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IDENTIFICATION OF OXIDATION PRODUCTS OF 5-AMINOSALICYLIC ACID IN FAECES AND THE STUDY OF THEIR FORMATION *IN VITRO*

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Abstract—The formation of three oxidant-derived products of 5-aminosalicylic acid (5-ASA) *in vivo* was demonstrated in patients with active ulcerative colitis as well as in healthy subjects. The products were isolated from faeces by preparative HPLC and their chemical structures were found to be oxidation products of 5-ASA using ^{1}H-NMR spectroscopy and mass spectrometry. Reactions carried out *in vitro* between 5-ASA and oxidants suggested to be present in the inflamed bowel verified that the hypochlorite-mediated oxidation of 5-ASA as well as the haemoglobin-catalysed H_2O_2-dependent oxidation of 5-ASA resulted in the formation of a single oxidation product of 5-ASA. This product was similar to, but not identical to any of the products identified in faeces from patients receiving 5-ASA. Oxygen radical-mediated oxidation of 5-ASA gave several products, different from the products isolated. Finally, it was verified that the products formed *in vivo* are not formed as a result of autooxidation of 5-ASA either in faeces extract or in pharmaceuticals.

5-Aminosalicylic acid (5-ASA ††) is an agent widely used in the treatment of inflammatory bowel diseases (IBD) [1]. The 4-aminophenol structure of 5-ASA possesses antioxidant properties [2] and consequently the compound may be susceptible to oxidative transformation *in vivo*. Metabolic studies of 5-ASA in humans have not so far revealed the formation of oxidant-mediated metabolites of 5-ASA [3, 4]. However, the existence of metabolic pathways alternative to the N-acetylation is suggested as a consequence of the low recoveries of 5-ASA found after administration of 5-ASA to healthy subjects [5, 6].

A theory that free oxygen radicals play an important role in the pathogenesis of IBD has been proposed [7]. Accordingly, an oxidative transformation of 5-ASA would be expected to occur in patients with active IBD due to reactive oxidants such as hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$) and hypoclorite anions (OCl^-), which are released by neutrophils present in the inflamed bowel [8, 9]. 5-ASA has been shown to be a potential scavenger of these oxidants [10–12] *in vitro*, but the identities of possible 5-ASA-derived products formed by reaction with the neutrophil oxidants have not been elucidated. A ferryl radical of haemoglobin [Hb(IV)$^{\cdot}$] shown previously to oxidize 5-ASA *in vitro* is also suggested to be present in the inflamed bowel [13].

Evidence of 5-ASA acting as an antioxidant *in vivo* has been indicated by Ahnfelt-Rønne *et al.* [14]. In patients with active ulcerative colitis (UC) they found reduced mucosal lipid peroxidation, disappearance of oxygen radical-mediated metabolites of 5-ASA and improvement in disease status following treatment with salazosulphapyridine, which is a compound capable of delivering free 5-ASA to the large bowel. However, the chemical structures of the oxygen radical-mediated metabolites of 5-ASA were not elucidated.

Recently, the autooxidation of 5-ASA has been studied in our laboratory [15]. This study revealed that 5-ASA is susceptible to autooxidative transformation resulting in polymeric species of 5-ASA, from which a trimeric species could be synthesized by oxidation of 5-ASA with hypochlorite.

Therefore, the aim of the present study was to investigate whether any oxidative transformation of 5-ASA *in vivo* results in the formation of products identical to the polymeric species and to investigate further whether $OH^{\cdot}$, OCl^- or Hb(IV)$^{\cdot}$ can mediate the formation of these polymeric species from 5-ASA.

MATERIALS AND METHODS

Chemicals

5-ASA (Mesalazine), Mesalazine tablets (500 mg) and Mesalazine enemas (1 g/100 mL) were provided by Ferring A/S (Vanløse, Denmark). The phar-

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†† Abbreviations: 5-ASA, 5-aminosalicylic acid; IBD, inflammatory bowel diseases; UC, ulcerative colitis; FAB, fast atom bombardment; ECNI, electron-capture negative ion.

