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Note

Assay of 5-aminosalicylate and its acetylated metabolite in biological fluids by high-performance liquid chromatography on dynamically modified silica

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Salicylazosulphapyridine (SASP) has for years been a cornerstone in the treatment of ulcerative colitis and Crohn's disease. Recently it has been suggested [1, 2] that the effect of this drug may be ascribed to the 5-aminosalicylate (5-ASA) moiety. This compound is formed by bacterial cleavage of SASP in the gut [3]. 5-ASA is mainly eliminated by acetylation, forming the metabolite acetyl-5-aminosalicylic acid (Ac-5-ASA) [4], which in turn is excreted by the kidneys. In order to measure these two substances in biological fluids, both spectrofluorimetry [5], colorimetry [6], and recently also an assay based on high-performance liquid chromatography (HPLC) [7] have been applied. The HPLC method, which is claimed to be both sensitive and specific, involves extraction and evaporation of the organic phase followed by redissolving in the mobile phase and injection on to the HPLC column. However, in our experience with this method, the recoveries from the extraction procedure only reached about 40% for 5-ASA and about 70% for Ac-5-ASA. Furthermore, 5-ASA, and particularly the internal standard *p*-aminosalicylate (PAS), were found to form interfering degradation products during the extraction and evaporation procedures. Finally, the assay as a whole is quite time-consuming, the chromatographic procedure alone lasting 30—40 min.

The aim of the present investigation was to develop an HPLC method involving dynamically modified silica [8, 9] in order to obtain a simple, rapid and sensitive method for the determination of 5-ASA and Ac-5-ASA in biological fluids. Furthermore, the assay should be reliable and specific enough for use in clinical experimental work.

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EXPERIMENTAL

Apparatus

A Waters liquid chromatograph consisting of a 6000A pump, a 710A WISP autoinjector, a 440 ultraviolet (UV) absorbance detector (254 nm), a 730 data module and a 720 system controller was used. The columns were thermostated in an LC 250/3 Kratos oven. Three different fluorescence detectors were used: a Perkin-Elmer LC 1000, a Perkin-Elmer 3000, or a Schoeffel FS 970 instrument.

For centrifugation an Ole Dich micro centrifuge was used.

Chemicals

5-Aminosalicylic acid, acetyl-5-aminosalicylic acid and the pharmaceutical preparations were kindly supplied by Ferring (Copenhagen, Denmark).

Acetonitrile HPLC S grade was obtained from Rathburn Chemicals (Walkerburn, Great Britain). All other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, G.F.R.).

Sample preparation

A 1-ml volume of plasma or urine was mixed with 4 ml of methanol. After standing for at least 30 min the mixture was centrifuged for 1 min at 15,000 *g* and 20 μl of the supernatant were injected onto the column. Faeces were collected in and extracted by 500 or 1000 ml of methanol per day depending on the amount of faeces delivered; 10 μl of the centrifuged methanol extract were injected on to the column. Ileostomy effluents were at each collection suspended in 50 ml of 0.9% sodium chloride. This suspension was treated in the same way as the plasma samples and 20 μl of the resulting supernatant were injected on to the column.

Chromatography

The column set-up has been described previously [8]. The analytical column was a Knauer column 120 × 4.6 mm I.D., packed with LiChrosorb Si 60 (5-μm particles) using the dilute slurry technique [10]. The guard column (100 × 4.6 mm I.D.) situated between the pump and the autoinjector was dry-packed with LiChroprep Si 60. Both columns were operated at 40°C. The mobile phase was acetonitrile + 0.2 *M* potassium phosphate (pH 7.5) + water (30:5:65) containing 1.25 m*M* N,N,N-trimethylhexadecylammonium bromide, and the flow-rate was 1.5 ml min^{-1}.

RESULTS AND DISCUSSION

Detection limits, reproducibility and recovery

The detection limits, i.e. the sensitivity of the method defined as three times the noise, were determined by the use of a standard solution containing 5-ASA and Ac-5-ASA. The minimal detectable quantities are given in Table I for the four detectors examined. The results show a detection limit of 20 ng of Ac-5-ASA per ml plasma when using a Perkin-Elmer 3000 detector.

The reproducibility and precision of the method were determined by adding

standard solutions to plasma samples. The results (Table II) show a recovery between 96 and 103%. The precision of the assay was excellent as the coefficient of variation never exceeded 5%. No degradation of the compounds during analysis has been observed. The use of the internal standard (PAS) was not found to improve the precision or reproducibility of the assay, and has, therefore, been omitted. The between-run and within-run coefficients of variation (n = 6) were, in the concentration range 25—5000 ng/ml, in all cases less than 5%.

