International Journal of Pharmaceutics, 88 (1992) 177–187

IJP 02910

Identification of major degradation products of 5-aminosalicylic acid formed in aqueous solutions and in pharmaceuticals

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(Received 17 April 1992)
(Accepted 9 May 1992)

Key words: 5-Aminosalicylic acid; Mesalazine; Oxidative polymerization; Degradation product; NMR; Structure determination

Summary

The formation of four major degradation products of 5-aminosalicylic acid (5-ASA) in buffered solutions at pH 7.0 was demonstrated by gradient HPLC analysis The isolation and structural elucidation of the resulting degradation products showed that the degradation of 5-ASA led to the formation of polymeric species by oxidative self-coupling of 5-ASA moieties. These results indicate that the degradation of 5-ASA follows the same mechanism as observed for the autooxidation of 4-aminophenol and 1,4-phenylenediamine. Some of the identified degradation products were found in 5-ASA-containing pharmaceuticals, which had not been stored as prescribed, but in diffuse daylight for up to 2 years.

Introduction

5-Aminosalicylic acid (5-ASA) has been employed for several years in the treatment of chronic inflammatory bowel disease (Bondesen et al., 1987). Because of the 4-aminophenol structure and antioxidative properties of 5-ASA, the compound is expected to undergo oxidative degradation. Consequently, this may lead to stability problems in pharmaceutical preparations, however, in the literature, published data concerning the stability of 5-ASA are scarce.

Cendrowska et al. (1990) suggested that the degradation of 5-ASA in pharmaceuticals may be observed visually as a discolouration of the pharmaceuticals due to the degradation of 5-ASA to 4-aminophenol. This compound is known to autooxidize rapidly in aqueous solutions to give strongly coloured compounds, but the investigators failed to detect any 4-aminophenol in the pharmaceuticals and furthermore the coloured products were not identified.

The instability of 5-ASA in biological samples has also been reported. Brendel et al. (1988) observed a marked decrease in the concentration

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of 5-ASA in plasma during storage, however, Tjørnelund and Hansen (1991) recently revealed that the observed decrease was related to the conjugation of 5-ASA with glucose forming *N*-β-D-glucopyranosyl-5-ASA. A considerable decrease in concentration of 5-ASA in faeces treated with $HgCl_2$ at ambient temperature as well as at −4°C has also been reported (Van Hogezand et al., 1988), but degradation products of 5-ASA were not found in this study, and the cause of the decrease in concentration of 5-ASA in faeces remains as yet unknown.

The aim of the present work was to study the degradation of 5-ASA in aqueous solutions, thereby establishing the identity of the major degradation products of 5-ASA, which may cause the discolouration of pharmaceuticals.

Materials and Methods

Chemicals

5-Aminosalicylic acid (Mesalazine), Mesalazine tablets 500 mg and enema 1000 mg/100 ml

TABLE 1

^{1}H-NMR data of 5-ASA and its four major degradation products

Assignment	Proton signal δ ppm (m, J, Hz)		Assignment	Proton signal δ ppm (m, J, Hz)		
	5-ASA	Product I		Product II	Product III	Product IV
5-ASA ring			5-ASA rings			
H-6	7 28 (d, 2 76)		H-2′	7.65 (d, 2.8)	7.69 (d, 2.6)	7.45 (d, 2.6)
H-2′		7.59 (d, 2.64)	H-2″	7.63 (d, 2.8)	7 69 (d, 2.6)	7 74 (d, 2 6)
			H-2‴			7 63 (d, 2.5)
H-4	6.95 (dd, 2.84, 8 64)					
H-6′		7 17 (dd, 2 6, 8 6)	H-6′	7 19 (dd, 2 7, 8 6)	7 45 (dd, 2.5, 8.8)	7.23 (dd, 2.4, 8.7)
			H-6″	7 17 (dd, 2 6, 8 5)	7 45 (dd, 2.5, 8.8)	7.53 (dd, 2.6, 8.8)
H-3	6.71 (d, 8.68)		H-6‴			7.42 (dd, 2 6, 8.8)
H-5′		6.81 (d, 8.5)				
Imidazole ring			H-5′	6 75 (d, 8.6)	6 98 (d, 8.7)	6 89 (d, 8.8)
H-2		7 96 (s)	H-5″	6.74 (d, 8.6)	6.98 (d, 8.7)	7.03 (d, 8.8)
			H-5‴			6.98 (d, 8.8)
H-4		7 61 (d, 8 4)	Quinoid ring			
			H-3	5 54 (s)	5 58 (s)	5 63 (s)
H-5		6 75 (d, 8 5)	H-6	5 96 (s)	5 58 (s)	5.90 (s)
			OH		9.33 [a] (s)	9 24 [a] (s)
			OH		9.33 [a] (s)	
		NOE		NOE		
		H-4 ↔ H-5		H-5′ ↔ H-6′	H-5′ ↔ H-6′	H-5′ ↔ H-6′
		H-5′ ↔ H-6′		H-5″ ↔ H-6″		H-5″ ↔ H-6″
						H-5‴ ↔ H-6‴
		H-2 ↔ H-2′				
		H-2 ↔ H-6′		H-3 ↔ H-2′	H-3 ↔ H-2′	H-3 ↔ H-2′
					H-3 ↔ H-2″	H-3 ↔ H-6′
				H-6 ↔ H-2″	H-6 ↔ H-6′	H-6 ↔ H-2″
				H-6 ↔ H-6″	H-6 ↔ H-6″	H-6 ↔ H-2‴
						H-6 ↔ H-6″
						H-6 ↔ H-6‴

