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Short Communication

High-performance liquid chromatographic assay for the determination of 5-aminosalicylic acid and acetyl-5-aminosalicylic acid concentrations in endoscopic intestinal biopsy in humans

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ABSTRACT

A high-performance liquid chromatographic method for the simultaneous determination of 5-aminosalicylic acid (5-ASA) and N-acetyl-5-ASA (Ac-5-ASA) concentrations in endoscopic mucosal biopsy homogenates is presented. The mean recoveries of 5-ASA and Ac-5-ASA from spiked blank biopsy homogenates ranged from 95.9 to 120% and from 92.5 to 100%, respectively. The coefficients of variation for 5-ASA and Ac-5-ASA were 0.7–8.6% and 1.4–12.9%, respectively. This method is useful for direct determination of topical availability of 5-ASA and Ac-5-ASA and probably an accurate parameter of drug bioavailability.

INTRODUCTION

Although salazosulfapyridine (SASP) has been used in the treatment of inflammatory bowel disease for 40 years, 5-aminosalicyclic acid (5-ASA) has only recently been recognized as its major anti-inflammatory component [1–3]. This contrasts with the presumed mode of action of the drug in rheumatoid arthritis [4]. The very low systemic availability of 5-ASA in SASP, coupled with considerable faecal excretion [5,6], suggests a topical mode of action. This hypothesis is further supported by the therapeutic efficacy of 5-ASA suppositories [7] and enemas [8] although a systemic effect has not been ruled out. Current practice is based on minimal systemic availability and maximal faecal concentration of 5-ASA, because of this presumed local mode of action [9]. Recently, several new preparations became available, which deliver 5-ASA into the terminal ileum and colorectum but avoid the use of sulfapyridine.

The determination of the concentrations of 5-ASA and its major metabolite N-acetyl-5-ASA (Ac-5-ASA) in plasma, urine and faeces showed no correlation

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with the clinical efficacy of the drug [10]. Published methods have dealt with indirect estimation of the effective intra-tissular 5-ASA concentrations. Therefore, we have developed a high-performance liquid chromatographic (HPLC) method for the simultaneous determination of 5-ASA and Ac-5-ASA concentrations in endoscopic biopsy specimens of terminal ileum and colorectum.

EXPERIMENTAL

Chemicals

5-ASA and 4-amino-2-hydroxybenzoic acid (4-ASA) were purchased from Fluka (Buchs, Switzerland). Ac-5-ASA was synthesized by the reaction of 5-ASA with acetic anhydride (Merck, Darmstadt, F.R.G.). N-Propionyl-5-ASA (Prop-5-ASA) and N-propionyl-4-ASA (Prop-4-ASA) were synthesized by the reaction of 5-ASA and 4-ASA with propionic anhydride (Aldrich, Steinheim, F.R.G.) and purified by recrystallization from water. HPLC-grade acetonitrile was purchased from Carbo-Erba (Milan, Italy) and HPLC-grade water was obtained by passing deionized water through an Elga-Stat apparatus. All other reagents were of analytical grade.

Apparatus

The chromatographic separations were carried out with an LKB 2150 pump (LKB, Bromma, Sweden) connected to a cartridge pre-column (10 mm × 4.6 mm I.D.) prepacked with 5-μm Spherisorb ODS-2 and an analytical cartridge column (150 mm × 4.6 mm I.D.) prepacked with 3-μm Spherisorb ODS-2, both obtained from Biorad-RSL (Eke, Belgium). Samples were injected with an autosampler (Hewlett-Packard, Model 1050, Waldbronn, F.R.G.). Detection was performed with a fluorescence monitor (Shimadzu, Model RF-535, Kyoto, Japan), excitation wavelength 315 nm, emission wavelength 430 nm. Detector signals were processed on an integrator (Hewlett-Packard, Model 3396A, Avondale, U.S.A.) and stored on an IBM compatible PC (Tulip, 's-Hertogenbosch, The Netherlands). An ultrasonic processor (Sonics & Materials, Danburg, CT, U.S.A.) was used to disrupt intact cells in crushed biopsy specimens.

