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Note

Stability of 5-aminosalicylic acid and 5-acetylaminosalicylic acid in plasma

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Sulfasalazine, 5-aminosalicylic acid (5-ASA) and various derivatives of 5-ASA are frequently used in the treatment of ulcerative colitis and Crohn's disease, as described by Bondesen et al. [1] in their excellent review article. Sulfasalazine is cleaved by gut bacteria into sulfapyridine and 5-ASA, the latter being the therapeutic moiety of sulfasalazine [2,3]. After absorption 5-ASA is rapidly metabolized, mainly to 5-acetylaminosalicylic acid (5-AcASA).

Several high-performance liquid chromatographic (HPLC) assays have been developed [4–10] for the determination of 5-ASA and 5-AcASA in biological matrices. However, the stability of the analytes under storage conditions prior to analysis has not been reported in most publications. To our knowledge only Fischer et al. [7] mentioned stability data for 5-ASA and 5-AcASA in plasma. We report in this paper detailed stability data for both compounds in plasma.

EXPERIMENTAL

Materials

5-ASA was purchased from Bayer (Leverkusen, F.R.G.) and 5-AcASA was synthesized by reaction of 5-ASA with acetic anhydride (Merck, Darmstadt, F.R.G.) under alkaline conditions. Sodium bisulphite and ascorbic acid (both of analytical grade) were from Merck. Glutathione was acquired from Sigma (Deisenhofen, F.R.G.). All other chemicals used were of analytical grade.

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Preparation of samples

Stock solutions of 10.0 mmol/l 5-ASA in dilute hydrochloric acid (water titrated to pH 1.0 with 1 *M* hydrochloric acid) and of 5-AcASA in ethanol were both diluted with water to give working standard solutions of 100–1 μmol/l. Appropriate volumes of these working standard solutions were mixed with pooled human plasma to obtain concentrations of 10.0, 1.0 and 0.1 μmol/l for both 5-ASA and 5-AcASA. Each sample was divided into aliquots, which were stored at −20°C and −80°C, respectively.

To investigate the effects of antioxidants, the working standard solutions were mixed with pooled human plasma containing 20 μl per ml of a 10% aqueous solution of sodium bisulphite, ascorbic acid or glutathione, respectively. Again the samples containing 10.0, 1.0 and 0.1 μmol/l of both 5-ASA and 5-AcASA were divided into aliquots, which were stored at −20°C.

Furthermore, samples containing 1.0 μmol/l of both 5-ASA and 5-AcASA were prepared by mixing appropriate volumes of the corresponding 10 μmol/l working standard solutions with pooled human plasma, which either contained 0.2% ascorbic acid (as described above) or had been titrated to pH 5.0 with 1 *M* hydrochloric acid or to pH 9.0 with 1 *M* sodium hydroxide, respectively. Aliquots of these samples were incubated at 37°C.

Assay

All assays were performed according to the procedure described in detail elsewhere [9].

RESULTS AND DISCUSSION

During the development of an HPLC assay for 5-ASA and 5-AcASA [9], we performed orientating stability tests for these analytes, because corresponding information from the literature was very limited. To our knowledge only Fischer et al. [7] have reported that they did not detect a decrease in the content of both compounds in plasma after storage at room temperature and at +4°C for seven days. Our preliminary experiments indicated that 5-ASA decomposed considerably in plasma even within four weeks when stored under the usual conditions, i.e. −20°C. This led to a more intensive investigation of the stability of both 5-ASA and 5-AcASA in this matrix.

Mean concentrations of 5-ASA and 5-AcASA in spiked plasma before and after storage at −20°C and −80°C are listed in Table I. At −20°C, ca. 25–40% of 5-ASA was decomposed after six weeks and up to 60% after three months, whereas losses of 5-AcASA were either negligible or much less. However, at −80°C both compounds were stable for at least six months.

Addition of various antioxidants led to an even more pronounced decomposition of 5-ASA during six weeks storage at −20°C compared with corresponding samples without any additive (Table II). The highest losses were observed in samples containing 0.2% ascorbic acid, where only ca. 25% of the original 5-ASA concentration was found. No influence of the antioxidants was detected with regard to 5-AcASA concentrations.