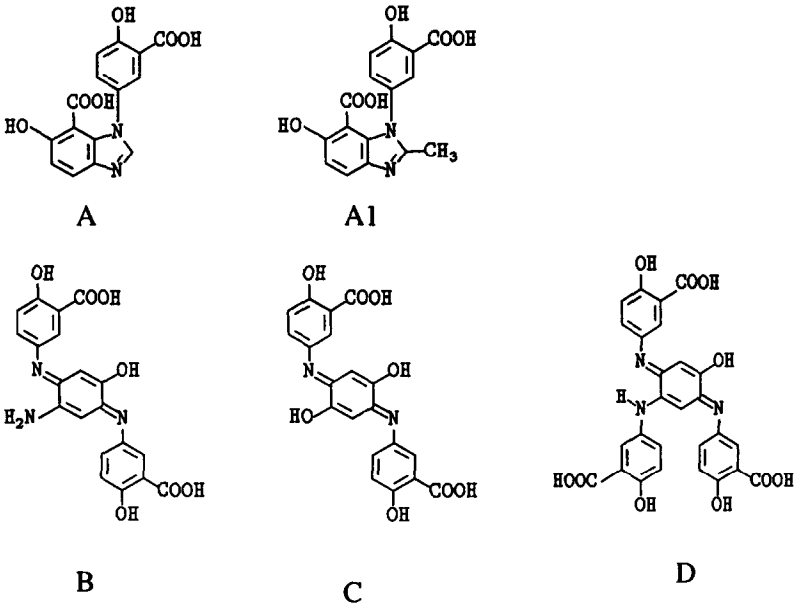

Fig. 1. Structures of known oxidation products of 5-ASA, named reference sample A, B, C and D, and the products identified in faeces extracts in the present study, named metabolites A, A1 and D. The chemical names of the metabolites are 1-(3-carboxy-4-hydroxy-phenyl)-7-carboxy-6-hydroxy-benzimidazol (A), 1-(3-carboxy-4-hydroxy-phenyl)-7-carboxy-6-hydroxy-2-methyl-benzimidazol (A1) and 5-(3-carboxy-4-hydroxy-phenylamino)-2-hydroxy-*N,N'*-bis-(3-carboxy-4-hydroxy-phenyl)-1,4-benzoquinone diimine (D). The chemical names of products B and C are 5-amino-2-hydroxy-*N,N'*-bis-(3-carboxy-4-hydroxy-phenyl)-1,4-benzoquinone diimine and 2,5-dihydroxy-*NN'*-bis-(3-carboxy-4-hydroxy-phenyl)-1,4-benzoquinone diimine, respectively.

maceuticals had been stored in the dark for 2 years in closed containers. Tetrabutylammonium bromide was purchased from Fluka Chemie AG (Buchs, Switzerland). Disodium salt of EDTA, ferroammonium sulphate [Fe(II)] and hydrogen peroxide 30% (H_2O_2) were purchased from Merck (Darmstadt, Germany). Sodium hypochlorite (NaOCl) was obtained from Janssen Chimica (Geel, Belgium). The concentrations of H_2O_2 and OCl^- were determined by iodometric titration. Reduced haemoglobin [Hb(III)] and salicyluric acid were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Salicyclic acid and gentisic acid were of pharmacopoiea quality. Other chemicals were of analytical reagent grade.

Syntheses of polymeric products of 5-ASA (reference standards), named A, B, C and D (Fig. 1), were performed in phosphate-buffered solutions of 5-ASA (8 mM) with Fe(II) ions (1 mM) and EDTA (2 mM) added. After storage for 8 weeks in diffuse daylight, the products were isolated in pure forms as described earlier [15]. *N*-Isobutyryl-5-ASA (internal standard) was synthesized according to Ref. 4.

Subjects

Eight patients with active UC treated with Mesalazine (5-ASA) were consecutively included in the study. Mesalazine was given as tablets or as enemas at a daily dose ranging between 0.5 and 4 g. Ten healthy subjects receiving Mesalazine tablets at a daily dose of 4 g were also included in the study. All subjects had been given written and oral information according to instructions provided by the local ethics committee. Subjects receiving steroids and salicylates other than 5-ASA were exluded from the study.

Collection and preparation of stools. Urine and faeces were collected separately during 24 hr and stored at 20° until used. The faeces samples obtained from patients were collected in 400 mL of methanol and faeces samples obtained from healthy subjects were suspended in 0.1 M of potassium phosphate buffer pH 6.0–methanol (50:50, v/v) in a volume corresponding to 4 mL/g faeces.

Screening of oxidant-derived products of 5-ASA

Gradient HPLC with UV diode array detection at three wavelengths 240, 310 and 410 was used in order to detect oxidant-mediated products of 5-ASA in biological fluids from subjects receiving 5-ASA, in blank faeces samples spiked with 5-ASA and in pharmaceuticals. Peaks of interest were evaluated by comparing chromatograms of biological samples with chromatograms of known oxidation products of 5-ASA or chromatograms of synthetic reaction mixtures of 5-ASA and oxidants.

UV spectra were recorded from 240 to 500 nm during the chromatographic run and were used as a preliminary identification of chromatographic peaks. The chromatographic system consisted of a Hewlett Packard (Palo Alto, CA, U.S.A.) 1090 liquid

chromatograph equipped with a column oven, a diode array detector and a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector with a 20-μL loop.

The separations were performed at 37° using a Knauer column, 120 × 4.6 mm (Berlin, Germany) packed with Spherisorb ODS-2, 5 μm particles from Phase Separation Ltd (Queensferry, U.K.). One hundred per cent of eluent A was applied for 0–5 min followed by a linear gradient from 100–50% eluent A within 5–55 min. Eluent A was a mixture of methanol–0.2 M potassium phosphate buffer pH 7.5–water (18:20:62 v/v) with 0.2% of tetrabutylammonium bromide added, and eluent B was 100% methanol.

Isolation of three oxidant-derived products (A, A1 and D) of 5-ASA from faeces

Treatment of sample. A faeces sample collected in methanol was obtained from a patient (patient 1, Table 2) with active UC and treated with 4 g of Mesalazine. After careful mixing, the sample was centrifuged and the supernatant was roto-evaporated to remove organic solvent. The remaining sample was mixed with 400 mL of 0.1 M ammonium hydrogencarbonate buffer pH 7.8 and centrifuged. Products A and A1 were isolated from a 200 mL aliquot of the resulting supernatant and product D was isolated from the remaining solution.