[a] Hydrogen exchange with D_2O

The assigned NMR data are shown together with the NOE observed between two protons. The protons were numbered according to the numbers given in Fig. 3.

were obtained from Ferring A/S (Vanløse, Denmark). Tetrabutylammonium bromide was purchased from Fluka Chemie AG (Buchs, Switzerland) and sodium hypochlorite from Janssen Chimica (Geel, Belgium). Na_2-EDTA, ammonium ferrosulphate (Fe(II)) and hexadeutero dimethylsulfoxide (DMSO-d_6) were obtained from Merck (Darmstadt,. Germany). All other chemicals were of analytical reagent grade.

Formation, isolation and structural characterization of four degradation products of 5-ASA

Product I Product I was isolated from a solution containing 10 mmol of 5-ASA, 1 mmol of EDTA and 1 mmol of ammonium ferrosulfate, dissolved in 1.0 l of 0.1 M ammonium hydrogen carbonate buffer pH 7.8. The solution was stored in diffuse daylight at room temperature for 1 month. The reaction mixture was then adjusted to pH 6.5 by the addition of acetic acid and centrifuged. A volume of 1.0 ml of acetic acid anhydride was added to the supernatant containing 5-ASA and product I as the major compounds. The *N*-acetylated 5-ASA was then removed from the supernatant by extraction with ethyl acetate. The remaining aqueous phase containing product I was roto-evaporated and redissolved in 50.0 ml of 0.1 M of ammonium acetate buffer pH 5.5. Product I was isolated by preparative HPLC using a mixture of methanol-0.2 M ammonium acetate buffer pH 5.5-water (22:10:68 v/v) as the eluent. The major peak was eluted with a capacity factor (k') of 2.3 and the remaining EDTA, 5-ASA and *N*-acetyl-5-ASA were eluted with k' values of 0.1, 0.2 and 1.1, respectively. The pooled fractions of the major peak were roto-evaporated to remove the organic solvent and then lyophilized. Using HPLC with UV diode array detection, the isolated product was found to be chromatographically pure and to be identical to the product observed in the parent ammonium hydrogen carbonate buffer.

Product I was characterized by ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2), elemental analysis (C 53.5%, H 3.1%, N 8.8%) and MS. The positive ion FAB spectrum (in glycerol) gave $[M + H]^+$ at m/z 315 and the negative ion FAB spectrum (in bis(2-hydroxyethyl)disulfide containing 8% of triethanolamine) gave $[M + 1]^-$ at m/z 313. A negative ion spectrum obtained under CI conditions with the desorption probe gave a prominent peak at m/z 539, probably due to thermal dimerization as a result of the loss of two molecules of CO_2.

TABLE 2

^{13}C-NMR data of 5-ASA and its four major degradation products

Assignment	δ (ppm)		Assignment	δ (ppm)		
	5-ASA I			II	III	IV
5-ASA ring			5-ASA rings			
COOH	171.9	171.3	COOH	171.0	171 3	171.7
			COOH	170.7	171.3	171.4
C-2	156.0		COOH			171.4
C-4′		155 3				
			C-4′	162.5	159.2	157.8
C-5	133.7		C-4″	161.5	159.2	158.8
C-1′		132.1	C-4‴			158 3
C-6	124.4		C-1′	128.6	131.8	126 0
C-2′		126 8	C-1″	128 5	131 8	132.4
			C-1‴			130.8
C-4	117 6					
C-6′		129 7	C-2′	126.1	128.8	130 3
			C-2″	126.1	128.8	129 3
C-3	117.6		C-2‴			129.6
C-5′		112.3				
			C-6′	125 8	125.6	118.1
C-1	115.3		C-6″	125.4	125.6	117 6
C-3′		118.9	C-6‴			117.7
Imidazole ring						
COOH		168.0	C-5′	120.2	118.0	113.7
			C-5″	120.1	118.0	113 4
C-2		144.9	C-5‴			113 3
C-3a		127.9	C-3′	117 0	114 1	123.9
			C-3″	116.8	114.1	123 3
C-4		122 6	C-3‴			123.3
			Quinoid ring			
C-5		116.3	C-1	no	179.4	179.8
			C-4	no	179 4	179 4
C-6		162.0				
			C-2	149.3	148.1	142.2
C-7		104.9	C-5	160 6	148.1	153 9
C-7a		137.1	C-3	96 8	94 6	96 0
			C-6	90 5	94.6	88.0

no, signals not observed.

Carbon atoms are numbered according to Fig. 3.

Product II This product was obtained by the oxidation of 10 mmol of 5-ASA, dissolved in 1.0 l of 0.1 M ammonium acetate buffer pH 6.5 with 10 mmol of hypochlorite. The hypochlorite was diluted with 0.1 M ammonium acetate buffer pH 6.5 before use. Product II separates immediately after mixing as a brown precipitate. The precipitate was isolated upon centrifugation and washed three times with 0.1 M ammonium acetate buffer pH 6.5 followed by centrifugation. The precipitate containing pure product II was lyophilized. The product was found to be chromatographically pure by analytical isocratic HPLC.

