ELSEVIER

Journal of Chromatography B, 661 (1994) 57–68

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Radical-derived oxidation products of 5-aminosalicylic acid and *N*-acetyl-5-aminosalicylic acid☆

C. Fischer*, U. Klotz

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstr. 112, D-70376 Stuttgart, Germany

First received 12 April 1994; revised manuscript received 11 July 1994

Abstract

5-Aminosalicylic acid is an agent effective in the treatment of chronic inflammatory bowel diseases. Its ability to scavenge radicals is considered to be a major factor responsible for its therapeutic efficacy. In this study oxidation products of aminosalicylates with hydroxyl radicals were produced. The compounds that could be discovered by gas chromatographic–mass spectrometric analysis originate from a 1,4-benzoquinone monoimine intermediate which subsequently undergoes multiple reactions such as hydrolysis, reductive 1,4-Michael addition, reoxidation and decarboxylation. Some of these products could represent metabolites formed under in vivo conditions and thus provide a tool for screening biological material from subjects under different clinical conditions.

1. Introduction

5-Aminosalicylic acid (5AS, **1**, Scheme 1) is an effective agent in the treatment of chronic inflammatory bowel diseases [1–4]. Following rectal or oral administration the drug is rapidly acetylated in the intestinal mucosa and liver to its major metabolite *N*-acetyl-5-aminosalicylic acid, Ac5AS (**2**) [5].

Its mode of action is still unclear, however, the ability to scavenge radicals is thought to be a major factor responsible for its therapeutic efficacy. Reactive radicals seem to play an important role in the development of chronic inflammatory bowel diseases (IBD). A cascade of biological processes is initiated by cellular oxidative stress such as inflammation or ischaemia-reperfusion injury which involves the activation of neutrophils, followed by the release of the superoxide radical anion $O_2^{-}\cdot$ and hypochlorous acid as a cellular response which subsequently leads to the formation of hydroxyl radicals [6,7]. Therefore, it is conceivable that the radical scavenging potential of 5AS could explain its clinical efficacy [8].

Recently, Jensen et al. [9,10] described oxidation products found in patients with active ulcerative colitis as well as healthy subjects, both taking 5AS. The identified oxidation products implicated the in vivo presence of formaldehyde or acetaldehyde which react with the postulated 1,4-quinoneimine intermediate to form benzimidazole compounds. Two of these substances

☆ Partly presented at the 35th Spring meeting of the German Society of Experimental and Clinical Pharmacology and Toxicology, Mainz, 1994.

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)0323-8

could be determined in faeces and urine samples of patients following intake of 5AS.

The aim of our study was to produce oxidation products of 5AS and Ac5AS with hydroxyl radicals generated by Fenton's reagent ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$; [11]) or $TiCl_3 + H_2O_2$ [12]) and to attempt the characterization of these products by high-performance liquid chromatography (HPLC) and capillary GC–MS. Some of these compounds could represent metabolites produced under in vivo conditions and thus provide a tool for screening biological material of subjects under different clinical conditions.

2. Experimental

2.1. Reference compounds

5AS was recristallized and Ac5AS was synthesized as described earlier [13]. 4-Aminosalicylic acid (4AS, Aldrich, Steinheim, Germany), gentisic acid (2,5-dihydroxy benzoic acid, GeA, Sigma, Deisenhofen, Germany), salicylic acid (2-hydroxybenzoic acid, SaA, Sigma) and 2,3,5,6-tetrachloro-1,4-benzene-diol (Sigma) were obtained in p.a. quality.

2.2. Solvents and reagents

All solvents were either from Rathburn Chemicals (Walkerburn, UK) or from J.T. Baker (Deventer, Netherlands) and of HPLC-grade purity. Ethyl chloroformate (ECF) was purchased from Aldrich. *N*-Methylbis(trifluoroacetamide) (MBTFA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were obtained from Fluka (Buchs, Switzerland). 2-Mercaptoethanol (Fluka) and $TiCl_3$ (ampoules, 15% in 10% HCl, E. Merck, Darmstadt, Germany) were used as received.

2.3. Reaction of 5AS or Ac5AS with Fenton's reagent, termination with HCl

5AS (1 mg, 6.5 μmol) or Ac5AS (1 mg, 5.1 μmol) were incubated (5 min at room temperature) with freshly prepared Fenton's reagent (1 ml) as described by Ahnfelt-Rønne et al. [14]. Briefly, $FeSO_4$ (13.9 mg $FeSO_4 \cdot 7H_2O$) was added to 10 ml 0.1 *M* K_2HPO_4 buffer containing 22 m*M* Na_2EDTA (75 mg Titriplex III, E. Merck) and 44 m*M* H_2O_2 (50 μl Perhydrol, 30%, E. Merck) and adjusted to pH 7.2 with H_3PO_4. Then, 1 ml was taken to react with the aminosalicylates for 5 min at room temperature. The reaction was terminated by addition of 300 μl of 10 *M* HCl. A 100-μl aliquot of the centrifuged clear supernatant (2 min at 10 000 *g*) was either injected directly onto the HPLC system or an aliquot of the whole mixture was derivatized for GC–MS analysis.

2.4. Reaction of 5AS with Fenton's reagent, termination with 2-mercaptoethanol

Following reaction with Fenton's reagent as described above the mixture was adjusted to pH 9 and 2.3 μl of 2-mercaptoethanol corresponding to 33 μmol (5 fold excess) was added.