Pre-concentration of products A and A1. Products A and A1 were pre-concentrated using a Pharmacia (Uppsala, Sweden) column 400 × 26 mm i.d. packed with 0.3–1.0 mm particles of Amberlite XAD-4 (Serva Chrom, Heidelberg, Germany). A flow rate of 5.0 mL/min was established by a Pharmacia peristaltic pump and a linear gradient was applied using a Pharmacia Ultrograd model 11300 gradient mixer. The gradient was performed from 100% eluent A (0.1 M ammonium acetate buffer pH 5.5) to 100% eluent B (methanol) during a period of 15 hr. The column was pre-conditioned with eluent A and after application of the sample, eluent A was further passed through the column for 1 hr before the gradient was started. Fractions of 25 mL were collected and tested by isocratic HPLC for the presence of products A and A1. Fractions 52 to 74 were pooled and concentrated by roto-evaporation. The concentrated solution was diluted with 0.1 M ammonium acetate buffer pH 5.5 and further purified by preparative HPLC.

Pre-concentration of product D. Product D was pre-concentrated from the faeces sample by the same method as described for products A and A1 except that eluent A was 0.1 M ammonium hydrogencarbonate buffer pH 7.8. Product D was present in fractions 91–109, which were pooled, roto-evaporated and finally freeze-dried. The freeze-dried sample was redissolved in 0.1 M ammonium hydrogencarbonate buffer pH 7.8 and subsequently purified by preparative HPLC.

Preparative HPLC. Products A, A1 and D were isolated in pure forms by preparative HPLC using a Merck (Darmstadt, Germany) Hitachi 655-A12 liquid chromatograph, a Rheodyne 7125 injector with a 1.5-mL loop and a Merck variable UV monitor 655 operated at 240 nm. The products were separated on a Knauer column (250 × 16 mm) packed with Polygosil-C-18 (Macherey-nagel, Düren, Germany) using a flow rate of 10.0 mL/min. Products A and A1 were eluted with a capacity factor (k') of 2.55 and 1.72, respectively, using an eluent consisting of water–methanol–tetrahydrofuran–0.2 M acetate buffer pH 5.5 (76:12:2:10, v/v). Product A1 was shown to be chromatographically pure by analytical HPLC and product A was shown to contain minor amounts of product A1.

Product D was eluted with a k' of 1.77 using an eluent consisting of methanol–water–0.2 M ammonium hydrogencarbonate buffer pH 7.8 (42:38:20 v/v). Product D was shown to be chromatographically pure by analytical HPLC.

Structure characterization of oxidant-derived products of 5-ASA isolated from faeces

^{1}H-NMR spectra of products A and A1 dissolved in dimethyl sulphoxide-d_6 were obtained with a Bruker AMX 400 WB spectrometer (Rheinstetten, Germany) using identical experimental parameters as those described in Ref. 15.

Fast atom bombardment (FAB) mass spectra of products A and A1, and electron-capture negative ion (ECNI) mass spectra (ECNI-MS) of product D were obtained on a Jeol JMS-AX505W mass spectrometer (Jeol Ltd, Tokyo, Japan) as described in Ref. 15.

Reaction of 5-ASA with various oxidants in vitro

Oxidation products obtained by the reaction of 5-ASA and various oxidants were studied by gradient HPLC as described above. The reactions outlined below were performed.

Hypochlorite-mediated oxidation of 5-ASA. This reaction was carried out by adding 200 μL of sodium hypochlorite (various concentrations from 20 to 160 mM) to 800 μL of a solution of 1 mM of 5-ASA in 0.1 M potassium phosphate buffer pH 7.4. After 10 min, the reaction mixtures were analysed by gradient HPLC.

Oxygen radical-mediated oxidation of 5-ASA. This reaction was carried out using the Fenton reaction [16]. The reaction mixture was made according to recommendations given in Ref. 17. Thus, a stock solution of 4 mM of Fe(II) ions and 6 mM of EDTA was prepared in distilled water just before use. A solution of 10 mM of 5-ASA was made in 0.2 M potassium phosphate buffer pH 7.4, and 1.6 M H_2O_2 was prepared in distilled water. The oxygen radical-mediated oxidation of 5-ASA was carried out by adding 100 μL of the solution of Fe(II)–EDTA to 800 μL of the solution of 5-ASA, and subsequently adding 100 μL of the solution of H_2O_2. The reaction mixture was analysed by gradient HPLC after 10 min, and 1, 4 and 24 hr of reaction.

Haemoglobin-catalysed H_2O_2-dependent oxidation of 5-ASA. This reaction was carried out as described in Ref. 13. A solution of 2 mL of 0.1 M phosphate buffer pH 7.0 containing sodium chloride (1 M), 5-ASA (2 mM), H_2O_2 (1 mM) and Hb(III) (500 μM) was incubated at 37° for 15 min. Following the incubation period, the haemoglobin was precipitated by the addition of 8 mL of methanol to the reaction mixture. The sample was then centrifuged and the supernatant was analysed by gradient HPLC.

