Journal of Chromatography, 385 (1987) 299–304
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1026

SIMULTANEOUS DETERMINATION OF 5-AMINOSALICYLIC ACID AND 5-ACETYLAMINOSALICYLIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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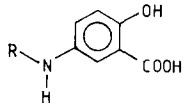
SUMMARY

A high-performance liquid chromatographic method has been developed for the simultaneous determination of 5-aminosalicylic acid (5-ASA) and its main metabolite 5-acetylaminosalicylic acid (5-AcASA) in plasma, with 4-acetylaminosalicylic acid as an internal standard. Prior to extraction into ethyl acetate, 5-ASA is derivatized to 5-carbobenzyloxyaminosalicylic acid (5-CboASA). The calibration graphs for both 5-ASA and 5-CboASA are linear between 0.1 and 20.0 μmol/l. The limit of detection is 0.02 μmol/l for 5-ASA and 0.05 μmol/l for 5-AcASA. At 0.1 μmol/l, the coefficients of variation were 8.1 and 9.8% for 5-ASA and 5-AcASA, respectively. At 1.0 and 10.0 μmol/l, the coefficients of variation were 4.1% or less. The mean bias ranged from −6.2 to −2.0% for 5-ASA and from +6.0 to 0% for 5-AcASA.

INTRODUCTION

5-Aminosalicylic acid (5-ASA) (Fig. 1A) is an effective drug for the treatment of ulcerative colitis and Crohn's disease[1–5], being administered either directly or as salazosulphapyridine, which is cleaved by gut bacteria to sulphapyridine and 5-ASA, the latter being the active principle of salazosulphapyridine[6,7]. After absorption, 5-ASA is metabolized mainly to 5-acetylaminosalicylic acid (5-AcASA) (Fig. 1B). During chronic treatment, oral administration of 250 mg of 5-ASA three times a day (tid) resulted, at steady state, in peak plasma concentrations of 0.3–3.2 μg/ml (2.0–



A: R = H
B: R = CH_3CO
C: R = $C_6H_5CH_2OCO$

Fig. 1. Chemical structures of (A) 5-ASA, (B) 5-AcASA and (C) 5-CboASA.

0021-9673/87/$03.50

21 μmol/l) and in trough levels of 0.02 μg/ml (0.13 μmol/l) or less for 5-ASA[8]. Mean plasma concentrations of 5-AcASA ranged from 0.4 μg/ml (2.0 μmol/l) (through level) to about 1.2 μg/ml (6.1 μmol/l) (peak level). Similar results were obtained during treatment with 500 mg of 5-ASA tid orally[9]. The plasma concentration of 5-AcASA ranged from 1.1 to 2.9 μg/ml (5.6–15 μmol/l) at steady state, whereas no 5-ASA could be detected in the plasma. In order to describe the pharmacokinetic behaviour of 5-ASA and its main metabolite with sufficient accuracy, an assay is required for the determination of low concentrations of 5-ASA in the presence of much higher concentrations of 5-AcASA.

Several high-performance liquid chromatographic (HPLC) assays have been developed in the past. Some avoid an extraction step for 5-ASA[10–12], which, being an amphoteric compound, cannot easily be extracted into organic solvents without derivatization. However, these assays lack sufficient sensitivity. Other assays incorporate acylation of the amino group of 5-ASA prior to extraction. Fischer *et al.*[13] acetylated 5-ASA and extracted the 5-AcASA into diethyl ether. Using this assay, each sample has to be worked up in duplicate, one with and one without the acetylation step, and the 5-ASA concentration is calculated as the difference between the two results. This procedure is time consuming and introduces large relative errors if low 5-ASA concentrations have to be determined in the presence of high, genuine 5-AcASA concentrations. Van Hogezand *et al.*[14] used propionic anhydride as an acylating reagent and determined 5-propionylaminosalicylic acid and 5-AcASA simultaneously. However, 4-propionylaminosalicylic acid, used as an internal standard, showed extensive tailing in the chromatogram, causing prolonged analysis times. We report here an HPLC procedure for the simultaneous determination of 5-ASA and 5-AcASA with benzyl chloroformate as derivatizing agent. The resulting 5-carbobenzyloxyaminosalicylic acid (5-CboASA) (Fig. 1C) is then measured, together with 5-AcASA.