Chromatographic conditions

The mobile phase consisted of 0.1 *M* acetic acid–acetonitrile–triethylamine (920:80:2, v/v), filtered through a 0.45-μm membrane filter (type HVLP 04700) (Millipore, Milford, MA, U.S.A.). A flow-rate of 1.5 ml/min was maintained and all analyses were performed in triplicate at room temperature.

Internal standard

Prop-4-ASA was choosen as internal standard (I.S.) because of its close chemical and structural similarity with the products of interest and for its good chromatographic properties. After recrystallization from water, Prop-4-ASA was fur-

ther purified to remove a small interfering chromatographic peak (retention time, t_R, Prop-4-ASA 7.3 min; t_R interfering peak 3.2 min). Therefore, several 20-μl aliquots of a solution containing 1 mg/ml Prop-4-ASA were analysed under the same chromatographic conditions. Eluent fractions of chromatographically pure Prop-4-ASA (I.S.) were collected. Before use, the I.S. solution was diluted four-fold to a final concentration of 57.6 μg I.S./ml.

Calibration graph

A stock solution, containing 5-ASA and Ac-5-ASA, was prepared by dissolving 10.24 mg of each standard in 100 ml of 0.01 *M* hydrochloric acid. This solution was optically clear and further diluted to a working solution with a concentration of 1024 ng/μl. Standard solutions of different concentrations were obtained by two-fold dilution of this working solution to 4 ng per 100 μl, and stored at -80°C.

For the calibration graph, 100 μl of each standard solution were added to 900 μl of phosphate buffer (0.05 *M*, pH 7.4) containing 100 μl of I.S. solution and assayed according to the analytical procedure. A calibration graph was constructed by linear regression for each analyte from the peak-height ratios of the respective analyte to the I.S. *versus* the amount of the analyte.

Sample preparation

Mucosal intestinal biopsies (mean wet weight = 5.02 mg, range 0.2–10.54 mg, $n = 239$), taken during ileocolonoscopy, were transferred to tared polypropylene tubes, accurately weighed ($\pm$ 0.02 mg), immediately frozen in liquid nitrogen and stored at -80°C until analysis. The whole procedure lasted less than 30 min.

Sample preparation started by immersing the polypropylene tube, containing the frozen biopsy, in liquid nitrogen. The biopsy was carefully crushed and ground with a stainless-steel rod. The rod was removed from the tube, and 20 μl of propionic anhydride, 100 μl of I.S. solution and 500 μl of phosphate buffer were added at room temperature. In order to remove quantitatively all the biopsy particles adhering to its surface, the rod was immersed again in the suspension, and finally flushed with 500 μl of phosphate buffer. Intact mucosa cells were disrupted ultrasonically (energy = 80 W) by inserting the microprobe into the suspension for 1 min. After vortex-mixing, all samples were left for 1 h at 37°C. Compounds of interest were extracted with 6 ml of acetonitrile after addition of 500 μl of 10% sodium chloride solution. Liquid phases were separated after cooling for 1 h at 4°C by centrifugation at 1500 *g* for 10 min. The organic layer was removed and evaporated to dryness under air. The residue was redissolved in 500 μl of mobile phase and filtered through a 0.45-μm disposable filter Spartan 13/20 (Schleicher and Schuell, Dassel, F.R.G.) into a conical 600-μl vial, and aliquots of 100 μl were injected for HPLC analysis.

RESULTS

As shown in Fig. 1A, the method results in three well separated peaks. The retention times for Ac-5-ASA, 5-ASA propionylated to Prop-5-ASA and Prop-4-ASA (I.S.) are 3.2, 5.7 and 7.3 min, respectively. A chromatogram of a blank biopsy indicates no interference by endogenous compounds (Fig. 1B). Fig. 1C represents a chromatogram of an ileal biopsy specimen from a patient treated with Claversal® 500 mg t.i.d. (Eudragit-L-coated slow-release 5-ASA preparation) during one week. This biopsy contained12.9 ng 5-ASA per mg wet weight and 51.9 ng Ac-5-ASA per mg wet weight.