TABLE I

CONCENTRATIONS OF 5-ASA AND 5-AcASA IN SPIKED PLASMA BEFORE AND AFTER STORAGE AT −20°C AND −80°C ($n=5$)

Time	Theoretical concentration (μmol/l)	Concentration of 5-ASA (μmol/l)		Concentration of 5-AcASA (μmol/l)	
		−20°C	−80°C	−20°C	−80°C
0	0.1	0.094		0.091	
	1.0	0.92		0.89	
	10.0	10.98		10.78	
Six weeks	0.1	0.072	0.087*	0.085	0.078
	1.0	0.76	0.97	0.96	0.95
	10.0	6.12	9.36	9.77	9.56
Three months	0.1	0.057	0.102	0.087	0.094
	1.0	0.55	0.91	0.84	0.89
	10.0	4.22	10.28	8.99	10.26
Six months	0.1	Not assayed	0.099	Not assayed	0.094
	1.0	Not assayed	0.90	Not assayed	0.91
	10.0	Not assayed	10.90	Not assayed	11.28

*$n=3$.

In order to investigate a possible pH effect, plasma samples containing 1.0 μmol/l of both 5-ASA and 5-AcASA were titrated to pH 5 and 9 and compared with untreated samples and also with those containing 0.2% ascorbic acid. Analyses were performed before and after incubation at 37°C for 24 h and four days. Small losses of 5-ASA could be detected in untreated samples and samples titrated to pH 9.0 only after four days incubation (Table III). Samples containing ascorbic acid showed ca. 65% decomposition of 5-ASA after 24 h and total decomposition after four days. At pH 5.0 no 5-ASA could be detected even after only 24 h incubation at 37°C, whereas 5-AcASA proved to be stable under these conditions. The decreased stability of 5-ASA in samples containing 0.2% ascorbic acid may therefore be explained by a slight change in the pH value.

The mechanism of the decomposition of 5-ASA remains unclear. Data from the literature indicate that 5-ASA can be oxidized by activated leukocytes [11] or can act as a radical scavenger [12]. Therefore it cannot be excluded that the decomposition of 5-ASA in plasma, even at −20°C, is caused by oxidative processes. No degradation products are detectable in the chromatograms under the assay conditions used when samples of pH 7 and above are worked up. However, when plasma (or aqueous) samples of pH 5 are processed, an additional peak of nearly the same retention time as 5-AcASA can be observed causing positively biased results for this compound (see Table III).

In conclusion, 5-ASA and 5-AcASA proved to be stable in plasma for at least six months only when the samples were stored at −80°C. We therefore recommend this storage condition when samples from clinical trials cannot be analysed immediately after collection.

TABLE II

CONCENTRATIONS OF 5-ASA AND 5-AcASA IN SPIKED PLASMA WITH AND WITHOUT ADDITION OF ANTIOXIDANTS BEFORE AND AFTER STORAGE AT −20 °C

Means of five analyses.

Time	Theoretical concentration (μmol/l)	Concentration of 5-ASA (μmol/l)				Concentration of 5-AcASA (μmol/l)			
		Without preservation	Sodium bisulphite (0.2%)	Ascorbic acid (0.2%)	Glutathione (0.2%)	Without preservation	Sodium bisulphite (0.2%)	Ascorbic acid (0.2%)	Glutathione (0.2%)
0	0.1	0.083*	0.092	0.085	0.090	0.087*	0.087	0.086	0.108
	1.0	0.88*	0.88	0.89	0.85	0.92*	0.83	0.84	0.89
	10.0	9.35*	10.33	10.14	10.15	10.04*	9.78	9.77	10.66
Six weeks	0.1	0.072	0.022	0.039	0.082	0.091	0.124	0.102	0.085
	1.0	0.71	0.41	0.24	0.58	0.93	0.98	0.99	0.90
	10.0	7.05	3.78	2.69	4.76	9.89	9.95	10.03	8.92

*Analysed five days after preparation of samples.

TABLE III

CONCENTRATIONS OF 5-ASA AND 5-AcASA IN PLASMA SPIKED WITH 1.0 μmol/l OF EACH COMPOUND AT VARIOUS pH VALUES OR ADDITIONALLY 0.2% ASCORBIC ACID BEFORE AND AFTER INCUBATION AT 37°C

Means of five analyses. N.D. = not detected; N.A. = not assayed.

Time	Concentration of 5-ASA (μmol/l)				Concentration of 5-AcASA (μmol/l)			
	Untreated	Ascorbic acid (0.2%)	pH 5.0 with hydrochloric acid	pH 9.0 with sodium hydroxide	Untreated	Ascorbic acid (0.2%)	pH 5.0 with hydrochloric acid	pH 9.0 with sodium hydroxide
0	0.96	0.91	0.82	0.96	0.94	0.89	0.87	0.94
24 h	0.94	0.36	N.D.	0.99	1.06	1.00	1.42	1.05
4 days	0.87	N.D.	N.A.	0.75	1.13	1.11	N.A.	1.10

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