ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1995) 175–180

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Simultaneous determination of 5-aminosalicylic acid, acetyl-5-aminosalicylic acid and 2,5-dihydroxybenzoic acid in endoscopic intestinal biopsy samples in humans by high-performance liquid chromatography with electrochemical detection ☆

G. Palumbo [a]*, G. Carlucci[b], P. Mazzeo [b], G. Frieri[c], M.T. Pimpo [c], D. Fanini [a]

[a] *Department of Internal Medicine, Chair of Pharmacology, University of L'Aquila, Via S. Sisto, 22E-67100 L'Aquila, Italy,*
[b] *Department of Chemistry, Chemical Engineering and Materials, University of L'Aquila, Via Vetoio, 67010 Coppito, Italy,*
[c] *Department of Internal Medicine, Chair of Gastroenterology, University of L'Aquila, Via S. Sisto, 22E-67100 L'Aquila, Italy*

Received for review 26 April 1995; revised manuscript received 30 June 1995

Abstract

A high-performance liquid chromatographic method for the simultaneous determination of 5-aminosalicylic acid (5-ASA), acetyl-5-aminosalicylic acid (Ac-5-ASA) and 2,5-dihydroxybenzoic acid (5-HSA) in human endoscopic intestinal biopsy with electrochemical detection has been developed and validated. A liquid–liquid extraction procedure was used to isolate these drugs from the biological material prior to analysis. The compounds were separated on an Erbasil S reversed-phase column using methanol–citric acid–sodium hydrogenphosphate–heptanesulfonic acid–disodium ethylenediaminetetraacetate (pH 3) as mobile phase. The method was linear from 1.0 to 300 ng ml^{-1} for 5-ASA, from 10 to 1000 ng ml^{-1} for Ac-5ASA and from 0.1 to 10 ng m^{-1} for 5-HSA. The limit of detection for 5-ASA and for Ac-5-ASA was 1 ng ml^{-1} and that for 5-HSA was 0.1 ng ml^{-1}. This procedure is suitable for pharmacological and clinical studies of 5-ASA.

Keywords: 5-Aminosalicylic acid; Metabolites; Liquid–liquid extraction; Human biopsy; High-performance liquid chromatography; Electrochemical detection

1. Introduction

Oral administration of sulfasalazine (SAZ) and other 5-aminosalicylic acid-containing drugs is used in the treatment of acute inflammatory bowel disease and in the maintenance of the clinical remission [1]. Pharmacokinetic studies have demonstrated that SAZ passes unmodified through the upper gastrointestinal tract until it reaches the colon, where it is metabolized by endogenous bacteria to yield sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) (Fig. 1(a)) [2].

☆ Presented at the Sixth International Symposium on Pharmaceutical and Biomedical Analysis, April 1995, St. Louis, MO, USA.

* Corresponding author.

0731-7085/95/$09.50
SSDI 0731-7085(95)01608-2

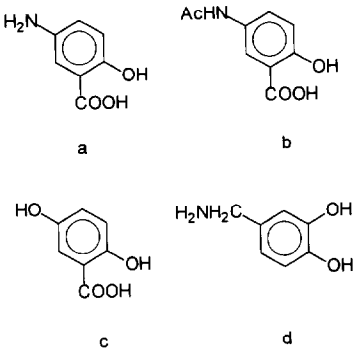

Fig. 1. Structures of (a) 5-ASA, (b) Ac-5-ASA, (c) 5-HSA and (d) DHBA (internal standard).

Luminal liberation of 5-ASA from various preparations has been indirectly investigated in several studies [3,4], generally by measuring urinary or faecal excretion, or plasma appearance of 5-ASA and its metabolites to estimate the kinetics and extent of its absorption.