Product II was characterized by ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2), elemental analysis (C 58.8%, H 3.8%, N 10.5%) and MS. The ECNI mass spectrum showed a $[M]^-$ at m/z 409.

Product III This product was formed in a solution containing 10 mmol of 5-ASA, dissolved in 1.0 l of distilled water. The pH was adjusted to 7.5 by the addition of potassium hydroxide. After 4 weeks, the product was isolated as a humus-like precipitate by the addition of acetic acid until the pH reached 5.5 and subsequent centrifugation. The precipitate was dissolved in 0.1 M ammonium hydrogen carbonate buffer pH 7.8 and injected onto the preparative HPLC system. The major peak of this solution was eluted with a k' of 1.3 using a mixture of methanol-0.4 M ammonium hydrogen carbonate buffer pH 7.8-water (42:20:38 v/v) as the eluent. The pooled fractions of the major peak were evaporated and lyophilized. The final product was found to be chromatographically pure.

Product III was characterized by ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2), elemental analysis (C 58.1%, H 3.5%, N 6.9%) and MS. The ECNI mass spectrum showed a $[M]^-$ at m/z 410.

Product IV A fourth product was formed as the major product when 10 mmol of 5-ASA, 1 mmol of Fe(II) and 2 mmol of EDTA were dissolved in 1.0 l of 0.1 M ammonium hydrogen carbonate pH 7.5, and stored for several weeks. The solution was acidified after 4 weeks by the addition of acetic acid and the resulting precipitate was redissolved in 0.1 M of ammonium hydrogen carbonate buffer pH 7.8. A volume of 1.5 ml of this solution was injected onto the preparative HPLC system and the main peak was eluted with a k' of 1.7 using a mixture of methanol-0.4 M ammonium hydrogen carbonate buffer pH 7.8-water (42:20:38 v/v) as the eluent. The pooled fractions were evaporated and lyophilized, the product being found to be chromatographically pure by isocratic HPLC.

Product IV was characterized by ^{1}H-NMR (Table 1), ^{13}C-NMR (Table 2), elemental analysis (C 59.0%, H 3.5%, N 7.5%) and MS. The ECNI mass spectrum showed a $[M]^-$ at m/z 545.

Analysis of degradation products of 5-ASA in aqueous solution

The degradation of 5-ASA was preliminarily studied in two solutions, A and B, having the same pH (7.0) and equal initial concentrations of 5-ASA (10 mM). Solution A contained 5-ASA dissolved in 0.1 M potassium phosphate buffer, solution B being similar to solution A except for the addition of Fe(II) (1 mM) and EDTA (2 mM). The solutions were then stored for 6 weeks in diffuse daylight at ambient temperature until analysis by gradient HPLC. The undiluted sample was injected onto the column.

Analysis of degradation products of 5-ASA in pharmaceuticals

Mesalazine tablets (500 mg) and enemas (1000 mg/100 ml), stored under conditions not in accordance with those prescribed for the drug, but in diffuse daylight for up to 2 years and with a significant change in colour were selected for analysis by isocratic HPLC. The identity of the peaks found in tablets and enemas was verified by matching the UV spectra of the peaks with those of products II–IV. 10 tablets were suspended in 50.0 ml of 0.1 M potassium phosphate buffer pH 7.5. After 1 h, the suspension was centrifuged and the supernatant was analysed by analytical HPLC. Analysis of the enemas was performed after centrifugation of one entire enema (100.0 ml). The precipitate isolated from the centrifuged enema was redissolved in 10.0 ml of mobile phase before analysis by analytical HPLC. The supernatant of the enema was concentrated before injection by carrying out the following steps; 10 μl of 4 M HCl was added to 2.0 ml of

the supernatant. The mixture was centrifuged and the resulting precipitate was redissolved in 200 μl of mobile phase.

Stability of degradation products at pH 7.5

The stability of degradation products (0.1 mg/ml), dissolved in 0.2 M phosphate buffer pH 7.5 was examined by analytical isocratic HPLC. The samples were analysed immediately after mixing and after 24 h at room temperature.

Chromatography

Analytical HPLC The chromatographic equipment consisted of a Hewlett Packard (Palo Alto, CA, U.S.A.) Model 1090 liquid chromatograph equipped with a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector with a 20 μl loop and a Hewlett Packard 1090 diode array detector, operating at three wavelengths: 240, 310 and 410 nm. UV spectra were recorded from 240 to 500 nm during the chromatographic run at the onset and at the apex of the peaks. The separations were performed at 37°C using a Knauer (Berlin, Germany) column, 120 × 4.6 mm, packed with 5 μm particles of Spherisorb ODS-2 (Phase Separation Ltd, Queensferry, U.K.).

Gradient HPLC was carried out using two eluents, A and B, and the following linear gradient profile: 100% A, 0–5 min; 100–60% A, 5–45 min; 60–15% A, 45–75 min. Eluent A was a mixture of methanol-0.2 M potassium phosphate buffer pH 7.5-water (25:20:55 v/v) with 0.2% of tetrabutylammonium bromide added. Eluent B was 100% methanol. The flow rate was 1.0 ml/min.