2.5. Reaction of 5AS with $TiCl_3 + H_2O_2$

5AS was reacted with $TiCl_3 + H_2O_2$ to explore the formation of radicals by electron-spin resonance. The aminosalicylate solution (100 m*M*) was continuously added to 100 m*M* solutions of $TiCl_3 + H_2O_2$ (1 ml of each). The tanned coloured solution was studied by HPLC and GC–MS.

2.6. Control experiments

Control experiments of Fenton's reaction were performed by excluding the addition of either 5AS, Fe^{2+} salt or H_2O_2.

2.7. HPLC separation

The reaction products were separated on an analytical Lichrosphor 100 RP-18 column (5 μm, 12.5 cm × 4 mm I.D. stainless steel column, coupled to an 2.5-cm precolumn filled with the same material, E. Merck). A combined isocratic (5 min) and gradient (20 min) solvent program with absorbance monitored at 225 nm was used as described by Ahnfelt-Rønne [14] with minor

modifications. Briefly the mobile phase was 0.1 *M* KH_2PO_4 (pH 4.5)–methanol (95:5, v/v) up to 5 min and was then linearily changed to 50:50 over a 20-min period. The flow-rate was 1 ml min^{-1} throughout.

2.8. Derivatization

The HPLC fractions obtained or the whole reaction mixtures were dried by a vigorous stream of nitrogen and the residues redissolved in 1 ml of methanol. The phosphate salts remaining from the mobile HPLC phase were removed by filtration through a glass pipette containing a silanized glasswool plug.

2.9. Procedure A: formation of ethyl ester N,O-ethyloxycarbonyl-derivatives (Et-EtOCO)

The reaction procedure was performed as described by Hušek [15]. A 100-μl volume of a mixture of acetonitrile–ethanol–water–pyridine (5:2:2:1, v/v) was added to the dried samples followed by 10 μl of ethyl chloroformate. After vigorously shaking for 20 s and standing for a further 10 min at room temperature, 2 ml of ethyl acetate–*n*-hexane (1:1, v/v) was added together with 0.5 ml of 1 *M* $NaHCO_3$ (pH 10) and the samples were vortex mixed. The samples were extracted twice and the organic phases were transferred to another vial. Traces of water were eliminated by addition of Na_2SO_4. The organic solvents were removed by a stream of nitrogen and the residue reconstituted in 50 μl of ethyl acetate. Aliquots of 1–2 μl were injected onto the GC–MS system.

2.10. Procedure B: formation of methyl ester trifluoroacetyl derivatives (MeTFA)

The dried samples were redissolved in one drop of methanol and 0.3 ml of ethereal diazomethane were added. After 10 min standing at room temperature the volatile compounds were removed by a stream of nitrogen. Acetonitrile (10 μl) and MBTFA (10 μl) were added and the samples were kept overnight at 40°C. Aliquots of 1–2 μl were injected onto the GC–MS system.

2.11. Procedure C: formation of tert.-butyldimethylsilyl derivatives (tBMDS)

Acetonitrile and MTBSTFA (10 μl of each) were added to the dried samples and allowed to stand overnight at 40°C. Aliquots of 1–2 μl were injected onto the GC–MS system.

2.12. GC–MS analysis

GC–MS analysis in the positive-ion electron-impact mode (PIEI) at 70 eV was performed with a Hewlett-Packard 5970 series mass-selective detector coupled to a Hewlett-Packard 5890 gas chromatograph. A HP1 capillary column was installed (25 m × 0.22 mm I.D., 0.33 μm film thickness, 1.5 × 0.32 mm retention gap). The GC was kept at 80°C for 1 min and subsequently the oven was heated to 310° at 30°C min. The Gerstel cold injection system was heated from 80°C to 310°C within 120 s from the start. The transfer line was kept at 300°C.

An additional Hewlett-Packard instrument HP 5985A equipped for analysis in the negative-ion chemical-ionization mode (NICI) was employed for some of the samples. A Rtx-200 capillary column (25 m × 0.32 mm I.D., 0.25 μm film thickness; Restec, Sulzbach, Germany) was installed and a temperature program was run starting with a 100°C hold for 1 min and increasing the temperature at 30°C/min up to 310°C. Injections were made in the splitless mode at 250°C. The transfer line was kept at 310°C and methane was used as the reagent gas.

3. Results

Reddish brown colouring was observed within seconds following the addition of 5AS to either Fenton's reagent or $TiCl_3 + H_2O_2$. The reaction mixtures were subjected to HPLC and GC–MS analysis after preceding derivatization.

3.1. HPLC screening for reaction products of 5AS

Separation of reference compounds 5AS, Ac5AS, GeA and SaA was achieved by re-

versed-phase HPLC (Fig. 1A). The displayed numbers refer to the corresponding mass spectra shown in Fig. 3. 5AS reacted with Fenton's reagent and the reaction was terminated by addition of either HCl or 2-mercaptoethanol (see chromatograms presented in Fig. 1B and 1C, respectively). Oxidation of 5AS with $TiCl_3 + H_2O_2$ provided peaks as shown in Fig. 1D. Moreover, Ac5AS was exposed to Fenton's reagent and the reaction terminated by addition of HCl (Fig. 1E).