EXPERIMENTAL

Materials

5-Aminosalicylic acid (5-ASA) was purchased from Bayer (Leverkusen, F.R.G.) and 4-aminosalicylic acid (4-ASA) from Merck (Darmstadt, F.R.G.). 5-Acetylaminosalicylic acid (5-AcASA) and 4-acetylaminosalicylic acid (4-AcASA) were synthesized by reaction of 5-ASA and 4-ASA, respectively, with acetic anhydride (Merck) under basic conditions. Acetonitrile (LiChrosolv) and ethanol (Uvasol) were purchased from Merck, ethyl acetate (Chromasolv) from Riedel-de-Haen (Seelze, F.R.G.) and benzyl chloroformate from Aldrich (Steinheim, F.R.G.).

Synthesis of 5-CboASA

In a 250-ml flask 1.53 g (10 mmol) of 5-ASA were dissolved by stirring in 30 ml of a 5% (w/v) solution of sodium hydrogen carbonate in water. With vigorous stirring, 1.24 ml of benzyl chloroformate was added at room temperature. After 30 min, the mixture was acidified to pH 1 with 1 *M* hydrochloric acid. The reaction mixture was extracted twice with 200 ml ethyl acetate. The organic layers were combined, washed once with 0.1 *M* hydrochloric acid and dried over sodium sulphate. The solution was evaporated to dryness and the residue recrystallized from 2-propanol.

IR (KBr): 3250 vs, 3100 s (broad), 1680 vs, 1620 s, 1590 s, 1540 vs, 1480 s, 1450 vs, 1250 vs, 1200 vs, 1080 s, 1060 vs. ^{1}H NMR ([$^2H_6$]dimethylsulphoxide) (δ in ppm): 5.12 (2) s; 6.89 (1) d, J = 9 Hz; 7.35 (5) s; 7.55 (1) dd, J_1 = 3 Hz, J_2 = 9 Hz; 7.97 (1) d, J = 3 Hz; 9.67 (1) s; 10.5–12.5 (1) broad; 12.5–15 (1) broad. Analysis: calculated, C 62.72, H 4.56, N 4.88%; found, C 62.73, H 4.52, N 4.99%.

Apparatus and chromatographic conditions

The analyses were performed with an M 6000 A pump (Waters Assoc., Eschborn, F.R.G.), connected to a Nucleosil 10 SB anion-exchange column (250 × 4.0 mm I.D.; 10 μm) from Macherey, Nagel & Co. (Düren, F.R.G.) and a Merck/Hitachi F 1000 fluorescence detector (excitation wavelength, 300 nm; emission wavelength, 470 nm; sensitivity, 0.5; time constant, 1.0 s). The column was heated to 50°C in a Temperature Control Module (TCM) column oven (Waters Assoc.). For automatic injection of the samples a WISP 710 B (Waters Assoc.) was used. Chromatograms were recorded with a Data Module integrator (Waters Assoc.). The mobile phase was 0.05 *M* sodium sulphate buffer (pH 4.0)–acetonitrile (1:1), which was pumped through the column at a flow-rate of 1.3 ml/min.

Preparation of samples

A 500-μl sample of plasma was mixed with 25 μl of a solution of 100 μmol/l of 4-AcASA (internal standard) in ethanol and 800 μl of acetonitrile in a polypropylene centrifuge vial. The mixture was shaken vigorously for 10 min and centrifuged at 4400 *g* for 5 min. The supernatant was decanted into a clean centrifuge vial, mixed with 10 μl of a 10% (v/v) solution of benzyl chloroformate in acetonitrile and incubated at 40°C in a water-bath for 30 min. Thereafter, 300 μl of 1 *M* hydrochloric acid were added, followed by 5 ml of ethyl acetate. This mixture was shaken on a reciprocating shaker for 10 min and then briefly centrifuged, then 5 ml of the organic layer were transferred into a clean vial and evaporated to dryness under nitrogen at 40°C. The residue was dissolved in 200 μl of eluent and 10–20 μl of the solution were injected into the HPLC system.