Isocratic HPLC of products II–IV was performed using a mixture of methanol-tetrahydrofuran-0.2 M potassium phosphate buffer pH 7.5-water (34:6:20:40 v/v) with 0.2% of tetrabutylammonium bromide added as the eluent.

Isocratic HPLC of product I was conducted using a mixture of acetonitrile-0.1 M potassium citrate buffer pH 6.5-water (18:10:72 v/v) with 0.2% of tetrabutylammonium bromide added as the eluent. The flow rate was 1.5 ml/min.

Preparative HPLC This was performed using a Merck Hitachi 655-A12 liquid chromatograph (Darmstadt, Germany) equipped with a model 655 variable-wavelength UV monitor operating at 240 nm. The major degradation products of 5-ASA were chromatographed on Polygosil C-18 (10 μm particles; Macherey-Nagel, Düren, Germany) column (250 × 16 mm), using a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector with a loop of 1.5 ml and a flow rate of 10 ml/min.

NMR

^{1}H-NMR and ^{13}C-NMR spectra of 5-ASA and its four major degradation products were obtained at ambient temperature using a Bruker AMX 400 WB spectrometer (Rheinstetten, Germany). The samples were dissolved in DMSO-d_6.

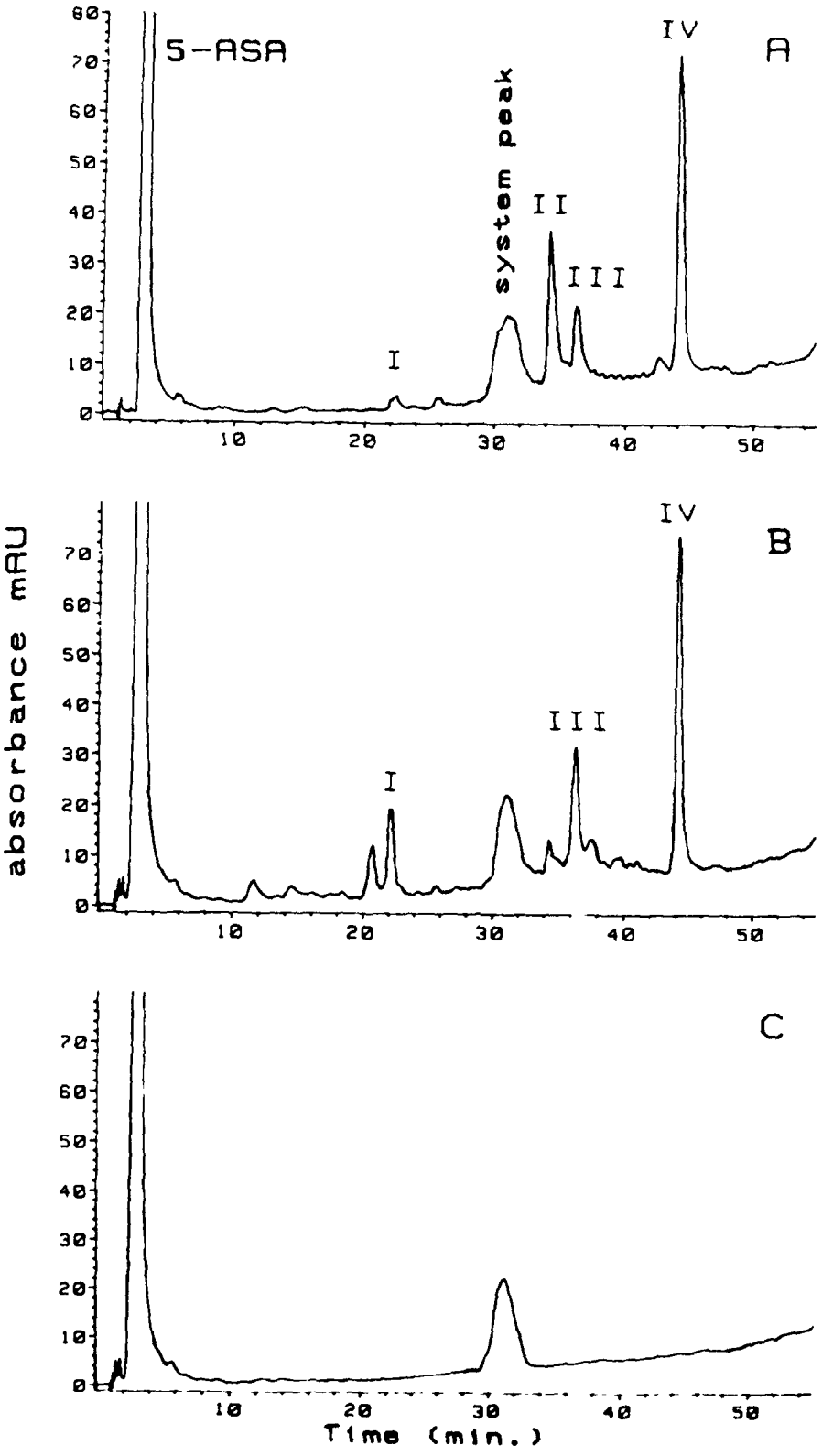
Nuclear Overhauser Effects (NOE) were determined using two-dimensional spectroscopy (NOESY). The experiments were carried out in the phase-sensitive mode using a microprogram from the Bruker library. The following experimental parameters were employed for product II–IV: ambient temperature; relaxation delay, 3.0 s; mixing time, 550 ms; sweep width, 4000 Hz; increments in F1, 512; data points in F2, 1024; data matrix of two-dimensional FT, 2048 × 2048; phase-shifted squared sinebell in both directions, SSB = 2. The parameters used for product I were as follows: temperature, 308 K; relaxation delay, 3.5 s; mixing time, 550 ms; sweep width, 1201.92 Hz; increments in F1, 128; points in F2, 256; data matrix after two-dimensional FT, 1024 × 1024; phase-shifted squared sinebell in both directions, SSB = 4. A CH correlated spectrum was recorded for product I, optimized for a $^{1}J_{CH}$ coupling of 140 Hz, an exponential linebroadening in F2 of 5 Hz and a squared sinebell in F1 (SSB = 2). The final number of data points in F1 and F2 was 1024 and 4096, respectively.