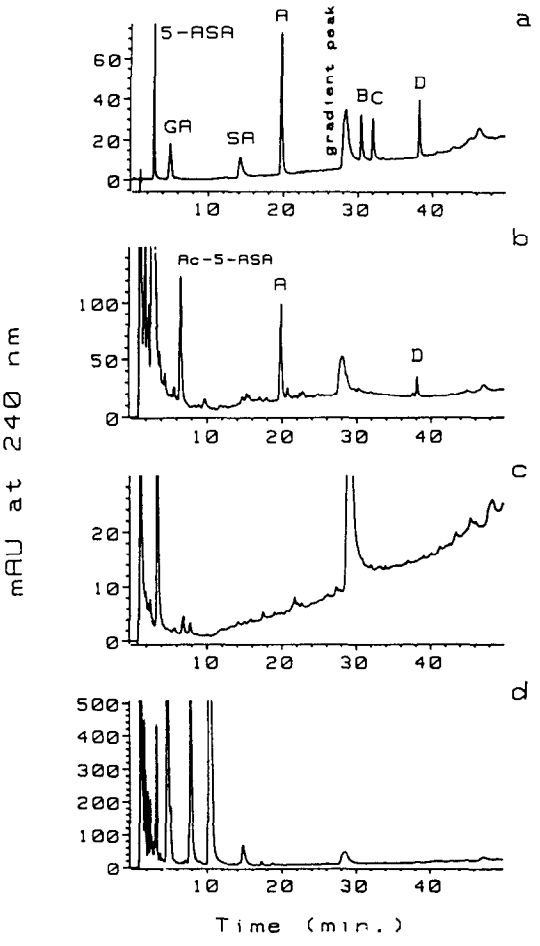

Fig. 2. Screening for oxidant-derived metabolites in biological samples by gradient HPLC. Chromatogram of four known oxidation products of 5-ASA (UV spectra are shown in Ref. 15) as well as gentisic acid (GA), salicylic acid (SA) and 5-ASA. (a) Chromatographic analysis of a faeces sample from a patient treated with 5-ASA, demonstrating the presence of two peaks, A and D, corresponding to reference samples A and D. (b) Blank faeces sample spiked with 1 g of 5-ASA. (c) Chromatogram of a urine sample from the same patient as chromatogram b. (d) A peak around 29 min is present due to the gradient elution.

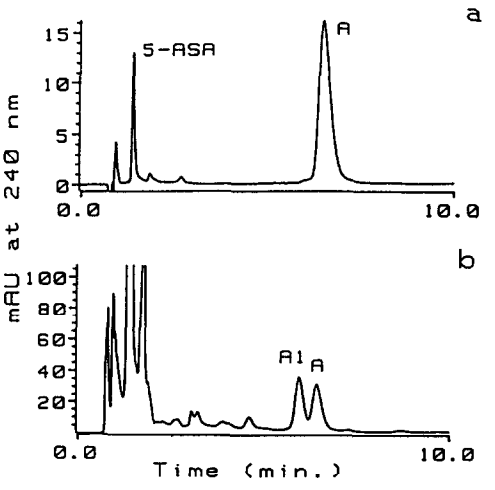

Fig. 3. The chromatogram of a faeces extract from a patient (b) demonstrates the presence of one product with retention time similar to reference sample A (a) and further one product having UV spectra identical to reference sample A. The metabolites were chromatographed on Spherisorb, ODS-2, using a mixture of acetonitrile–0.1 M potassium citrate buffer pH 6.0–water (18:10:72) with 0.2% of tetrabutylammonium bromide added as the eluent.

Survey of the possible formation of A, A1 and D in faeces and pharmaceuticals

Samples of blank faeces spiked with 5-ASA and samples of tablets as well as enemas were screened for the presence of A, A1 and D by the gradient HPLC system described above. The samples were also analysed by isocratic HPLC systems, which have lower detection limits for the products A, A1 and D. The isocratic HPLC systems are presented under the heading: Quantitative determination of A, A1 and D in faeces extract and urine.

Preparation of blank faeces samples spiked with 5-ASA. Blank faeces samples spiked with 5-ASA were prepared by adding 50.0 mL of premixed test solutions of 5-ASA (2 mM) and isobutyryl-5-ASA (200 μM) to 25.0 g of faeces. The test solutions were dissolved in each of the following media: 0.1 M potassium phosphate buffer pH 6.0 (solution a), methanol–0.1 M phosphate buffer pH 6.0 (50:50 v/v) (solution b) and methanol (solution c). The samples were analysed following 24 hr at room temperature and 1 month at −20°. A blank faeces sample prepared with 400 mL of phosphate buffer and spiked with 1 g of 5-ASA was also investigated for the presence of products A, A1 and D after 24 hr at room temperature.

The concentration of 5-ASA was determined in the faeces samples using reversed-phase (C-18) HPLC with fluorescense detection (exitation wavelength of 330 nm and an emission wavelength of 500 nm) and an eluent consisting of acetonitrile–0.1 M potassium citrate buffer pH 6.0–water (13:10:77 v/v) with 0.2% of tetrabutylammonium bromide added. The concentration of 5-ASA was calculated using isobutyryl-5-ASA as internal standard.

