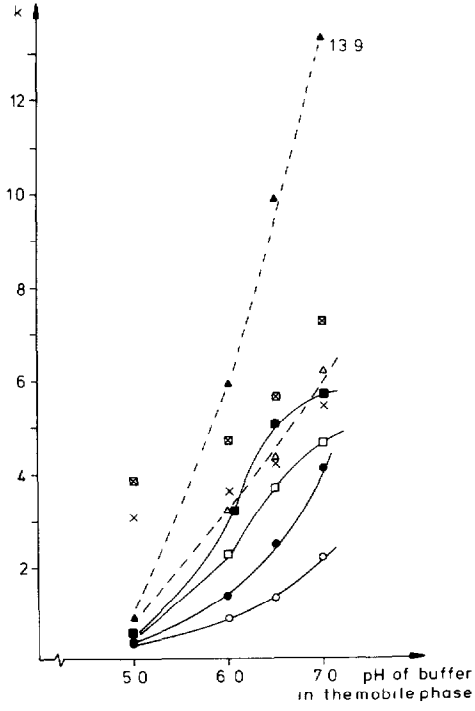

Fig. 3. Relationship between the pH of the buffer added to the eluent and the capacity factor (k') for the eight metabolites and conjugates. Column: as in Fig. 2; eluent: methanol–0.2 *M* potassium phosphate (pH 5–7)–water (45:10.45, v/v) with 5 m*M* CTMA added. Symbols as in Fig. 2.

matographed will decrease. Fig. 3 shows that large changes in selectivity also occur when the buffer pH is altered.

Ionic strength

In a previous paper [24] it was shown that a change in ionic strength had only a minor effect on the retention of non-ionic solutes, but a significant effect on the retention of anionic solutes. This effect was shown to increase with the

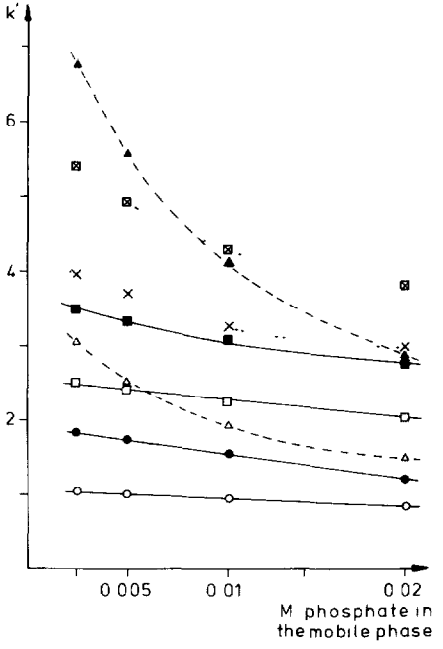

Fig. 4. Relationship between the phosphate concentration in the buffer added to the eluent and the capacity factor (k') for the eight metabolites and conjugates. Column: as in Fig. 2; eluent: methanol–0.2 *M* potassium phosphate (pH 6.5)–water [45:(1.25–10):(53.75–45), v/v] with 2.5 m*M* CTMA added. Symbols as in Fig. 2.

TABLE I

SELECTIVITY EXPRESSED AS α-VALUES FOR SEVEN METABOLITES OR CONJUGATES OF SASP RELATIVE TO THE METABOLITE Ac-5-ASA

	SP	Ac-SP	SP-OH	SP-O-GlcU	Ac-SP-OH	5-ASA	Ac-SP-O-GlcU
Polygosil 60	5.70	3.47	1.98	2.13	1.51	1.24	1.10
Hypersil	5.37	3.30	2.20	2.07	1.62	1.36	1.08
LiChrosorb Si 60	5.37	3.63	2.02	2.21	1.55	1.32	1.11
Spherosil XOA 600	6.19	3.66	2.13	2.24	1.60	1.38	1.11
Chromosorb LC 6	5.43	3.34	2.22	2.00	1.73	1.34	1.04

valency of the anionic solute. The effect may be used to regulate the selectivity when non-ionic and anionic solutes are to be separated (Fig. 4).

