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Note

High-performance liquid chromatographic assay of 5-aminosalicylic acid and its acetylated metabolite in biological fluids using electrochemical detection

E. NAGY*

Department of Neurology and Psychiatry, University of Debrecen, Medical School, 4012 Debrecen (Hungary)

I. CSIPO

3rd Department of Medicine, University of Debrecen, Medical School, 4012 Debrecen (Hungary)

I. DEGRELL

Department of Neurology and Psychiatry, University of Debrecen, Medical School, 4012 Debrecen (Hungary)

and

G. SZABO

3rd Department of Medicine, University of Debrecen, Medical School, 4012 Debrecen (Hungary)

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Sulfasalasin (SS) was first used in the therapy of ulcerative colitis in the early 1940s by Svartz [1]. It has also been applied in the therapy of other inflammatory bowel diseases [2,3] and rheumatoid arthritis [4].

In recent years several comparative clinical trials with SS and its primary metabolites sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) have demonstrated that 5-ASA is most likely the therapeutically active moiety of SS [2,5,6] and that SP accounts mainly for the side-effects [7]. Therefore, the treatment of inflammatory bowel diseases has been focused on the use of 5-ASA [8].

5-ASA is mainly eliminated by acetylation in the form of the metabolite acetyl-5-aminosalicylic acid (Ac-5-ASA), which is excreted by the kidneys.

Several high-performance liquid chromatographic (HPLC) methods have been used to measure the concentrations of 5-ASA and Ac-5-ASA in biological fluids. Fischer and Klotz [9] have utilized an ion-pair reagent in the sample prepara-

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tions. Other methods [10,11] require the prior acetylation or propionylation of 5-ASA to improve the fluorescence characteristics. Hansen [12] presented a method utilizing dynamically modified silica and an ion-pair reagent in the mobile phase. All these authors have applied fluorescence and/or UV detection.

Our investigations were aimed at developing an HPLC method involving electrochemical detection in order to achieve a simple, sensitive method for the determination of 5-ASA and Ac-5-ASA in biological fluids.

EXPERIMENTAL

Chemicals

5-ASA and Ac-5-ASA were synthetized in our laboratory and purified by recrystallization. All other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, F.R.G.).

Apparatus

The equipment used for this assay was a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 10 high-performance liquid chromatograph with a Rheodyne (Cotati, CA, U.S.A.) 7125 sample injector and a Model LC-4B thin-layer electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) equipped with a TL-5A cell. Separation was performed on a reversed-phase, Spherisorb C18-2 column (average particle size 5 μm; 250 mm $\times$ 4.6 mm I.D.; Latek, Eppelheim, F.R.G.). The potential was set at +0.80 V versus an Ag/AgCl reference electrode with a glassy carbon working electrode. Chromatograms were recorded with a Shimadzu (Kyoto, Japan) CR 1B integrator.

Chromatographic conditions

The mobile phase was a mixture of a buffer with an organic modifier [15% (v/v) methanol]. The buffer consisted of 0.12 mmol/l sodium octylhydrogensulphate, 0.05 mmol/l EDTA, 0.2 mol/l citric acid and 0.01 mol/l phosphate (Na_2HPO_4) solution, adjusted to pH 3.1 with orthophosphoric acid. The water used was demineralised and filtered through a 0.45-μm filter. The mobile phase was continuously degassed with nitrogen. The flow-rate was 0.8 ml/min, resulting in a pressure drop of about 21 MPa. Samples were analyzed isocratically at 28°C.

Sample preparation

Blood samples were collected in unheparinized tubes. Serum was separated after clotting by centrifugation and stored at −20°C until taken for analysis.

From two patients with intensive headaches 3.0 ml lumbar cerebrospinal fluid (CSF) were taken with their written consent. Routine neurological and electroencephalographic (EEG) examinations did not reveal any pathological signs.

A 1-ml volume of serum or CSF was mixed with 4 ml of methanol. After standing for at least 30 min at room temperature, the mixture was centrifuged for 5 min at 1000 *g* and the supernatant was dried under nitrogen at 40°C. The residue was dissolved in 1 ml methanol and 100 μl of the solution were injected onto the column.

Clinical study

Two patients suffering from chronic gastritis, enteritis and colitis were treated with Salofalk. Histological examinations from stomach, jejunum and rectum showed the mucosa (laminar propria) to be infiltrated.

On the first day of treatment they received 2.0 g of 5-ASA (Salofalk®) on an empty stomach, followed by 3×1.0 g daily. Five other patients (two patients with chronic gastroenterocolitis, one with ankylosing spondylitis, one with psoriatic arthropathy and one patient with ulcerative colitis), were treated with 3×1.0 g Salofalk daily to determine the steady-state concentrations of 5-ASA and Ac-5-ASA.

The sera of the first two patients were obtained after the start of the drug administration, and again after a prolonged administration of five to six days. This time serum was obtained from all seven patients.