Preparation of pharmaceuticals. Five tablets were dissolved in 25.0 mL of phosphate buffer pH 7.5 and the supernatant was analysed by HPLC. The enema was analysed without dilution.

Quantitative determination of A, A1 and D in faeces extract and urine

Products A and A1. Detection was by fluorescence at an exitation wavelength of 308 nm and an emission wavelength of 410 nm and the separation was performed on Spherisorb ODS-2, 5 μm particles, (120 × 4.6 mm) with a mixture of acetonitrile–0.1 M potassium citrate buffer pH 6.0–water (18:10:72 v/v) with 0.2% of tetrabutylammonium bromide added

Table 1. ^{1}H-NMR data of reference sample A, and metabolites A and A1

Assignments	Reference A δ ppm (m, J Hz)	Metabolite A δ ppm (m, J Hz)	Metabolite A1 ppm (m, J Hz)
5-ASA ring			
H-2′	7.59 (d, 2.64)	7.53 (d, 2.6)	7.32 (d, 2.6)
H-6′	7.17 (dd, 2.6, 8.6)	7.12 (dd, 2.6, 8.5)	7.01 (dd, 2.6, 8.5)
H-5′	6.81 (d, 8.5)	6.78 (d, 8.5)	6.69 (d, 8.5)
Imidazol ring			
H-2	7.96 (s)	7.92 (s)	
H-4	7.61 (d, 8.4)	7.58 (d, 8.5)	7.34 (d, 8.5)
H-5	6.75 (d, 8.5)	6.78 (d, 8.5)	6.72 (d, 8.5)
CH_3			1.91 (s)

The compounds were dissolved in dimethyl sulphoxide d_6.

as the eluent. The concentrations of products A and A1 in biological samples were determined from a standard curve (0.1–20 μg/mL) obtained using reference sample A. The detection limit at a signal to noise ratio of two was shown to be 0.01 μg/mL.

Product D. Detection was by UV at an absorption wavelength of 410 nm and the elution was performed on the column as above with a mixture of methanol–tetrahydrofuran–0.1 M potassium phosphate buffer pH 7.5–water (34:6:20:40 v/v), with 0.2% of tetrabutylammonium bromide added as the eluent. The content of product D in faeces samples was determined using reference sample D. The detection limit at a signal to noise ratio of two was shown to be 0.1 μg/mL.

Preparation of biological samples. Prior to analysis, the faeces and urine samples were centrifuged and the supernatants were diluted 1:4 or 1:128 with the HPLC eluent.

RESULTS

Screening for oxidant-mediated products of 5-ASA in faeces and urine

Gradient HPLC of a methanolic extract of faeces from a patient receiving 4 g of 5-ASA (Fig. 2b) gave two chromatographic peaks, named metabolites A and D, which exhibited retention times and UV spectra corresponding to reference samples A and D (Fig. 2a), respectively. Isocratic HPLC (Fig. 3) revealed that the peak of metabolite A consisted of two compounds, named A and A1, both having a UV spectrum identical to reference sample A. Polymeric products of 5-ASA were not detected in the urine sample (Fig. 2d) obtained from the same patient.

Identification of oxidant-mediated metabolites of 5-ASA

Metabolites A, A1 and D were isolated by preparative HPLC from a pre-concentrated faeces sample. Metabolites A and A1 were obtained in sufficient amounts (about 10 mg) to perform structure characterization based on ^{1}H-NMR. These data (Table 1) demonstrate the identity of metabolite A and reference A, the benzimidazole derivative of 5-ASA. FAB-MS data support M = 314 for both compounds.

The ^{1}H-NMR data of metabolite A1 indicate that it is structurally close to metabolite A, the difference being that A1 has a methyl group at C-2 in the benzimidazole ring. This is confirmed by the presence of a $(M + H)^+$ ion at m/z 329 in the positive ion FAB spectrum. Thus, the structure of metabolite A1 is that of the 2-methyl-benzimidazol derivative of 5-ASA shown in Fig. 1.

Metabolite D was isolated in a minor quantity (0.5 mg) and the structure characterization was performed exclusively by comparing the ECNI-MS spectrum of metabolite D with that of reference sample D as shown in Fig. 4.

Possible reaction pathways involved in the in vivo *formation of A, A1 and D*

Hypochlorite-mediated oxidation of 5-ASA. The equimolar reaction between 5-ASA and hypochlorite (Fig. 5a) resulted in the formation of a product identical to reference sample B, whereas the oxidation of 5-ASA with hypochlorite in excess (Fig. 5b) resulted in the formation of two unknown products of 5-ASA, U1 and U2. These products were shown to have a UV absorbance maximum at about 280 nm and to be non-fluorescent.

Oxygen radical-mediated oxidation of 5-ASA. The reaction between 5-ASA and Fenton radicals for 1 hr reduced the concentration of 5-ASA by 40%. The chromatographic product profile of the 1 hr reaction mixture showed several products in addition to reference sample B (Fig. 5c). However, if the reaction was carried out for an extended period of 4 hr or more product B was not observed in the chromatogram (Fig. 5d).