Despite its use for over 50 years, the mechanism of action of this drug remains uncertain. 5-ASA has been shown to be a potential scavenger of oxygen free radicals that play a significant role in the pathogenesis of inflammatory bowel disease [5], it can reduce leukotriene production and it can inhibit the cellular release of interleukin-1 [6]. All these properties are probably important in reducing the acute inflammatory response in inflammatory bowel disease. It has been suggested that 5-ASA may function by inhibiting prostaglandin synthetase or lipoxygenase and interfering with the synthesis of antibodies. The drug exerts its action intramucosally after absorption and, as seems likely, it is doubtful whether sufficient unchanged 5-ASA would be present within the mucosa to have any effect. The main metabolite of 5-ASA is Ac-5ASA (Fig. 1(b)), and the drug is found predominantly in this form in the body. The site of acetylation of 5-ASA was widely assumed to be the liver, but it has recently been shown that the human colonic epithelial cells are capable of acetylating 5-ASA [7] and because the *N*-acetyltransferase enzyme is cytosolic, 5-ASA must be taken up into the cell before acetylation. Alternative metabolic pathways of 5-ASA occur in patients with active inflammatory bowel disease owing to reactive oxidants such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals (HO$^{\cdot}$) and hypoclorite anions (OCl^-), which are released by neutrophiles present in the inflamed bowel [8]. 5-HSA (Fig. 1(c)) could be generated with this alternative pathway by oxidants present in the inflamed bowel. In addition, it has been shown that Ac-5-ASA and 5-HSA are the forms of the drug predominantly found in the colon cells of patients taking oral sulfasalazine [9]. Since the effect of 5-ASA in the treatment of inflammatory bowel disease is believed to be due to local action, the site and amount of drug release are highly important.

Several high-performance liquid chromatographic (HPLC) methods have been used to measure the concentrations of 5-ASA and Ac-5ASA in biological fluids [10–12], but none allows the simultaneous determination of 5-ASA, Ac-5-ASA and 5-HSA.

The present work was designed to develop an HPLC procedure with electrochemical detection (ED) allowing the simultaneous determination of 5-ASA, Ac-5-ASA and 5-HSA in intestinal endoscopic biopsy samples. This method is rapid, without handling and time-consuming purification steps, sensitive and specific enough to avoid predictable interferences.

2. Experimental

2.1. Chemicals

HPLC-grade methanol and analytical-grade citric acid were obtained from Carlo Erba (Milan, Italy). 1-Heptanesulfonic acid sodium salt, ethylenediaminetetraacetic acid and sodium hydrogenphosphate were obtained from Fluka (Buchs, Switzerland). 5-Aminosalicylic acid, 2,5-dihydroxybenzoic acid and 3,4-dihydroxybenzylamine (DHBA) (Fig. 1(d)), as internal standard, were purchased from Sigma Chemical (St. Louis, MO, USA). Acetyl-5-aminosalicylic acid was synthesized in the laboratory. Water (HPLC grade) was obtained by distillation in glass and purification through a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Chromatographic system and conditions

The HPLC system consisted of a Waters (Milford, MA, USA) Model 510 pump and a Waters Model U6K injection valve. The detector system consisted of a Coulochem (ESA, Bedford, MA, USA) Model 5100A electrochemical detector equipped with a Model 5021 conditioning cell and a Model 5011 analytical cell. The conditioning cell was placed between the column and the analytical cell; it was used to preoxidize or reduce the mobile phase and minimize the background noise due to the electroactive components in the mobile phase, and was set at +0.35 V. The analytical cell, connected to the conditioning cell, consisted of a dual detector cell unit. The electrodes of the analytical cell were set at +0.05 V for the first electrode and at −0.50 V for the second electrode. The signal generated by the second electrode was converted by a Model HP-3396-II integrator (Hewlett-Packard, Rome, Italy) into a chromatographic trace.

Separation was performed on an Erbasil S C18 analytical column (250 × 4.6 mm i.d.; particle size 10 μm) from Carlo Erba, protected with a Pelliguard precolumn (50 × 4.6 mm i.d.; particle size 40 μm) (Supelco, Bellefonte, PA, USA). The mobile phase was a binary mixture of 0.01 M Na_2HPO_4 (containing 0.1 mM EDTA, 0.1 M citric acid and 0.1 mM heptanesulfonic acid–methanol (85:15, v/v); the pH was adjusted at 3 with 0.1 M NaOH. The solution was filtered through GS 0.22 μm filters (Millipore) and methanol was filtered through WTP 0.5 μm filters (Whatman, Maidstone, UK). The mobile phase was sonicated before use and delivered at a flow rate of 1 ml min^{-1}.