Mass spectrometry (MS)

Mass spectra were taken on a Jeol JMS-AX505W mass spectrometer (Jeol Ltd, Tokyo, Japan) operating at 3 kV accelerating voltage and 10 kV conversion dynode voltage. Data processing was carried out with the Complement software running on an HP Apollo workstation.

Fast Atom Bombardment (FAB) spectra were obtained using xenon at 6 kV.

Electron-capture negative ion (ECNI) mass


5-ASA
A
B
C
I
II
III
IV
system peak
absorbance mAU
Time (min.)

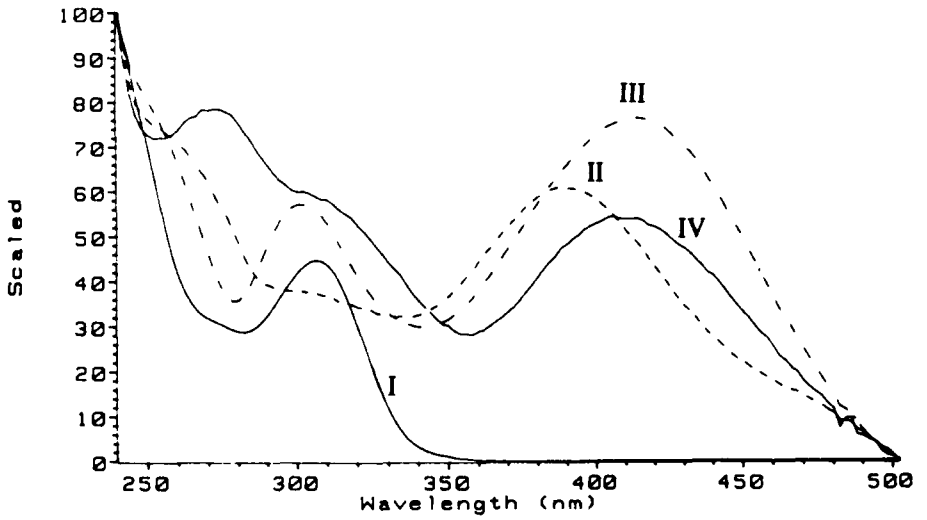


Fig 2 UV spectra of the four major degradation products (I–IV) of 5-ASA. The structures of the products are illustrated in Fig. 3.

spectra were recorded under normal isobutane CI conditions (source temperature, approx. 200°C; emission current, 100 μA; electron energy, 250 eV). The desorption chemical ionization probe was used for introducing the sample. The sample was loaded onto the platinum filament as a solution in DMSO. After evaporation of the DMSO in the mass spectrometer, filament heating at a rate of 1 A/min was initiated.

Results and Discussion

Formation of degradation products of 5-ASA in aqueous solutions at pH 7.0

In a preliminary investigation, the degradation of 5-ASA was studied in a phosphate-buffered solution (solution A). In this medium, ascorbate has been reported to undergo oxidative degradation (Buettner, 1986) and the rate of 'autooxidation' was shown to increase in the presence of iron-EDTA chelates. Therefore, the degradation of 5-ASA was also studied in solutions with Fe(II)-EDTA added (solution B). Storage of solutions A and B for 6 weeks resulted in the formation of several products, which were separated via gradient elution by HPLC and detected using the UV diode array (Fig. 1A and B). Since the degradation products were of unknown identity, a gradient HPLC method was chosen in an attempt to chromatograph degradation products with very different physico-chemical properties.

The resulting chromatograms of solutions A and B showed the elution of three major degradation products from 35 to 45 min and a minor degradation product after 21 min. The UV spectra recorded for products II–IV (Fig. 2) indicate the identity of highly coloured compounds consistent with the reported changes in colour of pharmaceuticals stored for several weeks (Cendrowska et al., 1990).