Haemoglobin-catalysed H_2O_2-dependent oxidation of 5-ASA. This reaction led to the formation of product B as demonstrated in Fig. 5e.

Autooxidation of 5-ASA in faeces samples. The concentration of 5-ASA in blank faeces samples prepared with phosphate buffer decreased by 4–10% within 24 hr at room temperature. 5-ASA was found to be stable in faeces samples stored at −20° as well as in faeces samples prepared with methanol as the decrease in the 5-ASA concentration was below the coefficient of variation of the analysis (3%). The

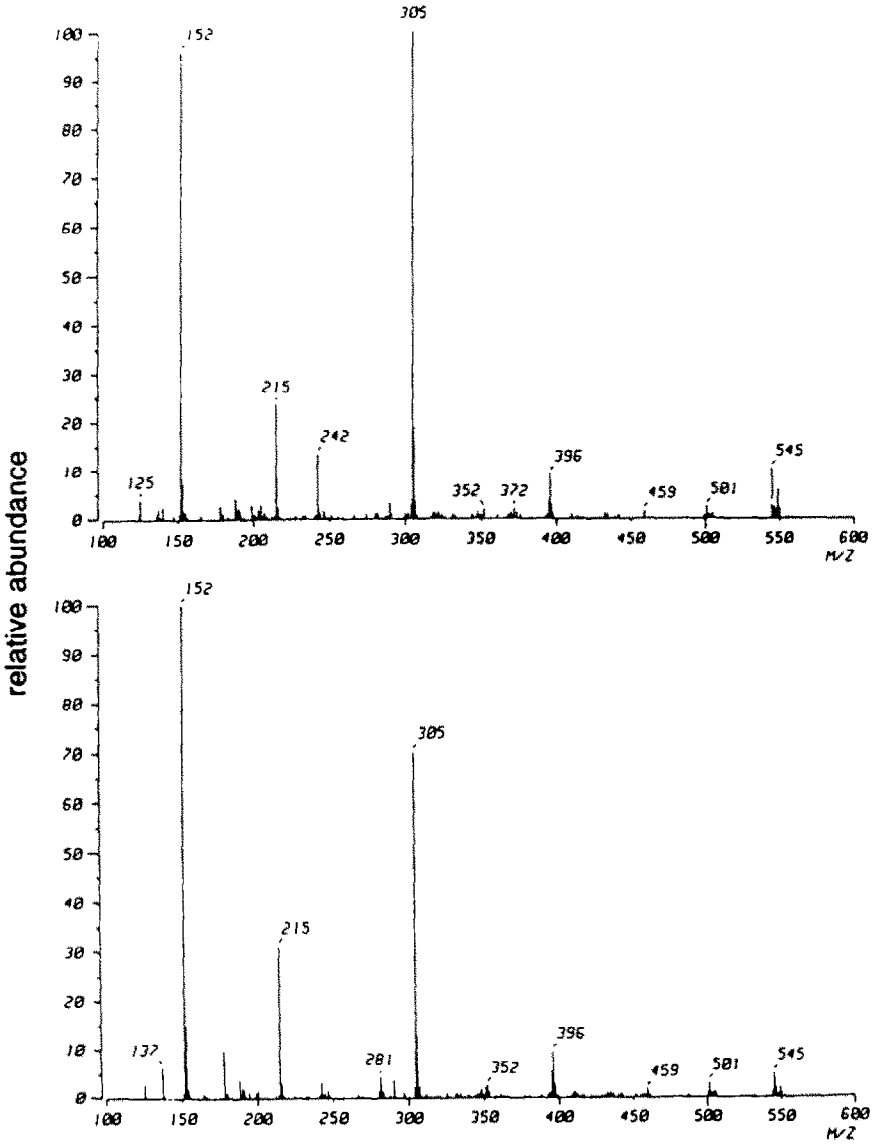

Fig. 4. Electron-capture negative ion spectra (ECNI-MS) of reference sample D (a) and metabolite D (b). The molecular ion is at m/z = 545 Da, which may indicate that both spectra contain product D. Furthermore, similar daughter ions are present in both spectra. These may be present due to loss of 5-ASA (m/z at 152) followed by loss of 2 mol of CO_2 (m/z at 305).

formation of metabolites A, A1 and D was not found in phosphate-buffered faeces samples stored for 24 hr at room temperature (Fig. 2c).

Autooxidation of 5-ASA in tablets and enemas. The chromatographic analysis of tablets and enemas stored under recommended storage conditions for 2 years did not indicate the formation of products identical to metabolites A, A1 and D.

Quantification of products (metabolites) A, A1 and D in faeces and urine

Metabolites A, A1 and D were detected in faeces samples obtained from patients as well as from healthy persons as shown in Table 2, but the metabolites were not detected in urine samples. The daily excretion of each of the metabolites A, A1 and D in the healthy subjects varied between 0.5 and 2.7 mg, 0.3 and 1.6 mg, and 0.2 and 1.3 mg, respectively. The excretion of metabolites varied significantly among patients. As an example, metabolite A was detected in only three out of eight persons. In addition a single patient excreted significantly higher levels of all the metabolites. Except for this patient, the daily excretion of metabolites A1 and D ranged between 0.5 and 2.6 mg, and 6.9 and 16.8 mg, respectively.