2.3. Standard solutions

Stock solutions of 5-ASA, Ac-5-ASA, 5-HSA and internal standard were prepared by dissolving 10 mg of each compound in 100 ml of methanol. These solutions could be stored at −80°C for over one month with no evidence of decomposition. Standard solutions, each containing the three drugs, were obtained by diluting the stock solutions with drug-free human plasma in the range 1–300 ng ml^{-1} for 5-ASA, 10–1000 ng ml^{-1} for Ac-5-ASA and 0.1–10 ng ml^{-1} for 5-HSA. For each solution the concentration of the other two drugs was kept constant at 50 ng ml^{-1}, while the internal standard was added at a constant level of 5 ng ml^{-1}. These standards were treated concurrently in the same manner as the samples to be analysed. Calibration graphs were obtained by plotting the peak-area ratios of each drug to internal standard against the drug concentrations obtained after extraction.

2.4. Sample preparation

All patients had been given oral information according to instructions provided by the local ethics committee. Subjects were dosed with the sustained-release 5-ASA formulation Asacol (6 × 400 mg per day). After overnight fasting of each patient, 3 l of PEG solution were used to obtain complete evacuation of the gut. All biopsy specimens taken from the colon of patients during colonoscopy were accurately weighed and placed on ice washed with ice-cooled saline, blotted with filter-paper, and stored at −80°C until assay. After thawing, the biopsy specimens were gently rinsed, dried for a few seconds on filter-papers and placed in tubes containing 2 ml of a 5 ng ml^{-1} solution of internal standard in methanol, in an ice-bath. After sonication for two cycles of 30 s ($W = 60$), the supernatants were collected by centrifugation (1800g, 10 min), filtered through a 0.5 μm WTP filter and evaporated to dryness with a stream of nitrogen under vacuum utilizing the Supelco drying attachment. The sample was then reconstituted with 100 μl of mobile phase and mixed with a vortex agitator. Aliquots of each sample (5 μl) were chromatographed utilizing the apparatus described above.

3. Results and discussion

Typical chromatograms for the analysis of biopsy samples with electrochemical detection are shown in Fig. 2. The compounds were well resolved and the baseline was free from interfer-