Calibration curves (5-ASA, Ac-5-ASA) were obtained with and without the presence of blank biopsy material, and the results compared by least-squares fitting, so as to evaluate the influence of the matrix on either the propionylation reaction of 5-ASA, or a possible transesterification of Ac-5-ASA. The curves resulted in an equation of $y = 1.002x - 0.004\,22$ with $r = 0.9997$ for 5-ASA and y

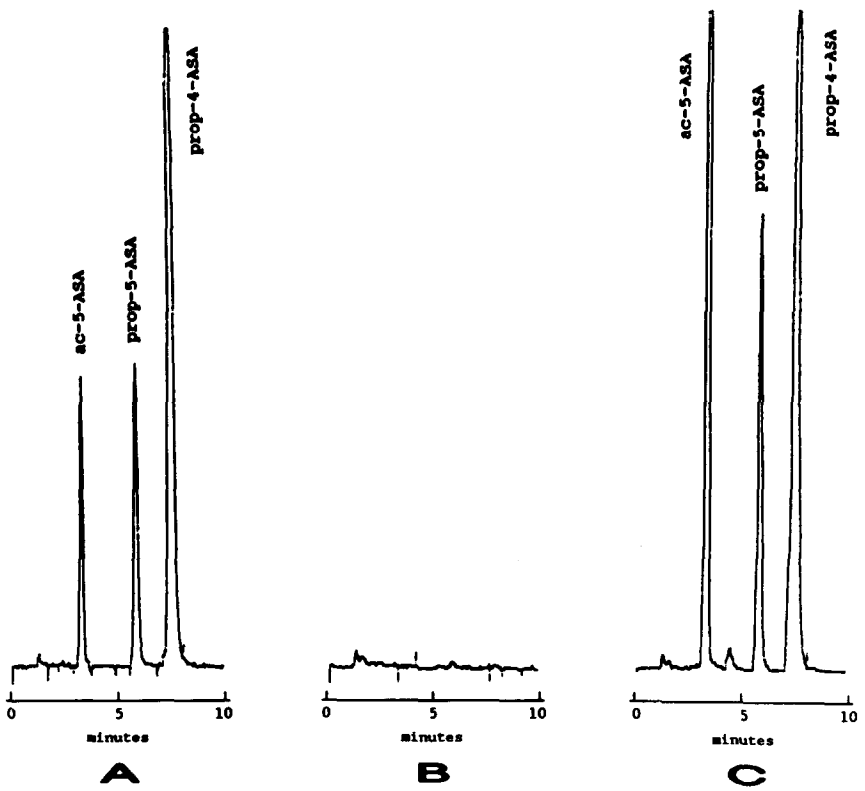

Fig. 1. Chromatograms of a biopsy homogenate spiked with 32 ng of 5-ASA and 32 ng of Ac-5-ASA (A), a blank biopsy homogenate (B), and ileal biopsy specimen from a patient treated during one week with Claversal 500 mg t.i.d. (C): measured concentrations: 12.9 ng/mg wet weight 5-ASA and 51.9 ng/mg wet weight Ac-5-ASA.

$= 0.995x - 0.006\,00$ with $r = 0.9995$ for Ac-5-ASA. The correlation was thus excellent, with no systematic or proportional systematic errors. For this reason, and in consideration of the unnecessary medical risks, no more blank biopsies were taken for obtaining the calibration curves. These calibration lines were linear in the range 4–1024 ng for each standard, with correlation coefficients of $r = 0.990$ and $r = 0.998$ for Prop-5-ASA and Ac-5-ASA, respectively.

Table I shows the intra-assay and inter-assay reproducibility of five replicates for different amounts of 5-ASA and Ac-5-ASA in spiked blank biopsies. The coefficient of variation (C.V.) ranges were 0.7–8.6 and 1.4–12.9%, respectively.

The recovery was determined by adding known amounts of native standards to blank biopsies. The recoveries ranged from 95.9 to 120% for 5-ASA and from 92.5 to 100% for Ac-5-ASA (Table II).