Assuming a similar value for the molar absorbance of the degradation products at 240 nm, the major degradation product formed in solutions A and B (Fig. 1) was product IV. Product I was only present in appreciable amounts in solu-

Fig 1 Separation of the degradation products of 5-ASA formed in aqueous solutions Chromatogram A shows the elution of solution A, containing 10 mM 5-ASA dissolved in 0.1 M phosphate buffer, pH 7.0, stored for 6 weeks and chromatogram B shows the elution of solution B after storage at 6 weeks. Solution B contained 10 mM 5-ASA dissolved in 0.1 M phosphate buffer, pH 7.0, with 1 mM Fe(II) and 2 mM EDTA added. Chromatogram C shows the elution profile of a freshly prepared solution of 5-ASA (10 mM). I–IV refer to products I–IV, respectively.

tions with Fe(II)-EDTA added. The degradation of 5-ASA was accelerated in the presence of Fe(II)-EDTA, which suggested the mechanism to be oxidative degradation of 5-ASA.

Thus, in an attempt to synthesize the degradation products, the direct oxidation of 5-ASA with a suitable oxidant would be useful. Dallegri et al. (1990) and Tamai et al. (1991) have reported 5-ASA to exhibit hypochlorite scavenging activity in biological systems, thereby indicating that hypochlorite is able to oxidize 5-ASA. Therefore, the chemical oxidation of 5-ASA in our work was carried out with hypochlorite as the oxidant. 5-ASA reacted rapidly with hypochlorite at pH 6.5 and pure product II separated spontaneously from the reaction mixture as a brown precipitate. The redissolved precipitate was shown to elute with a retention time and UV spectrum corresponding to product II, which was shown to be formed in aqueous solutions of 5-ASA stored for several weeks.

However, products I, III and IV were not obtainable in pure form by oxidation of 5-ASA. Preliminary studies of the autooxidation of 5-ASA in aqueous solutions showed that products I and IV were formed as the major degradation products when Fe(II)-EDTA was added to buffered solutions of 5-ASA and other investigations showed that product III was the major degradation product formed in solutions of 5-ASA in

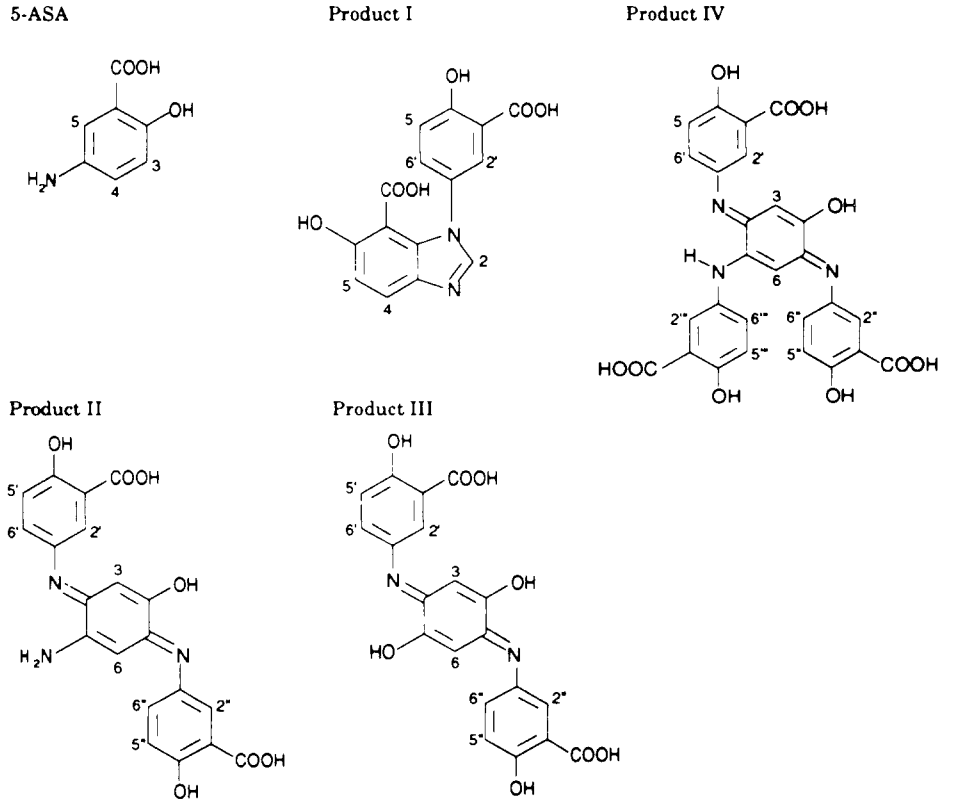


Fig. 3. Structures of 5-ASA and its four degradation products (I) 1-(3-Carboxy-4-hydroxyphenyl)-7-carboxy-6-hydroxybenzimidazole; (II) 5-amino-2-hydroxy-*N*,*N*′-bis(3-carboxy-4-hydroxyphenyl)-1,4-benzoquinone diimine, (III) 2,5-dihydroxy-*N*,*N*′-bis(3-carboxy-4-hydroxyphenyl)-1,4-benzoquinone diimine; (IV) 5-(3-carboxy-4-hydroxyphenylamino)-2-hydroxy-*N*,*N*′-bis(3-carboxy-4-hydroxyphenyl)-1,4-benzoquinone diimine

distilled water. Consequently, the products were isolated in pure form by preparative HPLC from solutions containing autooxidized 5-ASA.