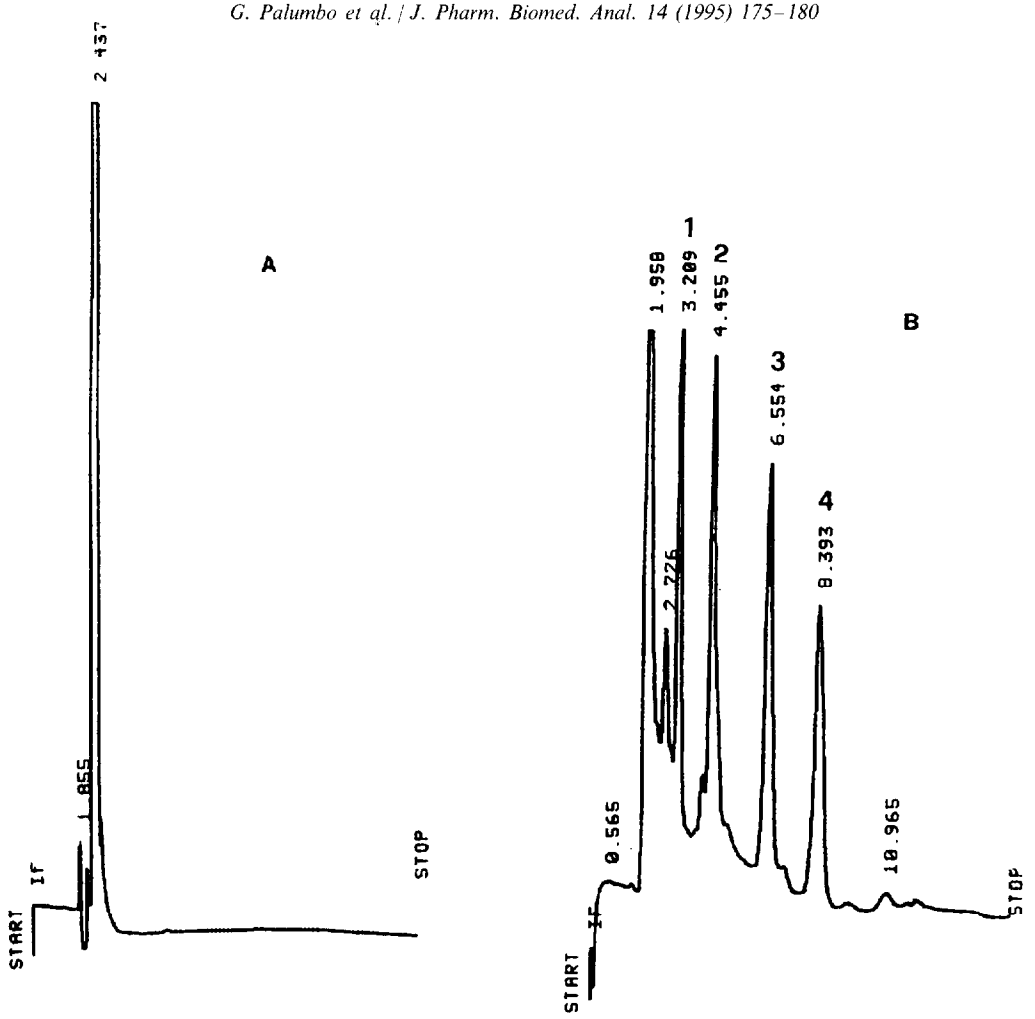

Fig. 2. Chromatograms of human plasma extracts. (A) Blank plasma; (B) blank plasma spiked with (2) internal standard (5 ng ml^{-1}), (1) 5-ASA (20 ng ml^{-1}), (4) Ac-5-ASA (200 ng ml^{-1}) and (3) 5-HSA (5 ng ml^{-1}).

ences at the positions of the analytes and internal standard. The retention times for 5-ASA, 5-HSA, Ac-5-ASA and internal standard were 3.2, 6.5, 8.3 and 4.4 min, respectively. The percentage recovery of the extraction procedure for 5-ASA, 5-HSA, Ac-5-ASA was determined at three concentrations ranging from 5 to 100, from 50 to 500 and from 1 to 10 ng ml^{-1}, respectively. Peak-area ratios obtained from the extracted biopsy samples were compared with those obtained by injection of standard solutions. The extraction efficiency was not significantly different over the whole concentration range. The lowest mean recovery values ($\pm$SD) were $96 \pm 3.5\%$ ($n = 7$), $97 \pm 2.3\%$ ($n = 7$) and $95 \pm 4.4\%$ ($n = 7$) for 5-ASA, 5-HSA and Ac-5-ASA, respectively. The extraction efficiency of the internal standard was $97 \pm 2.2\%$ ($n = 7$). All other recoveries were consistently higher than the above (Table 1).

In order to avoid unnecessary medical risks, and to evaluate the influence of intestinal tissue, 5-ASA, Ac-5-ASA and 5-HSA were added to only a small number of blank biopsies and their calibration graphs were compared with those ob-