DISCUSSION

Ahnfelt-Rønne *et al.* [14] were the first group to

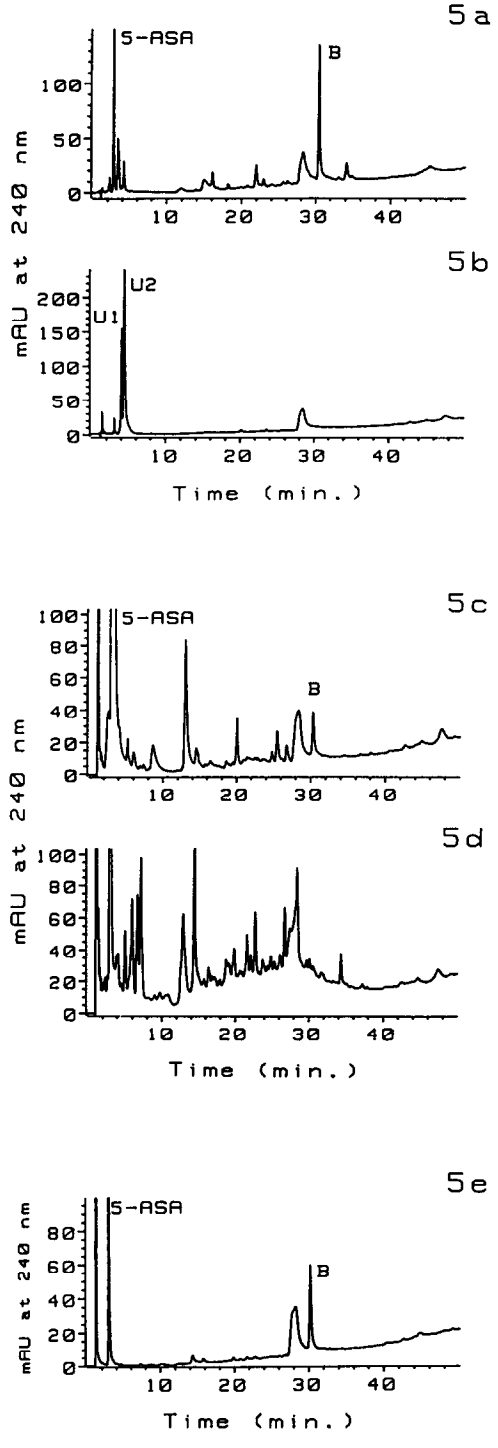

Fig. 5. Gradient HPLC analysis of the reactions between 5-ASA and various oxidants. The eqimolar reaction between 5-ASA and hypochlorite was shown to generate product B as a major oxidation product (a), and the reaction of 5-ASA with hypochlorite in excess was shown to generate two unknown products, U1 and U2 (b). The product profile of the reaction between 5-ASA and Fenton radicals obtained after 1 hr (c) and 24 hr of reaction (d) is shown. Product B was shown to be present in the 1-hr reaction mixture, but not in the 24-hr reaction mixture. The haemoglobin–H_2O_2-mediated degradation of 5-ASA resulted in the generation of product B (e).

Table 2. The excretion of metabolites A, A1 and D in patients as well as healthy subjects treated with 5-ASA

	A (mg)	A1 (mg)	D (mg)
Patients			
Tablets (mg)			
1000	0.3	0.8	6.9
1000	BD	0.9	8.2
1500	BD	0.5	BD
1500	BD	0.6	8.3
Enema (mg)			
1000	BD	1.0	7.2
1000	1.6	2.6	16.8
2000	BD	0.8	16.1
4000	29.4	36.4	51.5
Healthy subjects			
Tablets (mg)			
4000	1.4	1.2	0.4
4000	1.4	8.3	0.6
4000	1.5	0.4	0.2
4000	1.2	0.5	0.4
4000	0.7	0.4	0.3
4000	1.1	0.6	0.3
4000	0.6	0.3	0.3
4000	0.5	0.6	0.3
4000	1.2	1.3	0.6
4000	2.7	2.0	1.3
4000	0.5	1.6	0.4

5-ASA was given to patients as tablets or enemas at various daily doses, and to healthy subjects as tablets at a daily dose of 4 g.

find evidence for the *in vivo* formation of oxidant-mediated metabolites of 5-ASA in patients with active UC treated with sulphasalazine. These oxidant-mediated metabolites were demonstrated in faeces extracts by reversed-phase gradient HPLC using an eluent at low pH, and evidence for the chemical nature of the metabolites was obtained by comparing the UV spectra of coeluting peaks found in chromatograms of faeces extracts and reaction mixtures of oxygen radical-mediated oxidation of 5-ASA. The oxygen radicals were generated by the Fenton reaction.