Structure determination of major degradation products

Elucidation of the structure of the isolated products was mainly based on the data obtained from ^{1}H-NMR (Table 1) and the determination of NOE (Table 1). The results from ^{13}C-NMR (Table 2), mass spectrometry and elemental analysis were in support of the proposed structures of the major degradation products of 5-ASA which were thus established (Fig. 3). The structures of products II–IV are shown in the form of the *p*-quinone diimine isomer, despite the fact that some of the products may prefer other isomer forms, e.g., product IV may exist as the *p*-quinone diimine, *o*-quinone diimine or the *p*-quinone monoimine isomer.

The following observations were important for the determination of the structures. In general, the characteristic signals normally observed in the ^{1}H-NMR spectra (Table 1) and the ^{13}C-NMR spectra (Table 2) of 5-ASA were also present in the spectra of the four products.

Thus, in the ^{1}H-NMR spectra of product I it was possible to identify one intact 5-ASA moiety as well as another 5-ASA moiety substituted in position 6. In addition to these two 5-ASA moieties, a proton at δ 7.96 ppm and a carbon atom at δ 144.9 ppm were observed. The correlated CH spectrum (data not shown) showed that these atoms were linked. The observed δ values of the C atom and H atom may indicate a C-H group linked to oxygen (H-C = O) or nitrogen (H-C = N). However, the observed NOE between the proton at δ 7.96 ppm and the two aromatic protons in the *ortho* position to the amino group of the intact 5-ASA molecule (H-2′ and H-6′) indicates that the C-H group is linked to the amino group of the intact 5-ASA moiety. This is consistent with the presence of a H-C = N group in the structure. FAB-MS demonstrated a molecular mass of 314 Da, which correlates with the benzimidazole structure depicted in Fig. 3.

The NMR spectra of products II–IV indicated compounds with very similar structures. Two intact 5-ASA moieties in product II as well as III and three intact 5-ASA moieties in product IV were readily identified. The products also contained an additional ring system with two non-coupled protons having δ values in the range of 5.5–6.0 ppm, indicative of protons on alkenes or quinoid carbon. The ^{13}C-NMR data further supported the identity of a quinoid ring with two protons. The NOESY experiment revealed NOEs between quinoid protons and protons in the *ortho* position of the amino group of the intact 5-ASA moiety (H-2 and H-6). Consequently, these results indicate the existence of structures with a quinoid nature and structures with intact 5-ASA moieties linked to the quinoid ring via the amino group of the 5-ASA moieties. The peaks observed in the mass spectra at m/z 409, 410 and 547 for products II, III and IV, respectively, provide support for the structures given in Fig. 3.

Identification of degradation products in pharmaceuticals

To verify whether the isolated degradation products are formed in pharmaceuticals containing 5-ASA, selected tablets and enemas stored for 2 years in diffuse daylight were analysed for the presence of products II–IV as shown in Fig. 4.

The coloured degradation products were found in both tablets (Fig. 4D) and enemas (Fig. 4B and C), in addition to other compounds with UV absorbance at 410 nm. Thus, the compounds causing the discolouration of pharmaceuticals are mainly identical to the degradation products of 5-ASA found in this study. The total content of products II–IV in tablet preparations and in enemas was estimated to be 0.3 and 0.2%, respectively, of the initial concentration of 5-ASA.

Stability of the degradation products

Products I, III and IV were stable for 24 h in 0.1 M potassium phosphate buffer pH 7.5. After 24 h about 15% of the initial content of product II had been transformed to product III.

Degradation mechanism of 5-ASA

The nature of the degradation products of 5-ASA identified indicates that 5-ASA undergoes

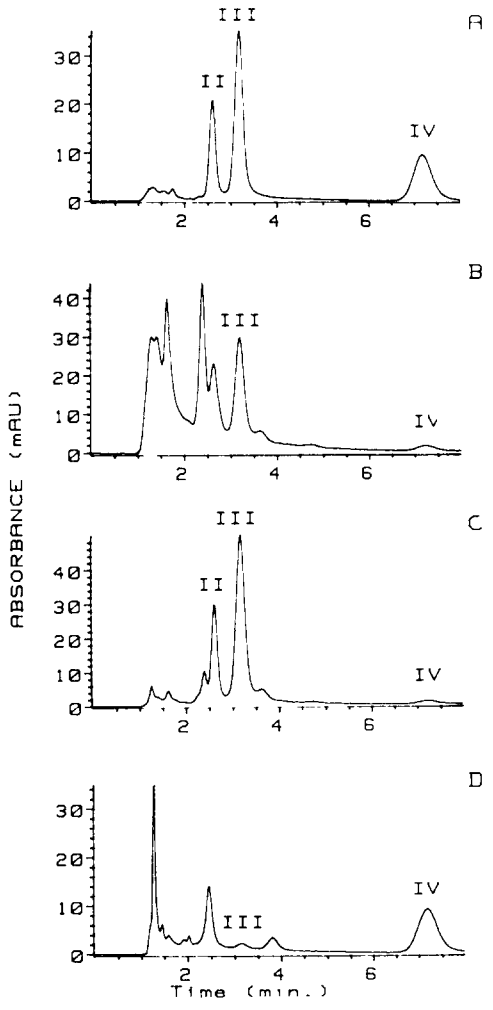


Fig. 4 Analysis of coloured products in Mesalazine tablets and enemas. The following chromatograms are shown (A) standard samples of products II–IV; (B) the concentrated supernatant of the enema; (C) the precipitate of the enema and (D) the centrifuged suspension of tablets.