3.2. Derivatization of reaction products

Derivatization was required prior to GC analysis. The reaction conditions described by Hušek [15] permitted the formation of ethyl esters (Et) and acylation of basic functions to the corresponding *N,O*-ethyloxycarbonyl derivatives or *N*-ethyloxycarbonyl derivatives $(EtOCO)_n$ using ethyl chloroformate. The reaction takes place under mild conditions. For example, 5AS is converted to its ethyl ester diacylated *N,O*-ethyloxycarbonyl derivative $Et(EtOCO)_2$ (Fig. 3: spectrum **1**) and to its ethyl ester monoacylated *N*-ethyloxycarbonyl derivative $Et(EtOCO)_1$, giving the molecular ions *m/z* 325 and 253 with retention times of 9.1 and 7.9 min, respectively. Both the mono- and bis-acylated derivatives are useful for identification of the compound. GC–MS total-ion current tracings of the different oxidation mixtures measured following derivatization with ECF are presented in Fig. 2 and the corresponding mass spectra in Fig. 3.

Two further derivatization procedures B and C (see Experimental) were applied in order to elucidate the structure of one of the novel compounds termed **4**. The spectra obtained were compared with the corresponding derivatives of 5AS.

3.3. Analysis of reaction products by GC–MS

In initial experiments the collected HPLC fractions were separated and each fraction subjected to derivatization reactions. This time-consuming process was replaced by direct derivatization of the whole reaction mixture followed by GC–MS analysis using capillary GC columns which provided high resolution power. The GC–MS analysis of ethyl ester and *N*- and *N,O*-acylated compounds are presented for the reaction of 5AS with Fenton's reagent following termination with HCl and with 2-mercaptoethanol in Fig. 2A and B, respectively, for reaction of 5AS with $TiCl_3 + H_2O_2$ in Fig. 2C, and for Ac5AS with Fenton's reagent and HCl termination in Fig. 2D. The numbers displayed at specified peaks refer to the corresponding mass spectra presented in Fig. 3.

3.4. Mass spectral characterization of reference compounds 5AS and Ac5AS (derivatization to ethyl ester N- and N,O-acyl derivatives)

The electron-impact spectra of the major GC–MS peaks are presented in Fig. 3 with numbers according to the suggested structures given in Scheme 1. Fig. 3A and B show the fragmentation of the reference compounds 5AS-$Et(EtOCO)_2$ and Ac5AS-$Et(EtOCO)_1$ derivatives. The molecular ions *m/z* 325 and 295 for the 5AS and Ac5AS derivatives, respectively accounted for ca. 10% relative abundance. The [M-118]-ion of the ortho ester *O*-acylated phenol moiety was a prominent fragment with 100% (*m/z* 207) and 65% (*m/z* 177) relative abundance for the 5AS and the Ac5AS derivative, respectively.

3.5. Reaction of 5AS with Fenton's reagent and termination with HCl

The dark brown solution was studied by HPLC (see Fig. 1B) and was shown to contain several compounds which were collected according to their prominent UV absorbing peaks. The HPLC peak obtained at 7.9 min was subjected to derivatization procedure A and identified by mass-spectral analysis to be a monochlorine-substituted analogue of 5AS (Scheme 1; Fig. 2A; Fig. 3: spectrum **4**) according to its fragmentation pattern which was analogous to that of the corresponding 5AS derivative but shifted by 34 mass units. It displayed the characteristic $^{35}Cl/^{37}Cl$ chlorine isotope abundances characteristic

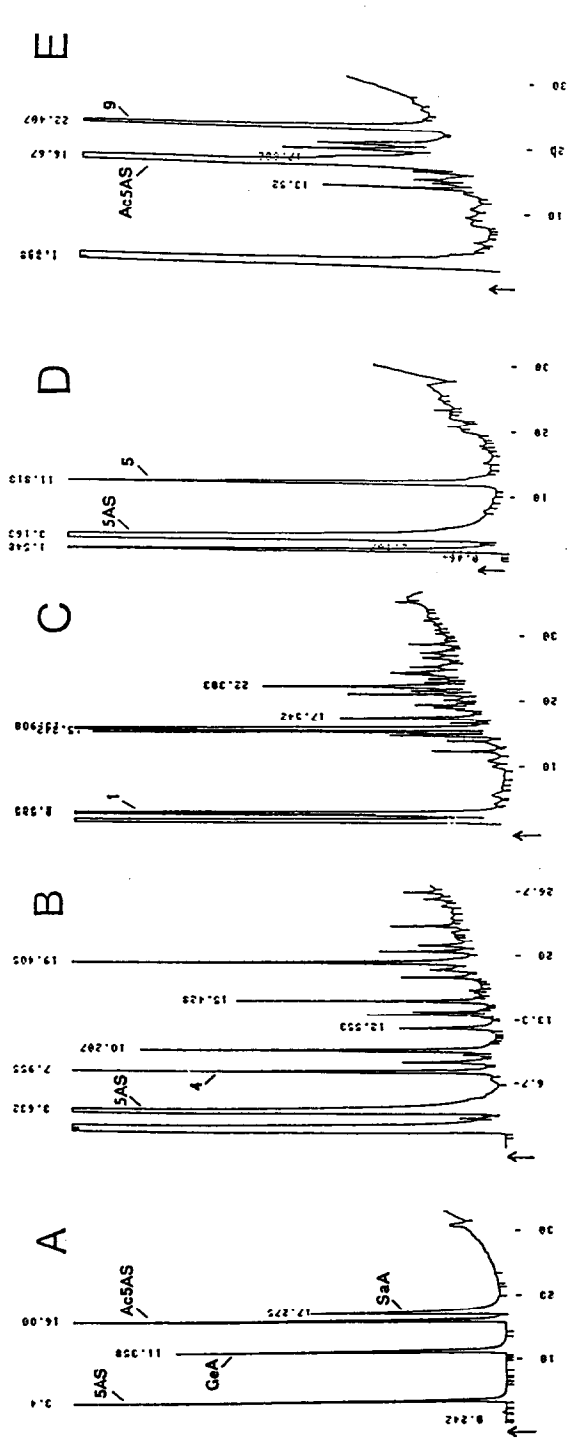


Fig. 1. Representative HPLC chromatograms. Number assigned to the peaks refers to identified compounds as shown in Scheme 1 and Fig. 3. (A) Reference compounds (5AS, GeA, Ac5AS, SaA); (B) Reaction of 5AS with Fenton's reagent, termination with HCl; (C) Reaction of 5AS with Fenton's reagent, termination with 2-mercaptoethanol; (D) Reaction of 5AS with $TiCl_3 + H_2O_2$; (E) Reaction of Ac5AS with Fenton's reagent, termination with HCl.