Calibration graph

Standard solutions of 1.0 mmol/l to 1.0 μmol/l of 5-ASA in dilute hydrochloric acid (water brought to pH 1.0 with 1 *M* hydrochloric acid) and standard solutions of 5-AcASA of identical concentrations in ethanol were prepared and stored in a refrigerator (the solutions are stable for at least 1 week). Appropriate volumes of standard solutions of both 5-ASA and 5-AcASA were adjusted to 500 μl with pooled human plasma to yield plasma concentrations of 0.1–20.0 μmol/l of each compound. The standards thus obtained were worked up according to the procedure described above and chromatographed. A calibration graph was constructed by linear regression for each analyte from the peak-area ratios of the respective analyte to the internal standard as a function of concentration units. The calibration graph was prepared daily.

RESULTS AND DISCUSSION

The objective for the development of a derivatization procedure for 5-ASA is

the preparation of a derivative that is extracted more easily than the parent substance into organic solvents. The reaction must lead to defined, uniform products, which are not likely to be present in real samples. Further, the reagent must react rapidly and completely with the analyte and should give rise to a minimum of by-products. We chose benzyl chloroformate, as the carbobenzyloxy moiety is a well known protective group for the amino function in peptide chemistry and the reaction of amino acids with benzyl chloroformate is a standard reaction by means of which high yields can be obtained in aqueous systems.

Fig. 2 shows chromatograms of a blank plasma standard, a standard containing 0.05 μmol/l of 5-ASA and 5-AcASA and a standard containing 5.0 μmol/l of both analytes. The chromatograms demonstrate that 0.05 μmol/l of each compound can be easily distinguished from a blank. Owing to a small interference peak, plasma concentrations of less than 0.05 μmol/l of 5-AcASA cannot be detected with sufficient statistical precision. With 5-ASA, however, chromatographic signals corresponding to 0.02 μmol/l could be detected routinely. Therefore, this concentration was accepted to be the limit of detection for this analyte. The calibration graphs were linear in the range 0.1–20.0 μmol/l, with correlation coefficients of $r = 0.9995$ for 5-ASA and $r = 0.9998$ for 5-AcASA.

The accuracy and precision of the assay for 5-ASA and 5-AcASA in plasma were assessed by adding known concentrations of 0.1, 1.0 and 10.0 μmol/l of each

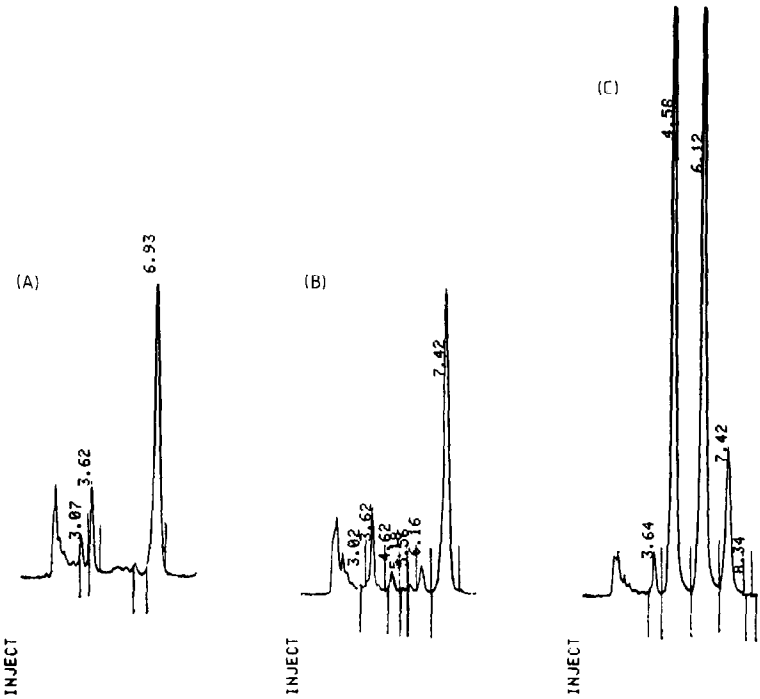


Fig. 2. Chromatograms of (A) blank plasma standard, (B) standard containing 0.05 μmol/l of 5-ASA and 5-AcASA and (C) standard containing 5.0 μmol/l of both compounds. Peaks at 4.62 and 4.58 min = 5-CboASA, peaks at 6.16 and 6.12 min = 5-AcASA and peaks at 6.93 and 7.42 min = internal standard.