The detection limit for the amount of 5-ASA and Ac-5-ASA was *ca.* 1 ng, at a signal-to-noise ratio 3:1.

The amounts of 5-ASA and Ac-5-ASA, obtained from the regressive line in reverse, were divided by the wet weight of the biopsy specimens in order to get results expressed as ng/mg wet weight.

TABLE I

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION FOR SEVERAL AMOUNTS OF NATIVE STANDARDS ADDED TO BLANK BIOPSIES

Intra-assay variability[a]			Inter-assay variability[b]		
Added amount (ng)	Detected amount (mean ± S.D.) (ng)	Coefficient of variation (%)	Added amount (ng)	Detected amount (mean ± S.D.) (ng)	Coefficient of variation (%)
5-ASA					
4	4.9 ± 0.16	3.4	4	5.0 ± 0.63	12.5
8	8.3 ± 0.06	0.7	8	8.2 ± 0.66	8.1
16	16.1 ± 0.24	1.5	16	15.5 ± 0.68	4.3
32	30.6 ± 1.07	3.5	32	31.5 ± 0.44	1.4
128	124.6 ± 2.64	2.1	128	128.5 ± 6.03	4.7
1024	1025.3 ± 5.35	0.5	1024	1041.1 ± 38.52	3.7
Ac-5-ASA					
4	3.7 ± 0.17	4.7	4	4.4 ± 0.57	12.9
8	6.9 ± 0.59	8.6	8	8.2 ± 0.46	5.7
16	15.1 ± 0.88	5.8	16	16.4 ± 0.36	2.2
32	30.0 ± 1.09	3.7	32	32.5 ± 1.35	4.2
128	125.9 ± 1.93	1.5	128	141.2 ± 7.06	5.0
1024	1034.2 ± 15.36	1.5	1024	1132.5 ± 47.57	4.2

[a] For five replicate analysis.

[b] For five consecutive days.

TABLE II

RECOVERIES OF 5-ASA AND Ac-5-ASA IN BIOPSY SPECIMENS

Added amount (ng)	Detected amount (mean, $n = 3$) (ng)	Recovery (%)
5-ASA		
4	4.8	120.0
8	8.5	106.0
16	16.9	105.3
32	32.6	101.9
64	61.4	95.9
128	132.6	103.6
256	259.1	101.2
Ac-5-ASA		
4	4.0	100.0
8	7.6	95.0
16	15.0	93.8
32	30.4	95.0
64	59.2	92.5
128	123.4	96.4
256	239.9	93.7

DISCUSSION

HPLC is generally used for the determination of 5-ASA and Ac-5-ASA in serum, urine and faeces. Few determinations of concentrations in biopsy homogenates have been reported [11,12]. The low mass of endoscopic biopsy specimens and the low concentrations require a sensitive and selective detection method. Because 5-ASA is chemically unstable, storage of biopsy specimens and standard solutions at −80°C was necessary to avoid deterioration of the samples [13]. Small glass homogenization tubes (Dounce) were avoided because of possible catalytic reactions with glass impurities and the relatively high temperature during homogenization. Therefore only polypropylene tubes were used throughout the assay.

Propionylation of 5-ASA, as described by Van Hogezand [14], provided complete derivatization with no noticeable side-products. Only a slight modification was applied: we used a liquid–liquid extraction with acetonitrile and added triethylamine to the mobile phase as an ion-pairing agent, resulting in excellent peak symmetry. The recovery studies from blank biopsies indicated that the method was appropriate for a rapid and quantitative determination of 5-ASA and its metabolite Ac-5-ASA in intestinal biopsy specimens.

Coefficients of variation were determined on different blank biopsies spiked

with known amounts of 5-ASA and Ac-5-ASA, because a single biopsy specimen was to small to be split in several fragments. The intra-assay and the inter-assay coefficients confirm the value of the analytical technique.

The method was used for the determination of intramucosal 5-ASA and Ac-5-ASA concentrations in ileocolonic biopsies in patients treated with different oral 5-ASA preparations. These results will be presented in a separate publication.

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