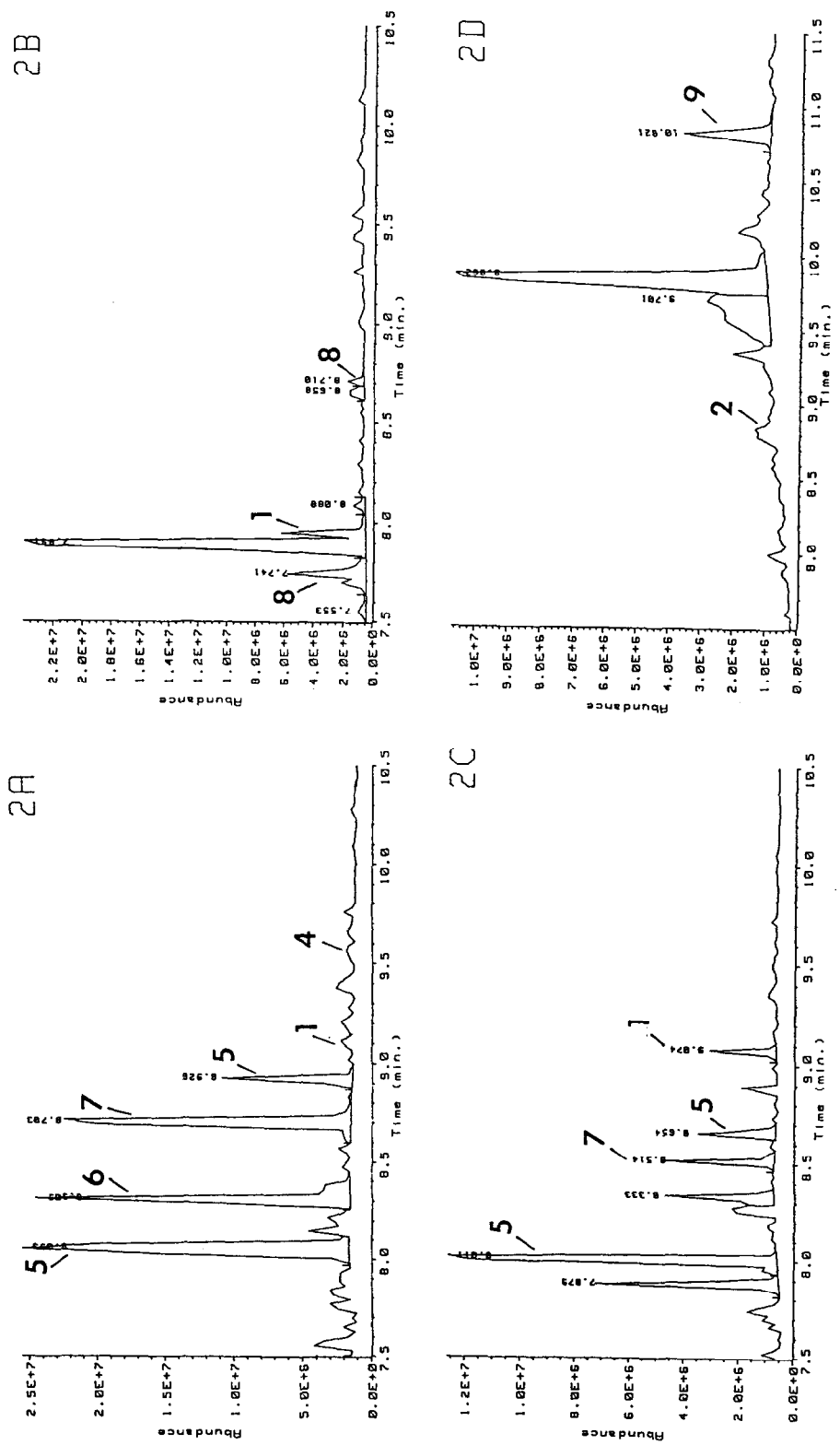


Fig. 2. GC–MS total-ion current tracings following derivatization procedure A. The numbers assigned to the peaks refer to the corresponding compounds depicted in Scheme 1 and Fig. 3. (A) Reaction of 5AS with Fenton's reagent, termination with HCl; (B) Reaction of 5AS with Fenton's reagent, termination with 2-mercaptoethanol; (C) Reaction of 5AS with $TiCl_3 + H_2O_2$; (D) Reaction of Ac5AS with Fenton's reagent, termination with HCl.