CSF samples were taken on the sixth day from the first two patients. The concentrations of 5-ASA and Ac-5-ASA in the samples were determined using a calibration curve. In the case of serum samples a solution consisting of 2.0 μg/ml 5-ASA and 1.5 μg/ml Ac-5-ASA was used as a control standard solution. In the case of CSF 0.2 μg/ml 5-ASA and 0.25 μg/ml Ac-5-ASA was used.

RESULTS

In order to determine the concentrations of 5-ASA and Ac-5-ASA in serum and CSF it was necessary to establish an optimal operating potential for the working electrode. Fig. 1 shows the current–voltage curves for 5-ASA and Ac-5-ASA. Under the above chromatographic conditions 5-ASA can be detected at +0.55 V where its potential curve reaches a constant level. Ac-5-ASA is hardly measureable at +0.55 V. Its potential achieves maximum intensity at +0.80 V. We have chosen +0.80 V for the simultaneous determination of 5-ASA and Ac-5-ASA, because both compounds can be dectected with high sensitivity at this potential. The value of the background current is 3 nA using the mobile phase described above. This low value makes quantitative, selective determinations possible.

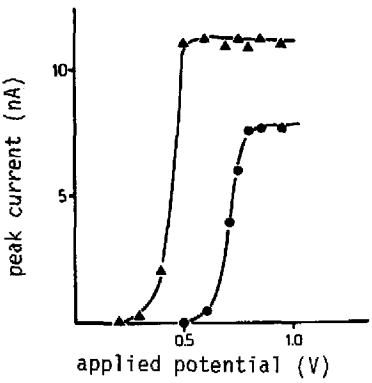

Fig. 1. Current–voltage curves for 5-ASA (▲) and Ac-5-ASA (●). The optimal operating potential is +0.80 V, using an Ag/AgCl reference electrode.

As Klotz et al. [13] reported earlier, both 5-ASA and Ac-5-ASA are bound to plasma proteins. By adding 2 μg/ml standards to eleven normal serum samples we found that the recovery of total 5-ASA was 41 ± 10% and that of Ac-5-ASA 58 ± 6%.

During our studies no changes were observed in the standard water solution, nor in CSF containing 0.20 μg/ml 5-ASA and 0.25 μg/ml Ac-5-ASA. Consequently, standards of 5-ASA and Ac-5-ASA were prepared in serum (range 0.1–4.0 μg/ml) and in water (range 0.01–0.40 μg/ml) for the determination of their levels in serum and CSF, respectively.

The calibration graphs for both compounds were linear over the concentration range studied. Linear regression equations ($n=11$) for 5-ASA and Ac-5-ASA calibration curves are $y=5.87x-0.32$, $r=0.994$ ($p<0.001$) and $y=4.04x-0.11$, $r=0.993$ ($p<0.001$), respectively.

Using electrochemical detection, concentrations of 5-ASA could be measured more sensitively at +0.80 V than those of its acetylated metabolite. Detection limits were 0.15 ng 5-ASA and 0.20 ng Ac-5-ASA per injected volume at a signal-to-noise ratio of 3:1.

Stability tests for both compounds in serum were performed at room temperature or at 4 °C. After seven days there was no change in the serum levels and no additional peaks were observed in the chromatograms. Some typical chromatograms are shown in Fig. 2, demonstrating adequate resolution under the chromatographic conditions. The retention times of 5-ASA and Ac-5-ASA were 6.4 and 10.3 min, respectively. We have investigated the levels of 5-ASA and Ac-5-ASA in serum samples of two patients after oral administration of a single 2.0-g