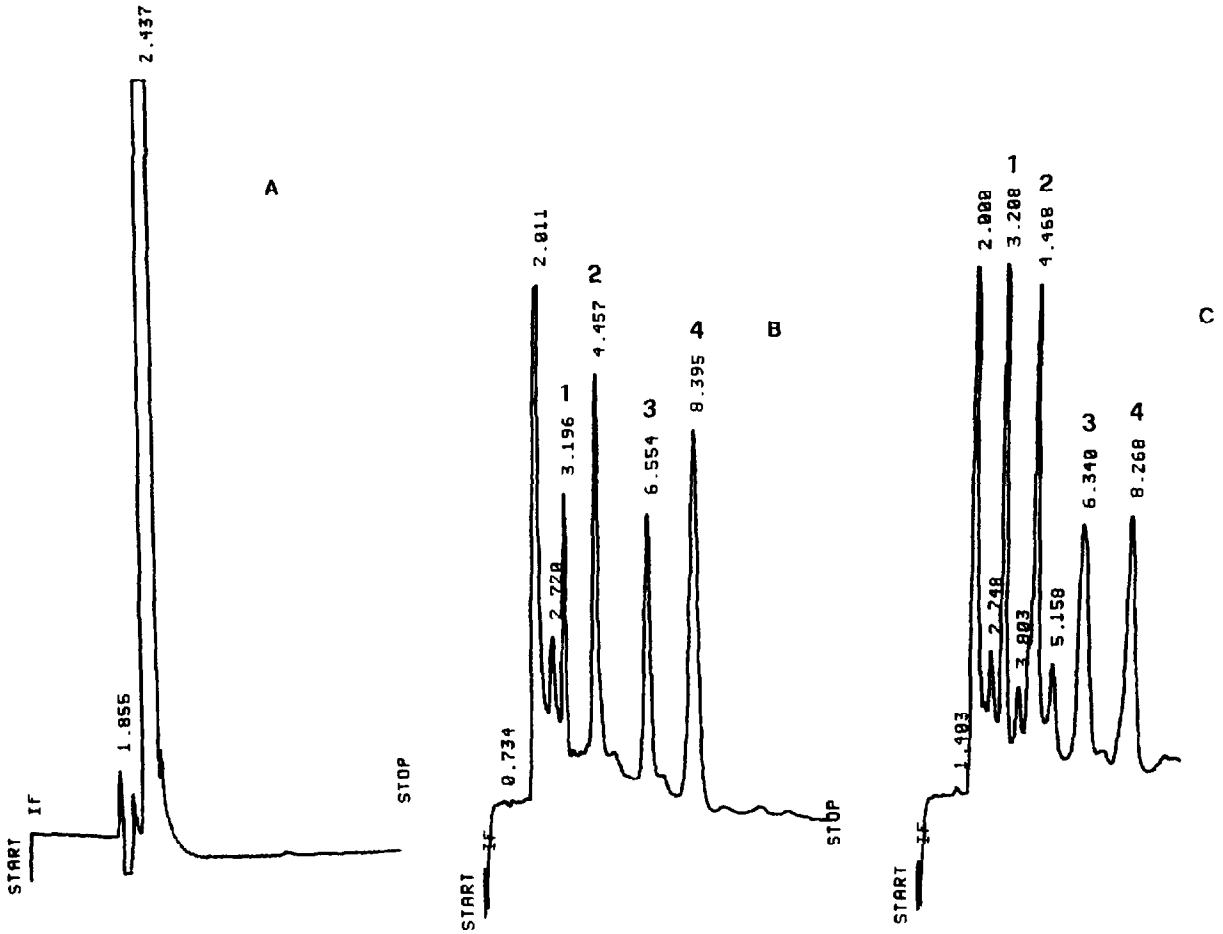

Fig. 3. Chromatograms of human biopsy extracts. (A) Blank biopsy; (B) blank biopsy spiked with (2) internal standard (5 ng ml^{-1}), (1) 5-ASA (10 ng ml^{-1}), (4) Ac-5-ASA (250 ng ml^{-1}) and (3) 5-HSA (2 ng ml^{-1}); (C) biopsy sample containing 5-ASA (27 ng mg^{-1}), Ac-5-ASA (192 ng mg^{-1}) and 5-HSA (3 ng mg^{-1}).

tained by diluting the analytes into drug-free plasma samples or drug-free biopsy homogenates. This comparison gave a good correlation coefficient ($r \geqslant 0.997$) for all the analytes, allowing plasma samples to be used for all subsequent analyses.

The assay was validated by analysing seven 5-ASA, Ac-5-ASA and 5-HSA standards. Each datum was the average of a minimum of five determinations. The equations obtained through regression analysis of data for the above standard solutions were $y = -2.8 \times 10^4 + 1.4 \times 10^3 x$ ($r = 0.998$) for 5-ASA, $y = 2.9 \times 10^4 + 7.9 \times 10^2 x$ ($r = 0.999$) for Ac-5ASA and $y = 6.9 \times 10^4 + 2.1 \times 10^4 x$ ($r = 0.995$) for 5-HSA, where y is the peak-area ratio in arbitrary units of the HP-3396-II system used and x is the concentration (ng ml^{-1}). The accuracy of the assay was good and the reproducibility was within acceptable limits. The extraction efficiencies of analytes were over the 95%. The precision, expressed as the relative standard deviation (RSD) of the mean value found, and accuracy in terms of relative error (RE) are reported in Table 2. The detection limits for 5-ASA and Ac-5-ASA were 1 ng ml^{-1} and that for 5-HSA was ng ml^{-1} at a signal-to-noise ratio of 5. The accuracy and precision of the method were determined by processing spiked