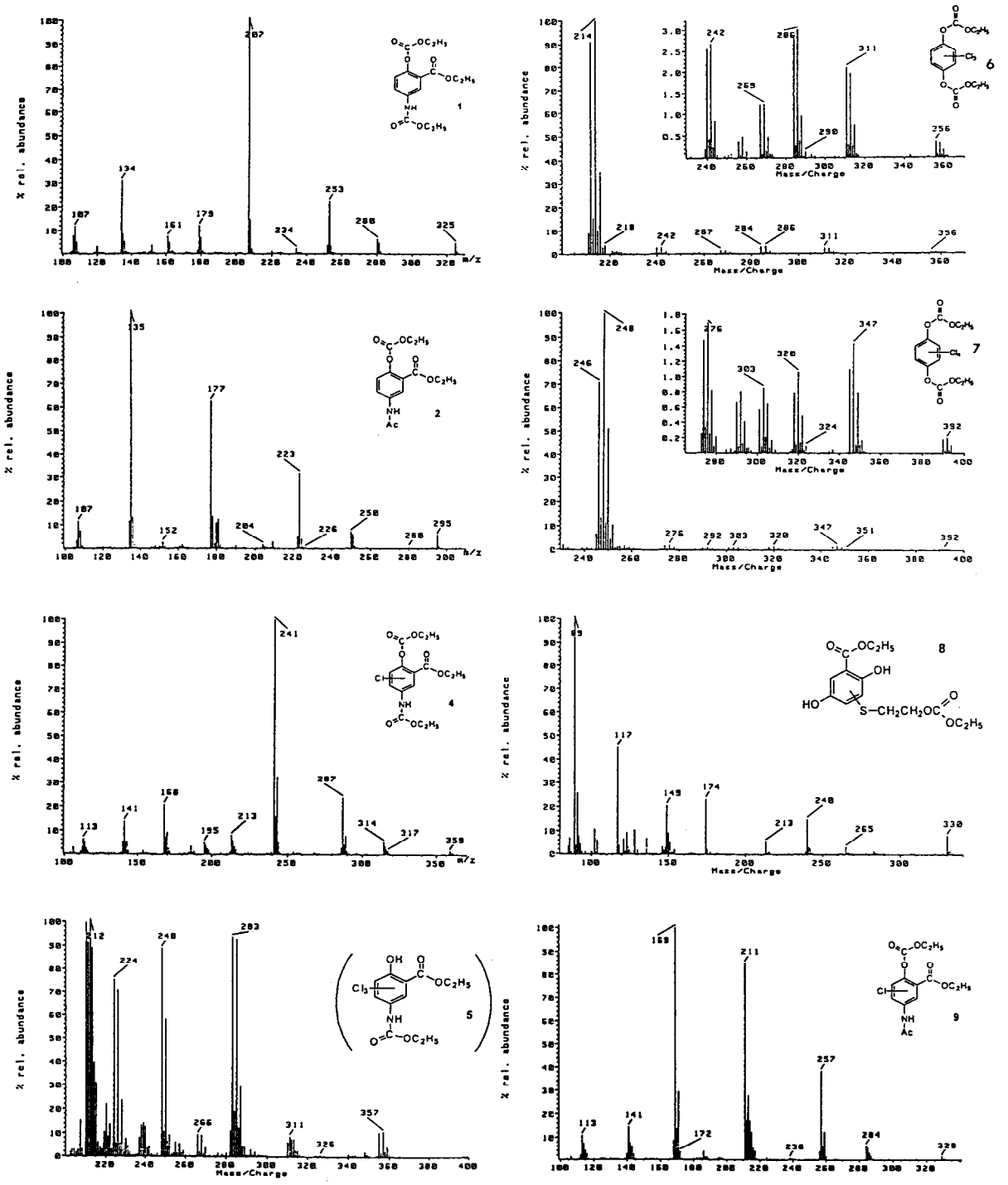
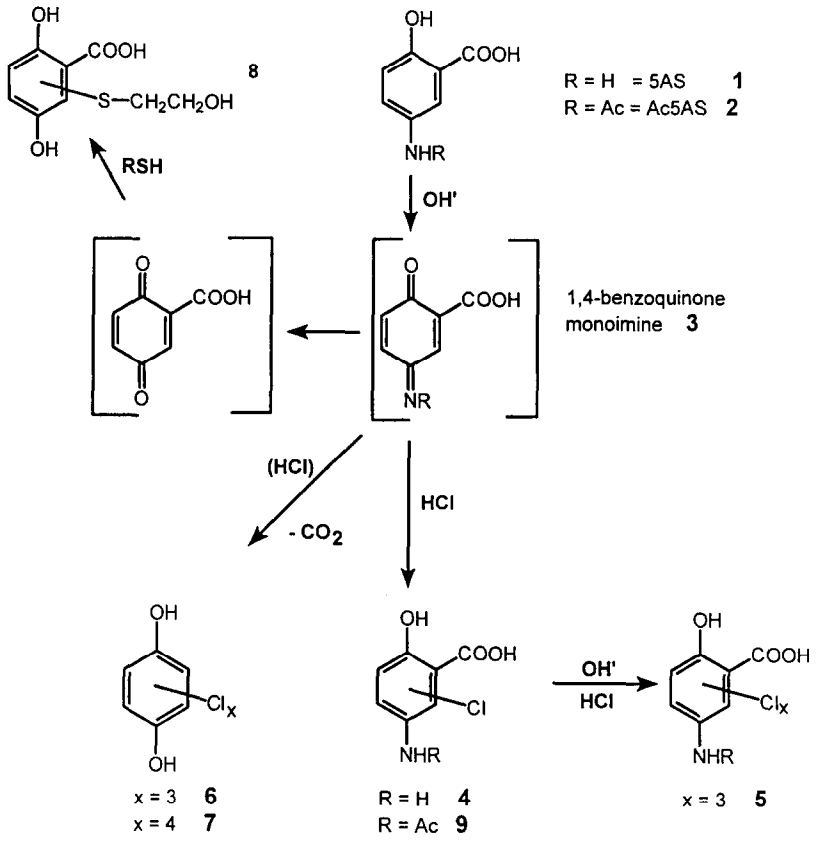