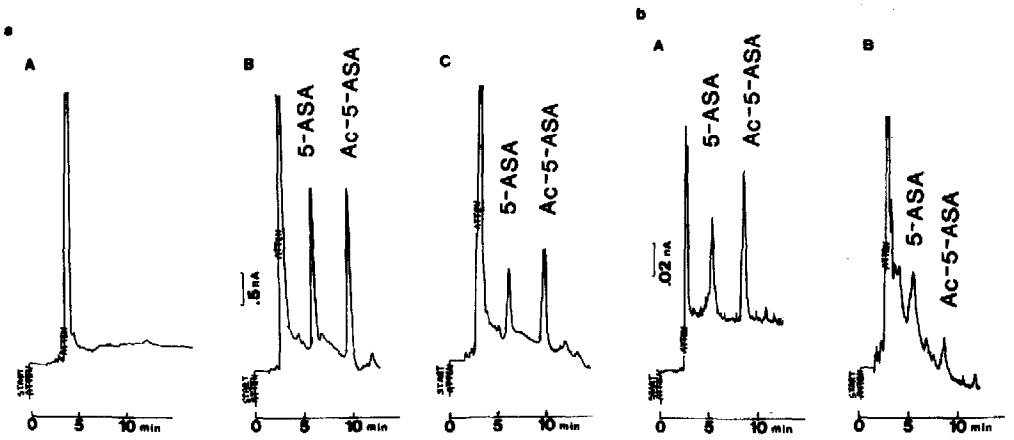

Fig. 2. (a) Typical chromatograms showing (A) blank serum, (B) 1.0 μg/ml 5-ASA and 1.25 μg/ml Ac-5-ASA in control serum and (C) 5-ASA (0.40 μg/ml), Ac-5-ASA (0.50 μg/ml) in patient serum. (b) HPLC profiles of (A) 0.10 μg/ml 5-ASA and 0.15 μg/ml Ac-5-ASA solution and (B) patient CSF sample. Conditions: injection volume, 100 μl; column, Spherisorb C18-2, 5 μm, 250 mm × 4.6 mm; mobile phase, 15% methanol, 0.12 mmol/l sodium octylhydrogensulfonate, 0.05 mol/l EDTA, 0.2 mol/l citric acid, 0.01 mol/l dibasic sodium phosphate (pH 3.1); flow-rate, 0.8 ml/min; electrochemical detector, Model LC-4B; operating potential, +0.80 V using an Ag/AgCl reference electrode.

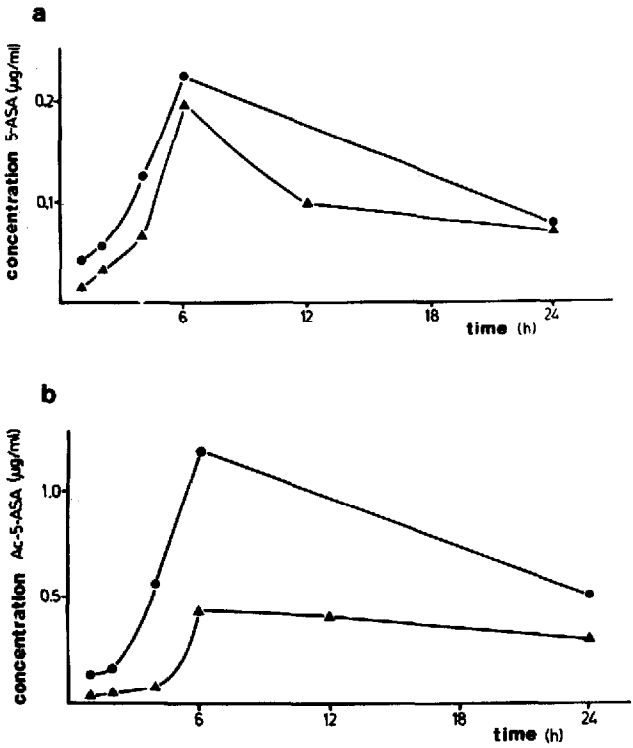

Fig. 3. Plasma concentrations of 5-ASA (a) and Ac-5-ASA (b) after oral administration of 2.0 g 5-ASA. (●) Patient A; (▲) patient B.

TABLE I

SERUM CONCENTRATIONS OF 5-ASA AND Ac-5-ASA IN PATIENTS TREATED WITH SALOFALK

Patient No.	Salofalk dose (g/day)	5-ASA (µg/ml)	Ac-5-ASA (µg/ml)
1	3.0	0.32	1.60
2	3.0	0.19	2.10
3	3.0	0.28	1.77
4	3.0	0.21	1.25
5	3.0	0.15	0.77
6	3.0	0.13	1.51
7	3.0	0.33	1.60

dose of Salofalk. Fig. 3 demonstrates concentration–time curves of 5-ASA and Ac-5-ASA measured in serum samples after drug administration.

The maximum concentrations of the drug and its metabolite could be detected after 6 h. The steady-state serum concentrations of 5-ASA and Ac-5-ASA after prolonged administration for five to six days was also measured. The steady-state serum concentrations of seven patients were found to be in the 01.3–0.33 µg/ml

range for 5-ASA and in the 0.77–2.1 μg/ml range for Ac-5-ASA (Table I). CSF levels in samples taken at the same time were 0.040 μg/ml for 5-ASA and 0.025 μg/ml for Ac-5-ASA.

DISCUSSION

A simple, reproducible assay for the simultaneous determination of 5-ASA and its main metabolite Ac-5-ASA in serum and CSF has been evaluated. For the determination of the above two compounds a reversed-phase analytical column with an eluent of pH 3.1 was used. Optimal retention times for compounds containing the amino group were achieved by addition of suitable amounts of organic solvent and sodium octylhydrogensulphate as ion-pair reagent.

To obtain optimum resolution it was important to perform the experiments at different pH levels, because with increasing pH the retention times decreased. The low detection limits of 5-ASA and Ac-5-ASA, as well as the simple sample preparation, made determinations of 5-ASA and Ac-5-ASA possible at low concentrations in biological fluids.

The therapeutic effects of 5-ASA represent a new field, as demonstrated in two cases. There was a marked improvement in gastroenterologic symptoms and in one case the neurologic symptoms showed some improvement. In chronic inflammatory bowel diseases symptoms of neurologic diseases are frequently experienced. It is thought that some symptoms may arise from the relative increase in mediators (leukotrienes).

Treatment with 5-ASA could be detected not only in the serum, but also in the CSF. Further investigations in this field are necessary.

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