Column temperature

An increase in the column temperature leads to shorter retention times but no changes in selectivity are seen.

TABLE II

MINIMUM DETECTABLE QUANTITIES AT A SIGNAL-TO-NOISE RATIO OF 5 OF THE EIGHT METABOLITES AND CONJUGATES

The chromatographic system is given in Fig. 6. N.F. = no fluorescence.

Sample	Minimum detectable quantity (ng)					
	UV detection		Fluorescence detection		Electrochemical detection	
	254 nm	315 nm	315 nm/430 nm	315 nm/500 nm	1000 mV	600 mV
5-ASA	1	0.5	1	0.1	0.1	0.01
Ac-5-ASA	1	2	0.1	0.5	0.5	> 100
SP	0.2	0.5	N.F.	N.F.	5	> 100
Ac-SP	0.2	0.5	0.5	2.5	> 100	> 100
SP-OH	5	10	N.F.	N.F.	1.0	1.0
Ac-SP-OH	5	10	1	5	1.0	1.0
SP-O-GlcU	5	10	N.F.	N.F.	> 100	> 100
Ac-SP-O-GlcU	5	10	1	5	> 100	> 100

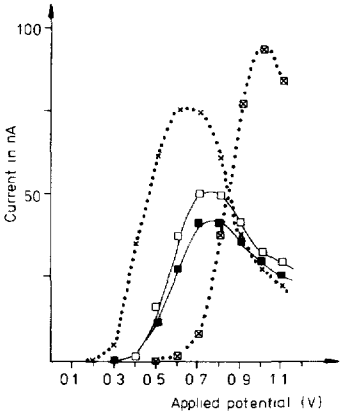

Fig. 5. Hydrodynamic voltammogram for four metabolites of SASP. Symbols as in Fig. 2.

Silica packing material

The reproducibility of the selectivity between the eight solutes when using different brands of silica column packing materials was found to be very good (Table I).

Detection

When samples of biological origin are analysed, selective and sensitive detection is often required. The common method of detection by UV absorption therefore often has to be supplemented by other methods. The two most convenient alternative methods for routine HPLC analysis are fluorescence detection and electrochemical detection. All three methods of detection have to be used if simultaneous determination of all eight metabolites of SASP is to be obtained at low levels in plasma. The minimum detectable quantities are given in Table II. SP and SP-O-GlcU have to be detected by UV absorption and Ac-5-ASA, Ac-SP, Ac-SP-O-GlcU should be detected by their fluorescence at 430 nm with excitation at 315 nm. 5-ASA, SP-OH and Ac-SP-OH are selectively detected by their electrochemical activity at +600 mV vs. Ag/AgCl. A plot of the relative electrochemical response versus the voltage across the cell is shown

TABLE III

ANALYSIS OF BLANK PLASMA SAMPLES SPIKED WITH SIX METABOLITES OF SASP

Values are in nanograms per millilitre of plasma.

Sample	Added (ng/ml)	Found (mean, $n=6$) (ng/ml)	Recovery (%)	Intra-assay C.V. (%)	Method of detection
5-ASA	19.1	17	89	9.1	Electrochemical
	76.4	80	105	4.2	
	382	373	98	3.7	
Ac-5-ASA	19.2	18	94	7.2	Fluorescence
	76.9	74	96	3.8	
	389	379	97	2.3	
SP	927	921	99	5.5	UV
	4636	4730	102	4.7	
Ac-SP	452	450	100	2.3	Fluorescence
	2258	2160	96	2.1	
SP-OH	100	105	105	3.8	Electrochemical
	500	490	98	3.6	
Ac-SP-OH	114	104	91	3.9	Electrochemical
	572	560	98	4.1	