Fig. 3. PIEI spectra following derivatization procedure A. The numbers assigned to the compounds refer to the corresponding compounds depicted in Scheme 1 and Figs. 1 and 2. (1) 5AS Et$(EtOCO)_2$; (2) Ac5AS Et $(EtOCO)_1$; (4) **4** Et $(EtOCO)_2$; (5) **5** Et $(EtOCO)_1$; (6) **6** $(EtOCO)_2$; (7) **7** $(EtOCO)_2$; (8) **8** Et $(EtOCO)_1$; (9) **9** Et $(EtOCO)_1$.



Scheme 1. Tentative reaction pathway following hydroxyl radical induced oxidation of 5AS.

for monochlorine-substituted compounds. In addition, the structure of this novel compound was verified using the other two derivatization proceduces B and C (see Experimental). The *t*BDMS$_2$ derivatives revealed loss of M−15, M−57, M−115, M−131, M−56−115 and small molecular ions *m/z* 381 and 415 for 5AS and compound **4**, respectively. The MeTFA$_2$ derivatives showed loss of M−31, M−59, M−97, M−114 and molecular ions *m/z* 359 and 393 with ca. 30% relative abundance for 5AS and compound **4**, respectively.

The Et(EtOCO$_2$) derivative of compound **4** was additionally studied in the NICI mode. The molecular ions *m/z* 359 and 361 constitute the most abundant peaks for the corresponding ^{35}Cl and ^{37}Cl isotopes, respectively. Fragments *m/z* 279 (−Cl−45), 252 (−Cl−72) and 234 (−Cl−91) indicate the loss of chlorine and therefore provide evidence that chlorine was inserted into 5AS. The oxidation experiment was repeated three times, and compound **4** was recovered in a reproducible way as one of the major components when analysed by HPLC analysis.

The dominant GC–MS peak at 8.1 min following derivatization procedure A was ascribed to the ethyl ester of a trichlorine-substituted analogue of 5AS (Fig. 3: spectrum **5**) as judged from its molecular ions *m/z* 283, 285 and 287 and the chlorine isotope pattern. The compound additionally reacted to its ethyl ester monoacyl derivative which is shifted by 72 mass units (*m/z* 355,357,358; GC retention time of 8.9 min). Spectra could be obtained in the NICI mode which ascertained the molecular ions and verified the multiple chlorine substitution by the isotope pattern and fragments with loss of HCl.

The GC peaks at 8.3 and 8.7 min (Fig. 3: mass

spectra **6** and **7**) probably correspond to the structures **6** and **7** as shown in Scheme 1. The tetrachlorine containing compound **7** was commercially available and showed identical retention times and mass spectra when measured in the PIEI and NICI modes as its ethyl ester *O,O*-diacyl derivative. The measurement in the NICI mode supported the proposed molecular ions and showed the typical isotope pattern of multiple chlorine substitution. Compound **6** which was 34 mass units smaller compared to compound **7** is in agreement with a trichloro substituted benzene-1,4-diol compound.

3.6. Reaction of 5AS with Fenton's reagent and termination with 2-mercaptoethanol

The mixture obtained following reaction of 5AS with Fenton's reagent was adjusted to pH 9 and 2-mercaptoethanol was added in excess as described by Eckert et al. [16]. The corresponding HPLC chromatogram is shown in Fig. 1C. The GC–MS total-ion current of the whole reaction mixture which was subjected to derivatization procedure A is shown in Fig. 2B. The disulfides of 2-mercaptoethanol could be detected in the form of its $(EtOCO)_2$ derivative (7.89 min), its $(EtOCO)_1$ derivative (6.8 min) or in its underivatized form (5.1 min) besides the unreacted parent compound 5AS (retention time 7.95 min). In addition, a thio-substituted gentisic acid (**8**) was discovered as its ethyl ester (7.7 min) and as its ethyl ester monoacyl derivative (8.7 min) with molecular ions *m/z* 258 and 330, respectively (Scheme 1, Fig. 3: mass spectrum **8**). Characteristic ions which refer to the thio moiety are *m/z* 89, 103, 117 and 149. The fragments M−90 comprises the gentisic acid part of the molecule (*m/z* 258 − 90 = 168, and *m/z* 330 − 90 = 240 for the two derivatives, respectively).

3.7. Reaction of 5AS with $TiCl_3 + H_2O_2$

An intensive red coloured solution was obtained. Analysis by HPLC (Fig. 1D) revealed a prominent peak at 11.8 min besides unreacted 5AS (retention time 3.2 min). This result could be confirmed by GC–MS analysis where unreacted 5AS (Fig. 2C) and a second compound, its ethyl ester or ethyl ester EtOCO derivative (GC retention times of 8.0 and 8.9 min), were detected. The later compound showed a mass spectrum identical to that found for compound **5**. One of the minor compounds (8.6 min) proved to be substance **7**. The structures of several minor peaks could not be identified.