oxidative degradation with the formation of 5-ASA polymers. This mechanism of degradation involves the initial oxidation of 5-ASA to the 2-carboxy-1,4-benzoquinone monoimine and the further coupling of the quinone monoimine with the nucleophilic amino group in 5-ASA. This process is consistent with the autooxidation mechanism observed for 1,4-phenylenediamine (Corbett 1969, 1972) and 4-aminophenol (Brown and Corbett, 1979). The oxidative transformation of these compounds resulted in the formation of the trimeric species 2,5-diamino-*N*,*N'*-bis(4-aminophenyl)-1,4-benzoquinone diimine and 2-hydroxy-5-amino-*N*,*N'*-bis(4-hydroxyphenyl)-1,4-benzoquinone diimine, respectively, however, the formation of a dimeric or a tetrameric species was not reported in those works.

In contrast, a dimeric species of 5-ASA was found in detectable amounts in phosphate-buffered solutions of 5-ASA. The dimeric species of 5-ASA isolated in this work was determined to have a benzimidazole structure, which reduces its ability to undergo further oxidative transformation into a trimeric species.

A tetrameric species of 5-ASA was found in considerable amounts in solutions having a neutral pH value. The formation of this tetramer may have involved a Schiff base reaction of the *p*-quinone isomer of product II with a 5-ASA moiety.

On the basis of the present results, the degradation of 5-ASA may initially involve the formation of a dimeric species of 5-ASA, which is transformed into a benzimidazole structure (product I) by reaction with a suitable acid. Alternatively, the dimeric species is oxidatively coupled with a 5-ASA moiety giving the trimeric species (product II). This product is unstable and is hydrolysed to product III, which is further transformed to product IV by a Schiff base reaction with a 5-ASA moiety.

The present results establish that 5-ASA is degraded oxidatively at neutral pH to highly coloured compounds, which may also be found in 5-ASA-containing pharmaceuticals when storage is not under the prescribed conditions. The degradation was found to be accelerated in the presence of metal chelates. However, elucidation of the factors contributing to the instability of 5-ASA requires further investigation of the influence of oxygen, light and metals on the instability of 5-ASA.

Acknowledgements

The Alfred Benzon Foundation, the Technology Council and the PharmaBiotec Research Center are gratefully acknowledged for donating

the NMR apparatus. Niels A. Midgaard is thanked for skilful technical assistance.

References

Bondesen, S, Rasmussen, S.N, Rask-Madsen, J., Nielsen, O.H., Lauritzen, K., Binder, V., Hansen, S.H. and Hvidberg, E.F, 5-Aminosalicylic acid in the treatment of inflammatory bowel disease. *Acta Med. Scand*, 221 (1987) 227–230.

Brendel, E., Meineke, I., Stüwe E. and Osterwald, H., Stability of 5-aminosalicylic acid and 5-acetaminosalicylic acid in plasma. *J Chromatogr.*, 32 (1988) 358–362.

Brown, K C and Corbett, J.F., Oxidation of *p*-aminophenol in aqueous solution. *J. Chem. Soc Perkin II*, 1 (1979) 308–311

Buetnner, G.R., Ascorbate autooxidation in the presence of iron and copper chelates. *Free Radical Res. Commun.*, 1, 6 (1986) 349–353.

Cendrowska, I., Drewnowska M., Grzeszkiewicz A. and Butkiewicz K., Investigation of the stability of 5-aminosalicylic acid in tablets and suppositories by high-performance liquid chromatography. *J. Chromatogr.*, 509 (1990) 195–199.

Corbett, J.F., Benzoquinoneimines. III. The structure of Bandrowski's base. *J Soc Dyer. Colour* 85 (1969) 71–73.

Corbett, J.F., The autooxidation of *p*-phenylenediamine. *J. Soc Cosm. Chem*, (1972) 683–693

Dallegri, F., Ottonello, L., Ballestrero, A., Bogliolo, F., Ferrando, F. and Patrone, F., Cytoprotection against neutrophil derived hypochlorous acid. a potential mechanism for the therapeutic action of 5-aminosalicylic acid in ulcerative colitis. *Gut*, 31 (1990) 184–186.

Tamai, H., Kachur, J.F., Grisham, M.B. and Gaginella, T S., Scavenging effect of 5-aminosalicylic acid on neutrophil-derived oxidants *Biochem Pharmacol.*, 41 (1991) 1001–1006

Tjørnelund, J.T and Hansen, S H., Stability of 5-aminosalicylic acid and its metabolites in plasma at −20°C. Formation of *N*-β-D-glucopyranosyl-5-aminosalicylic acid. *J Chromatogr. Biomed Appl.*, 570 (1991) 224–228.

Van Hogezand, R.A., Schaik, A., Van Hess, P.A.M. and Van Tongeren, J.H.M, Stability of disodium azodisalicylate (olsalazine) and metabolites in urine and faeces stored at different temperatures. *Eur J. Drug Metab Pharmacokinet.*, 13 (1988) 261–265