TABLE I

MINIMUM DETECTABLE QUANTITIES OF PAS, 5-ASA AND Ac-5-ASA MEASURED BY THREE DIFFERENT FLUORESCENCE DETECTORS AND BY A UV DETECTOR

Apparatus	Wavelength (nm)		Minimum detectable quantity (ng) injected on to column		
	Excitation	Emission	PAS	5-ASA	Ac-5-ASA
Perkin-Elmer LC 1000	315*	430	5	20	0.8
Perkin-Elmer 3000	315	430	0.5	2	0.08
Schoeffel FS 970	315	418**	0.4	0.1	0.06
Waters 440	254		1	5	2

*14-nm bandpass filter.
**Cut-off filter.

TABLE II

ANALYSIS OF PLASMA SAMPLES WITH STANDARD ADDITION OF PAS, 5-ASA AND Ac-5-ASA

The linear regression equation for the Ac-5-ASA calibration is $x = 1.03y - 8.49$, with $r = 1.000$.

Drug	Concentration (ng/ml)	Mean amount found (n = 6) (ng/ml)	Precision (C.V., %)	Recovery (%)
PAS	100	96	4.1	96
PAS	1000	984	2.0	98
5-AS	500	478	3.5	96
5-AS	5000	5150	2.2	103
Ac-5-ASA	25	23	5.1	92
Ac-5-ASA	100	101	2.5	101
Ac-5-ASA	500	512	2.0	102
Ac-5-ASA	1000	977	0.9	98
Ac-5-ASA	5000	4865	1.4	97

Specificity

The use of fluorescence detection provides a high degree of selectivity, and no interfering peaks from endogenous metabolites have been observed in any of

the sample materials analysed. A number of drugs have been tested but only other salicylates have been found to interfere. Salicylate has a longer retention time resulting in an extended analysis time to avoid interference with the next sample. The low detection limit for 5-ASA using the Schoeffel detector (Table I) is due to the use of a cut-off filter on the emission side. However, this results in less selectivity, which, on the other hand, in this particular case is an advantage. Some typical chromatograms are shown in Fig. 1.

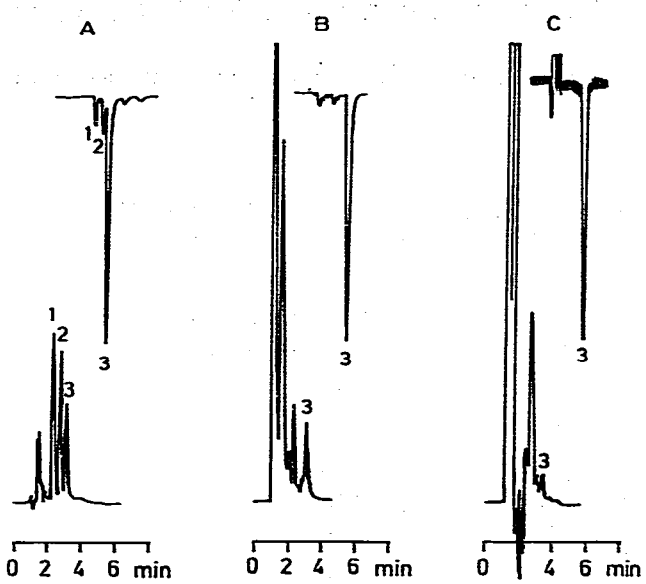

Fig. 1. Typical chromatograms of Ac-5-ASA in urine and plasma. Bottom traces: ultraviolet (254 nm) absorbance. Top traces: fluorescence detection (Perkin-Elmer 3000) at excitation 315 nm/emission 430 nm. (A) Standard mixture. 1 = PAS; 2 = 5-AS; 3 = Ac-5-ASA. (B) Urine sample. (C) Plasma sample (0.3 μg of Ac-5-ASA per ml of plasma).

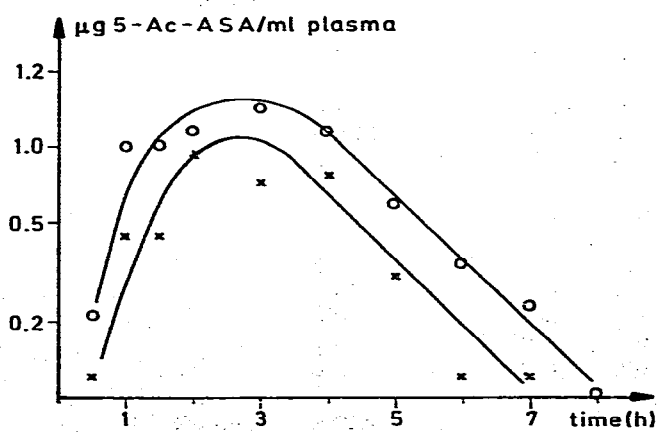

Fig. 2. Plasma concentrations of Ac-5-ASA in two patients after oral administration of 0.5 g of 5-ASA (slow release). (×), patient A; (○), patient B. See also Table III.