3.8. Reaction of Ac5AS with Fenton's reagent and termination with HCl

Reaction of Ac5AS with Fenton's reagent resulted in the chromatogram shown in Fig. 1E. GC–MS analysis demonstrated the presence of unreacted Ac5AS (Fig. 2D: peak at 9.7 min; Fig. 3: spectrum **2**). The fragmentation pattern of the compound eluting as the GC peak at 10.8 min was comparable to that of the Ac5AS-$Et(EtOCO)_1$ derivative but the ions detected were shifted by *m/z* 34 (Fig. 3: spectrum **9**). The M−118 fragment (*m/z* 211 for the ^{35}Cl isotope) indicated an ethyl ester with an *ortho* standing acylated phenol moiety, and the base-peak *m/z* 169 derived from this ion revealed additional loss of *m/z* 42 which is characteristic for the *N*-acetyl moiety. Therefore this compound was identified as compound **9**.

The major product obtained by GC–MS analysis (peak at 9.7 min) generated a mass spectrum that was 42 mass units higher than the corresponding derivative of compound **5**. The molecular ions *m/z* 325 and 327 ($^{35}Cl/^{37}Cl$) in combination with the isotope pattern found suggested a trichlorine-substituted analogue of Ac5AS.

3.9. Control experiments

No oxidation products of 5AS could be recovered when either H_2O_2 or Fe_2SO_4 were omitted. Likewise, Fenton's reagent alone gave no HPLC peaks under the conditions tested.

An oxidation experiment using gentisic acid and Fenton's reagent did not result in the formation of chlorine-substituted analogues, but only unreacted gentisic acid was found by HPLC analysis. The main GC–MS peak at 9.1 min

found following derivatization procedure A revealed a molecular ion of m/z 326 which appeared to be the ethyl ester O,O-diacyl derivative of GeA, $Et(EtOCO)_2$, and the corresponding monoacyl derivative of GeA, $Et(EtOCO)_1$, with molecular ion m/z 254 (GC retention time 7.8 min). Other peaks which occurred in small intensities could not be identified.

4. Discussion

Numerous GC–MS peaks in different quantities could be detected when 5AS reacted either with Fenton's reagent or a mixture of $TiCl_3 + H_2O_2$ (see Fig. 2A and C). The amount of unreacted 5AS was higher with $TiCl_3 + H_2O_2$ when compared with Fenton's reagent suggesting a more vigorous oxidizing potential of the later reagent. The relative intensities of the detected compounds substantially depend on the reaction conditions employed and the redox potential of the products formed.

Scheme 1 outlines a tentative reaction pathway to explain the origin of the identified compounds. All structures can be deduced from a 1,4-benzoquinonemonoimine intermediate (**3**) which subsequently undergoes multiple reactions involving reductive 1,4-Michael addition of HCl to **4**, repeated reoxidation and reductive addition of HCl to produce **5** or an additional decarboxylation step leading to **6** and **7**. Compound **7**, the 2,3,5,6-tetrachloro-1,4-benzenediol, is commercially available and its GC retention time as well as its PIEI and NICI spectra were identical to those of the compound found.

Grootveld and Halliwell [17] described the decarboxylation of salicylate to form catechol as a minor product when exposed to hydroxyl radicals generated by a Fenton system. The multiple-chlorine substitution of the 1,4-benzene-diols **6** and **7** suggest that a 1,4-benzoquinone compound was the precursor. The labile compounds could be stabilized by reaction with HCl, hydrolysis, reoxidation and decarboxylation.

The site of attack of the 1,4-benzoquinone intermediate **3** by nucleophils cannot be concluded from mass spectra but it probably follows 1,4-Michael addition. Thus, the position of the chlorine substitution in compounds **4** and **6** can be verified only if reference compounds were available.

Hydrolysis of quinoneimines to their corresponding quinones constitutes a common reaction [18]. In our experiments hydrolysis occurred generating compounds **6** and **7** and the sulfhydryl-substituted gentisic acid compound **8**. In contrast, hydrolysis was obviously prevented in case of compounds **4** and **5**. Eckert et al. [16] reported on multiple-substituted thioadducts when 4-aminophenol was oxidized and subsequently treated with 2-mercaptoethanol. The authors pointed out that thio-conjugates of glutathione were formed under in vivo conditions which are further metabolized and excreted as mercapturic acids in urine. Thus, screening for aminosalicylate-derived thioethers formed with cysteine residues after oxidation to the 1,4-benzoquinoneimine intermediate **3** seems to be of interest in patients under 5AS therapy.

Formation of a *N*-acetyl-1,4-quinoneimine which occurred during metabolism of acetaminophen, was proposed by Miner and Kissinger [19]. In vitro generation of this quinoneimine from the corresponding *N*-acetyl-4-aminophenol was achieved by electrochemical oxidation. Evidence for the proposed quinoneimine as an intermediate was indirectly obtained by subsequent reaction with different sulfhydryl nucleophils. Several thio adducts could be recovered by HPLC analysis. Likewise, in our experiments a mono- and a multiple-chlorine substituted product could be observed when Ac5AS reacted with Fenton's reagent. These compounds substantiate the conclusion that 1,4-aminophenols are oxidized to 1,4-benzoquinoneimines which further add nucleophils.

Some oxidation experiments were performed using 4AS. This compound has been shown to be effective in the treatment of ulcerative colitis in a preliminary trial [20]. The reaction either with Fenton's reagent or with $TiCl_3 + H_2O_2$ yielded moderate yellow coloured solutions. Following HPLC analysis mainly unreacted 4AS was de-

tected (retention time 7.8 min) besides a later peak at 12 min which gave a mass spectrum corresponding to compound **5** when derivatized following procedure A. Hence, compared to 5AS, 4AS was attacked only to a limited extent by hydroxyl radicals.