Table 1
Extraction recoveries of 5-ASA, Ac-5-ASA and 5-HSA from spiked plasma samples ($n = 7$)

Compound	Concentration ($ng\ ml^{-1}$)	Mean recovery (%)	±SD
5-ASA	10	96	3.5
	50	97.4	2.5
	100	97.8	3.2
Ac-5-ASA	50	95	4.4
	100	96.3	3.6
	500	97	2.7
5-HSA	0.5	97	2.3
	2.0	97.6	3.1
	5.0	97.2	2.0

Table 2
Precision and accuracy of the determination of 5-ASA, Ac-5-ASA and 5-HSA

Compound	Concentration ($ng\ ml^{-1}$)	Concentration found ($ng\ ml^{-1}$)	RSD (%)	RE (%)
5-ASA	10	9.6	4.6	3.9
	50	49.1	2.7	1.8
	100	98.8	2.9	1.2
Ac-5-ASA	50	49.4	2.3	1.2
	100	98.8	1.4	1.2
	500	488.2	1.0	2.4
5-HSA	0.5	0.47	6.3	6.3
	2.0	1.9	4.1	4.2
	5.0	4.7	2.9	5.7

biopsy samples at three concentrations with respect to a calibration graph.

The method described allows the determination of 5-ASA, Ac-5-ASA and 5-HSA in human intestinal biopsy samples and the choice of the electrochemical detection mode permits its application to pharmacokinetic studies and in therapeutic monitoring. This study enabled the role of the two metabolites to be examined, especially Ac-5-ASA, because there is no clear evidence that Ac-5-ASA inside the mucosa is inactive. In conclusion, this study can allow comparisons to be made of the clinical condition of patients with the direct intestinal biopsy concentrations of the drug and its metabolites and thus eventually to establish the "in situ" real therapeutic range of the drug which is expected to act locally.

Acknowledgements

This research was supported by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (quota 40%) and the Consiglio Nazionale delle Ricerche.

References

[1] C.P. Willoughby, J.K. Aronson, H. Agback, N.O. Bodin and S.C. Truelove, Gut, 23 (1982) 1081–1087.
[2] U. Klotz and K.E. Maier, Dig. Dis. Sci., 23 (Suppl. 12) (1987) 46S–50S.
[3] M.C.M. Rijk, A. van Schaik and J.H.M. van Tongeren, Scand. J. Gastroenterol., 23 (1988) 107–112.
[4] S.N. Rasmussen, S. Bondersen, E.F. Huidberrg, S.H. Hansen, U. Binder and S. Halskov, Gastroenterology, 83 (1982) 1062–1070.
[5] J. Brynskov, N. Tvede, C.B. Andersen and M. Vilien, Gut, 33 (1992) 55–58.
[6] W.H. Betts, M.W. Whitehouse, L.G. Cleland and B. Vernon-Roberts, J. Free Rad. Biol. Med., 1 (1985) 273–280.
[7] A. Ireland, J.D. Priddle and D.P. Jewell, Clin. Sci., 78 (1990) 105–111.
[8] E.G. Janzen, L.T. Jandrisits and D.L. Barber, Free Rad. Res. Commun., 4 (1987) 115–123.
[9] A. Ireland, J.D. Priddle and D.P. Jewell, Clin. Sci., 72 (Suppl. 16) (1987) 79P.
[10] S.H. Hansen, J. Chromatogr., 226 (1981) 504–509.
[11] E. Nagy, I. Csipo, I. Degrell and S. Szabo, J. Chromatogr., 425 (1988) 214–219.
[12] M. De Vos, H. Verdievel, R. Schoonjans, R. Beke, G.A. De Weerdt and F. Barbier, J. Chromatogr., 564 (1991) 296–302.