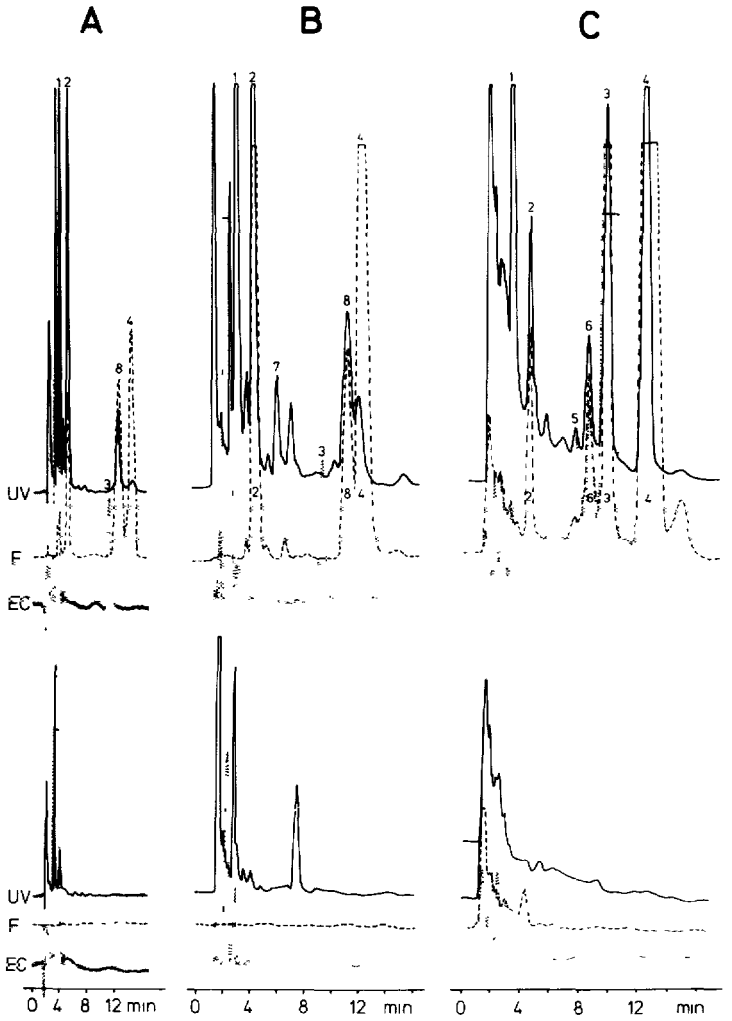

Fig. 6. Chromatograms of representative plasma (A), urine (B) and faeces (C) samples from a patient in steady state receiving 3 g of SASP per day. Column: as in Fig. 2; eluent: as in Fig. 2 with 3.75 m*M* CTMA added; detection: UV, 254 nm; fluorescence (F), excitation at 315 nm and emission at 430 nm; electrochemical (EC), +600 mV; detector sensitivity: (A) UV, 0.01 a.u.f.s.; F, high HV×10; EC, 16 nA full scale; (B and C) UV, 0.16 a.u.f.s.; F, medium×10; EC, 256 nA full scale The upper part of the figure shows sample chromatograms and the lower part shows chromatograms of blanks. Peaks: 1 = SP; 2 = Ac-SP; 3 = 5-ASA; 4 = Ac-5-ASA; 5 = SP-OH; 6 = Ac-SP-OH; 7 = SP-O-GlcU; 8 = Ac-SP-O-GlcU.

in Fig. 5. The decrease in response at higher voltage is probably due to the presence of 3.75 m*M* bromide in the eluent, as the bromide ion has a half-wave potential of *ca.* +1.0 V.

Applications

The method developed has been used in several clinical trials for analysis of the metabolites in plasma, urine and faeces from patients given a single dose of SASP, as well as in multiple dose studies [25].

The results from recovery studies after addition of the metabolites to plasma are given in Table III. The recoveries are all close to 100%, and the intra-assay coefficients of variation (C.V.) are good. The linearity of the detector response for each metabolite was tested in the plasma concentration range 1.0–100 μg/ml (for 5-ASA and Ac-5-ASA from 0.05 μg/ml). The correlation coefficient was in no case less than 0.997. Quantitative measurements of SP-O-GlcU and Ac-SP-O-GlcU were performed relative to a urine sample in which the glucuronides after cleavage with β-glucuronidase were determined relative to standards of SP-OH and Ac-SP-OH.

Examples of representative chromatograms of plasma, urine and faeces samples from a volunteer in steady state given 3 g of SASP per day are shown in Fig. 6.

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