TABLE I

ACCURACY, PRECISION AND MEAN RECOVERIES FOR 5-ASA AND 5-AcASA IN PLASMA

Compound	*Actual plasma concentration (μmol/l)*	*n*	*Mean concentration found (μmol/l)*	*Coefficient of variation (%)*	*Bias (%)*	*Recovery (%)*
5-ASA	0.1	5	0.098	8.1	−2.0	77.4
5-ASA	1.0	5	0.938	4.1	−6.2	64.8
5-ASA	10.0	5	9.66	2.9	−3.4	72.6
5-AcASA	0.1	5	0.100	9.8	0	67.6
5-AcASA	1.0	5	1.06	1.6	+6.0	70.2
5-AcASA	10.0	5	10.26	2.1	+2.6	75.5

analyte to blank human plasma and subsequently processing the samples thus obtained. The coefficients of variation ranged from 8.1 to 2.9% for 5-ASA and from 9.8 to 1.6% for 5-AcASA (Table I). The mean bias was −6.2% or less for 5-ASA and +6.0% or less for 5-AcASA. 5-AcASA did not react with benzyl chloroformate under the conditions of this assay. Mean recoveries for 5-ASA and 5-AcASA were

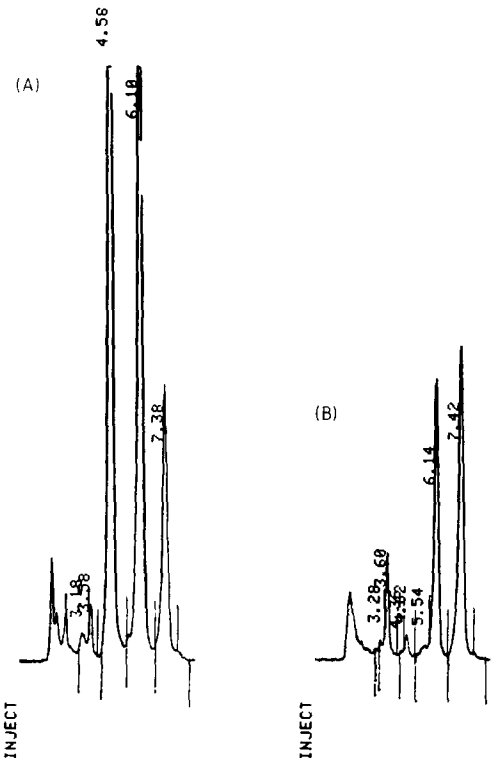


Fig. 3. Chromatograms of plasma samples taken (A) 3 h and (B) 24 h after a single oral dose of 500 mg of 5-ASA. (A) 5-ASA concentration = 4.64 μmol/l; 5-AcASA concentration = 3.49 μmol/l; (B) 5-ASA concentration = 0.06 μmol/l; 5-AcASA concentration = 0.99 μmol/l.

determined by comparison of chromatograms of spiked plasma samples after work-up with those of standard solutions of 5-CboASA and 5-AcASA of equivalent concentrations, injected directly into the HPLC system. They ranged from 64.8 to 77.4% for 5-ASA and from 67.6 to 75.5% for 5-AcASA.

Several buffer systems were investigated as the eluent. The sodium sulphate buffer produced the best results with respect to selectivity and analysis time. The cation used in the buffer system influences the chromatography considerably. Triethylammonium decreases the retention times drastically compared with sodium or potassium but impairs the selectivity. Higher buffer concentrations do not alter the chromatogram substantially. However, they may cause problems regarding the miscibility of the buffer system with the organic solvent.

The method was used for the analysis of plasma samples from a 24-h pharmacokinetic study in healthy subjects receiving 500 mg of 5-ASA orally. Fig. 3 presents chromatograms of samples taken at 3 and 24 h after dosing. The 24-h sample contained 0.06 μmol/l of 5-ASA in the presence of 0.99 μmol/l of 5-AcASA. The results demonstrate that the method described here is sufficiently sensitive to measure 5-ASA and 5-AcASA up to 24 h after oral doses of 500 mg of 5-ASA during pharmacokinetic profiling of the drug. The method is rapid and permits the analysis of at least 40 samples plus standards in a normal working day. It may therefore be particularly useful in extensive pharmacokinetic studies.

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