The oxidation of 5AS as its ethyl ester was described by van Euler et al. [21]. The authors isolated a green substance with a quinoid structure when using silver oxide as the oxidation reagent, and a yellow azo compound when using H_2O_2. Both products as well as additional polymeric compounds are not suitable for GC analysis and consequently could not be found during our investigations.

5AS is subject to complex reactions when dissolved in aqueous media [22]. It was shown by cyclic voltammetry and flow electrolysis that 5AS easily undergoes a two-electron/two-proton oxidation process consistent with the formation of the corresponding 2-carboxy-1,4-quinoneimine compound **3** (Scheme 1). The described quinoneimine was not isolated but could be indirectly postulated from the different experiments. Subsequently, further reactions of the reactive 1,4-quinoneimine led to complex structures resulting in various polymeric species which presumably will not be volatile enough to be identified by GC analysis.

5. Conclusions

Aminosalicylates, e.g. 5AS will form several products when exposed to hydroxyl radicals. The compounds that were discovered so far by GC–MS analysis originate from a 1,4-benzoquinone monoimine intermediate (structure **3**, R = H or R = *N*-acetyl) which subsequently undergoes multiple reactions such as reductive 1,4-Michael addition with SH-reagents (e.g. compound **8**) or HCl, hydrolysis and reoxidation (e.g. compounds **4**, **5**, **9**) or additional decarboxylation (compounds **6** and **7**). The in vitro results obtained will allow a better estimation of the reactivity of aminosalicylates in terms of their radical scavenging properties and antiinflammatory potency. It is likely that the quinoneimines identified here also occur as in vivo intermediary metabolites of 5AS if this drug is acting as a radical scavenger and thus exert their therapeutic efficacy in IBD where toxic radicals are generated by activated neutrophils in the intestinal mucosa. Some of the detected products might be useful markers for the identification of yet unknown metabolites of 5AS in biological material including urine and feces of patients treated with 5AS.

Acknowledgements

This work was supported by the Robert Bosch Foundation, Stuttgart. We wish to thank Mrs B. Mauch and Mrs H. Köhler for excellent technical assistance and Mrs Dr. U. Hofmann for helpful suggestions. We thank Dr. P. Such, Bruker Analytische Meßtechnik, Rheinstetten, for supplying us with samples following reaction with $TiCl_3$ and H_2O_2.

References

[1] O.I. Aruoma, M. Wasil, B. Halliwell, B.M. Hoey and J. Butler, *Biochem. Pharmacol.*, 36 (1987) 3739.
[2] M.B. Grisham, K. Ware, S. Marshall, T. Yamada and I.S. Sandhu, *Dig. Dis. Sci.*, 37 (1992) 1383.
[3] A.B.R. Thomson, *Aliment. Pharmacol. Therp.*, 5 (1991) 449.
[4] L.R. Sutherland, G.R. May and E.A. Shaffer, *Ann. Int. Med.*, 118 (1993) 540.
[5] U. Klotz, *Clin. Pharmacokin.*, 10 (1985) 285.
[6] B.J. Dull, K. Salata, A. van Langenhove and P. Goldman, *Biochem. Pharmacol.*, 36 (1987) 2467.
[7] J.G. Williams and M.B. Hallett, *Biochem. Pharmacol.* 38 (1989) 149.
[8] H. Allgayer, S. Rang, U. Klotz, P. Böhne, J. Retey, W. Kruis and R. Gugler, *Dig. Dis. Sci.*, 39 (1994) 145.
[9] J. Jensen, C. Cornett, C.E. Olsen, S. Bondesen, J. Christensen, L.A. Christensen, J. Tjørnelund and S.H. Hansen, *Biochem. Pharmacol.*, 45 (1993) 1201.
[10] J. Jensen, C. Cornett, C.E. Olsen, J. Tjørnelund and S.H. Hansen, *Int. J. Pharm.*, 88 (1992) 177.
[11] H.J.H. Fenton and H.J. Jackson, *J. Chem. Soc.*, 75 (1899) 1.
[12] C.R.E. Jefcoate and R.O.C. Norman, *J. Chem. Soc.* (B), (1968) 48.
[13] C. Fischer, C.O. Meese and U. Klotz, *Biomed. Mass Spectrom.*, 11 (1984) 539.

[14] I. Ahnfelt-Rønne, O.H. Nielsen, A. Christensen, E. Langholz, V. Binder and P. Riis, *Gastroenterology*, 98 (1990) 1162.
[15] P. Hušek, *FEBS Lett.*, 280 (1991) 354.
[16] K.-G. Eckert, P. Eyer, J. Sonnenbichler and I. Zetl, *Xenobiotica*, 20 (1990) 333.
[17] M. Grootveld and B. Halliwell, *Biochem. J.*, 237 (1986) 499.
[18] D. Hawley and R.N. Adams, *J. Electroanal. Chem.*, 10 (1965) 376.
[19] D.J. Miner and P.T. Kissinger, *Biochem. Pharmacol.*, 28 (1979) 3285.
[20] A.L. Ginsberg, N.D. Davis and L.E. Nochomovitz, *Gastroenterology*, 102 (1992) 448.
[21] H. van Euler, H. Hasselquist and U. Lööv, *Ark. Kemi, Mineral. Geol.*, 25 A (1948) 1.
[22] R. K. Palsmeier, D.M. Radzik and C.E. Lunte, *Pharm. Res.*, 9 (1992) 933.