Application to clinical material

The assay has been applied to various biological materials (plasma, urine, faeces and ileostomy effluents) obtained from healthy volunteers and ileostomy patients receiving 500 mg of 5-ASA. Details of these clinical experiments are published elsewhere [11], but examples from this study are presented in Table III and Fig. 2. They concern the results of measurements of 5-ASA and Ac-5-ASA in the ileostomy effluents and urine and concentration—time curves for plasma Ac-5-ASA in two patients. The assay proved suitable for pharmacokinetic studies. The apparent half-life of elimination ($t_{½\beta}$) for Ac-5-ASA in plasma is about 70 min.

Once the method has been set up in the automated mode ten samples can be analysed per hour.

TABLE III

TOTAL AMOUNT OF 5-ASA AND Ac-5-ASA EXCRETED IN ILEOSTOMY EFFLUENT AND URINE FROM TWO PATIENTS AFTER ADMINISTRATION OF 0.5 g 5-ASA (SLOW RELEASE)

Patient	Time (h)	Amount of sample*			Amount eliminated (mg) 5-ASA	Ac-5-ASA	Total amount expressed as 5-ASA (mg)	
A	0—3	87.5	g	IE	0.0	0.0	0.0	
	3—4	3.5	g	IE	22.4	2.5	24.3	
	4—5	127.3	g	IE	240.2	91.9	312.3	
	5—6	33.1	g	IE	1.5	14.0	12.5	
	6—7	66.4	g	IE	1.8	19.3	16.9	
	7—8	84.7	g	IE	1.0	9.0	8.1	
	8—24	919	g	IE	0.0	0.0	0.0	
	0—1	45	ml	U	0.0	4.7	3.7	
	1—5	225	ml	U	0.0	81.1	63.6	
	5—8	180	ml	U	0.0	9.1	7.1	
	8—24	696	ml	U	0.0	0.0	0.0	
							448.5	(89.7%)**
B	0—3	13.7	g	IE	0.0	0.0	0.0	
	3—4	9.9	g	IE	79.7	22.7	97.5	
	4—5	14.2	g	IE	69.5	32.0	94.6	
	5—6	18.2	g	IE	75.7	38.5	105.9	
	6—7	1.9	g	IE	1.0	0.8	1.6	
	7—8	72.7	g	IE	23.7	40.9	55.8	
	8—24	426	g	IE	0.0	0.0	0.0	
	0—1	25	ml	U	0.0	6.6	5.8	
	1—5	675	ml	U	0.0	84.3	66.1	
	5—8	165	ml	U	1.4	13.5	10.6	
	8—24	313	ml	U	0.0	9.2	7.2	
	24—48	643	ml	U	0.0	0.0	0.0	
							429.8	(86.0%)**

*IE = ileostomy effluent; U = Urine.

**Percentage recovery.

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REFERENCES

1 A.K. Azad Khan, J. Piris and S.C. Truelove, Lancet, ii (1977) 893.
2 P.A.M. van Hees, J.H.M. van Tongeren, J.H. Bakker and H.J.J. van Lier, Lancet, ii (1978) 277.
3 M.A. Peppercorn and P. Goldmann, J. Pharmacol. Exp. Ther., 181 (1972) 55.
4 H. Schröder and D.E.S. Cambell, Clin. Pharmacol. Ther., 13 (1972) 532.
5 K.-A. Hansson, Acta Pharm. Suecica, 10 (1973) 153.
6 H.J. Pieniazek, Jr. and T.R. Bates, Res. Commun. Chem. Pathol. Pharmacol., 12 (1975) 571.
7 C. Fischer and U. Klotz, J. Chromatogr., 162 (1979) 237.
8 S.H. Hansen, J. Chromatogr., 209 (1981) 203.
9 S.H. Hansen, P. Helboe, M. Thomsen and U. Lund, J. Chromatogr., 210 (1981) 453.
10 U. Lund and S.H. Hansen, J. Chromatogr., 161 (1978) 371.
11 S.N. Rasmussen, E.F. Hvidberg, S. Bondesen, S.H. Hansen, V. Binder and S. Halskov, submitted for publication.