In the present study, the reaction between 5-ASA and Fenton-generated radicals was also followed by gradient reversed-phase HPLC, but the pH of the eluent was 7.5, as preliminary studies of the reaction indicated the formation of products, of which some will precipitate at low pH. Contrary to the results reported by Ahnfelt-Rønne *et al.* [14], no oxygen radical-mediated products of 5-ASA were found in faeces extracts from 5-ASA-treated patients with active UC. These contrasting results could arise due to differences in disease activities of patients participating in the two investigations, to differences in the medication or to differences in performing the HPLC analysis and the Fenton reaction. Furthermore, the degradation of 5-ASA by Fenton-generated radicals did not result in salicylic acid or gentisic acid as suggested previously by Dull *et al.* [18]. In fact, no stable Fenton-mediated products of 5-ASA could be demonstrated, as products initially

formed disappeared after an extended period of reaction. As an example, product B was found to be an intermediate in the oxygen radical-mediated degradation of 5-ASA.

Haemoglobin has been reported to function as a biological Fenton reagent [19]. The oxidant formed by the interaction of reduced haemoglobin and H_2O_2 is considered not to be the hydroxyl radical, but more likely the ferryl radical, Hb(IV)$^{\cdot}$ [20]. The ferryl radical has been observed previously to oxidize 5-ASA with the formation of a golden-brown compound [13]. This observation was verified in the present study, as product B with λ_{max} 390 nm could be identified as the resulting product of the interaction between 5-ASA and Hb(IV)$^{\cdot}$. Product B was also observed to be formed by the equimolar reaction between 5-ASA and hypochlorite. The formation of a golden-brown product by hypochlorite-mediated oxidation of 5-ASA has in fact been reported previously [21]. However, Laffafian *et al.* [22] have just reported the generation of a hypochlorite-mediated compound of 5-ASA, which in contrast to product B possesses fluorescent activity. The fluorescent compound was shown to be formed by the reaction between 5-ASA and excess hypochlorite as well as in biological systems by activated neutrophils and myeloperoxidases/hydrogen peroxide. MS analysis of the fluorescent compound suggested a structure of 5-nitrososalicylic acid. The oxidation of 5-ASA by excess hypochlorite was also carried out in the present work, but the generation of a fluorescent product could not be verified as two non-fluorescent products with λ_{max} 280 nm were found in the chromatograms.

In summary, the present results demonstrate that two-electron oxidation of 5-ASA by hypochlorite or Hb(IV)$^{\cdot}$ results in product B, and one-electron oxidation by Fenton-generated radicals results in unidentified products formed by reaction pathways alternative to the two-electron oxidation. Neither of the resulting radical-mediated products of 5-ASA (Fig. 5c and d) nor the polymeric product B was identified in stools from 5-ASA-treated patients (Fig. 2b and d). However, three polymeric products of 5-ASA, A, A1 and D, with chemical natures similar to product B were identified in the present study. These products (metabolites) were isolated by preparative HPLC from a pre-concentrated faeces extract, and NMR and MS analyses provided evidence for their chemical structures. Thus, two benzimidazol derivatives of 5-ASA, A and A1 (dimeric species), and one tetrameric species of 5-ASA, D, were identified in faeces extracts but not in urine. Products A, A1 and D may be formed by oxidation of 5-ASA, but the sources mediating the oxidation *in vivo* are unknown. The products A, A1 and D have been observed to be formed as well as product B by autooxidation of 5-ASA *in vitro* [15]. Thus, partial oxidation of 5-ASA may result in the formation of the identified products (metabolites) in faeces, partly because the oxidation of 5-ASA may stop with the formation of dimeric species of 5-ASA due to possible stabilization upon the formation of benzimidazol derivatives by reaction with suitable acids [23]. Alternatively, the possible formation of product B *in vivo* may be proposed to result in the formation of product D due to hydrolysis of product B to product C (structure in Fig. 1) followed by coupling of product C to a 5-ASA molecule.

Metabolites A, A1 and D were detected in faeces extracts from patients with active UC undergoing 5-ASA therapy as well as in healthy subjects receiving 5-ASA. The level of excretion of the metabolites was low and a maximum of 1% of the ingested dose of 5-ASA was converted to each of the metabolites. The number of patients and healthy subjects included in this study was too low to determine any significant difference in the level of excretion of the metabolites between the two groups.

The fact that the products (metabolites) A, A1 and D are found in patients as well as healthy subjects indicates that the metabolites could be generated via pathways alternative to the oxidation of 5-ASA by oxidants present in the inflamed bowel. Potential alternative pathways are autooxidation of 5-ASA in faeces samples and in pharmaceuticals. van Hogezand *et al.* [24] have previously reported a significant decrease in the concentration of 5-ASA in faeces samples stored at room temperature (80%) as well as at −20° (40%) for 1 day. However, in the present work the concentration of 5-ASA was shown to decrease only slightly in phosphate-buffered faeces samples and, consequently, no degradation products of 5-ASA could be demonstrated in these samples. Furthermore, no products were detected in pharmaceuticals stored as recommended. Bacterial metabolism of 5-ASA may also constitute an alternative mode of generation of the metabolites. Actually, the 5-ASA moiety has been shown to be oxidized during aerobic bacterial metabolism to a *cis*-4-amino-6-carboxy-2-oxohexa-dienoate [25], which indicates that bacterial metabolism of 5-ASA by aerobic bacteria involves ring opening of the molecule. However, the metabolism of 5-ASA by anaerobic bacteria is unknown.

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