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Characterization of ^{60}Co γ-radiation induced radical products of antipyrine by means of high-performance liquid chromatography, mass spectrometry, capillary zone electrophoresis, micellar electrokinetic capillary chromatography and nuclear magnetic resonance spectrometry

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Abstract

Monitoring the amount of oxidative damage, caused by free radicals, is a major problem in free radical and aging research. Antipyrine is proposed as an exogenous marker for the biomolecular monitoring of oxidative stress. In this paper the characterization of the ^{60}Co γ-radiation products of antipyrine is described. Since mainly hydroxyl radicals are generated under these experimental conditions, hydroxylated derivatives can be expected. The reaction kinetics of antipyrine and hydroxyl radicals were examined. The results show pseudo first order reaction kinetics. In vivo hydroxyl radicals are one of the major causes of oxidative stress. The separation of the different derivatives was performed with high-performance liquid chromatography (HPLC) and with micellar electrokinetic capillary chromatography (MECC). Antipyrine and the radiolysis products were baseline separated with MECC and HPLC. The conversion of antipyrine, after exposure to different doses of γ-radiation, was determined with HPLC and with the more customized and cheaper analytical technique MECC. The conversion of antipyrine after exposure was calculated after analysis with MECC and with HPLC. The correlation coefficient between both techniques was 0.9984. The reaction products were characterized with nuclear magnetic resonance (NMR) and mass spectrometry (MS). The dissociation constants (pK_a values) of the radiation products were determined by means of capillary zone electrophoresis (CZE). The results identified the radiolysis products as *ortho*-, *meta*- and *para*-hydroxylated antipyrine. The *ortho*- and *meta*-hydroxylated isomers are different from the enzymatic metabolites formed in man.

Keywords: Antipyrine

1. Introduction

The average lifespan of man in modern western society has increased greatly since the beginning of this century. Because of the decreasing quality of life during the aging process, obvious reasons can be found why it is necessary to understand the biochemistry of aging. Free radicals are known to be one of the causes of aging and age-related diseases [1–4]. It

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is, however, still difficult to determine oxidative damage originating from free radicals.

To monitor the individual amount of oxidative damage, a marker that determines free radical damage is needed. Several methods, using endogenous markers, are described in scientific literature: e.g., the determination of pentane and butane in human breath [5–7], the state of lipidperoxydation [8–11] and DNA adducts (e.g., 8-hydroxydeoxyguanosine) [12–14]. A review on "the measuring of oxidative stress in vivo" is given by Hageman et al. [15]. The most commonly used test, determines the amount of malondialdehyde formed during oxidative stress [16,17]. However Draper et al. [18] and Cherif et al. [19] showed that the amount of oxidative damage can be misinterpreted if malondialdehyde is used as a marker.

Grootveld and Halliwell [20] used an exogenous marker, salicylate, and related the amount of 2,3- and 2,5-dihydroxybenzoates to oxidative damage. These molecules are not known as human metabolites and are considered as hydroxylated radical products of salicylate. Also phenylalanine [21], 4-nitrophenol [22], dopamine [23] and tryptophan [24] have been applied as markers for oxidative stress. In this research, the radiation products of antipyrine were characterized. Antipyrine is an antipyretic drug that is still being used to measure the total hepatic oxidase activity [25,26]. The properties of antipyrine make it a suitable marker for oxidative stress. One of the advantages of antipyrine is the well known metabolic pathway (see Fig. 1) since it has been studied for many years [27–30]. In the literature only two phenolic metabolites are described, 4′-hydroxyantipyrine [31] and 4,4′-dihydroxyanitpyrine [32]. *ortho*-Hydroxyantipyrine and *meta*-hydroxyantipyrine are not described in literature as human metabolites.

In a situation of oxidative damage, an excess of free radicals will react with antipyrine, as well as with biomolecules. Due to the reactive phenyl group in antipyrine, the reaction rate constant with hydroxyl radicals is in the order of 10^{10} l mol^{-1} s^{-1} [33]. In order to see which free radical products of antipyrine might be found in the human body during oxidative stress, in vitro experiments with a solution

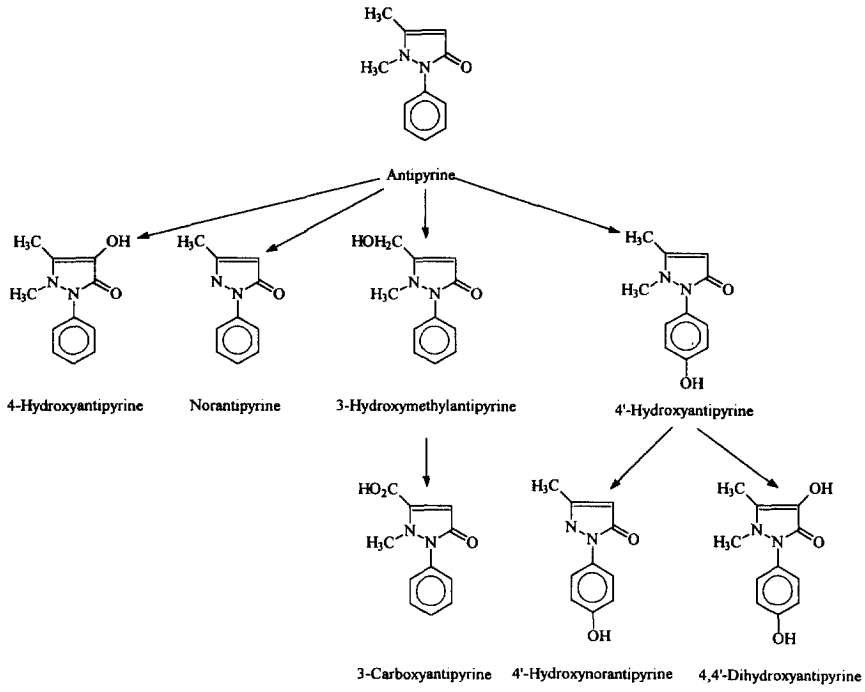

Fig. 1. Enzymatic metabolites of antipyrine in man [27–30].

of antipyrine in water have been carried out. This solution of antipyrine was exposed to a ^{60}Co γ-source in order to generate free hydroxyl radicals.

In this paper MECC, as well as HPLC were used as separation techniques for antipyrine and its free radical products. MECC has several advantages compared to HPLC: easy method development, fast analysis, high efficiency, small sample volume and low organic solvent consumption. However, MECC has a higher detection limit (10^{-5} *M*) for antipyrine and its radical products compared to HPLC (10^{-8} *M*).

2. Experimental

2.1. Sample pretreatment by ^{60}Co γ-radiation

For all experiments, a 1 m*M* antipyrine solution in demineralized water was used. This solution was exposed to γ-radiation under normal atmospheric conditions at the Pilot Plant for Food Irradiation (PROVO, Wageningen, Netherlands). ^{60}Co was used as a γ-radiation source. The dose varied from 0 to 10 kGy, the dose rate was 1.5 kGy/h. For the characterization of the radiation products, a dose of 4 kGy with a dose rate of 1.5 kGy/h was used. The temperature during the radiation was ca. 20°C.

2.2. Materials

Antipyrine (99%) was obtained from Janssen (Geel, Belgium) and sodium dodecyl sulfate (SDS) (>99%) was obtained from Sigma (Zwijndrecht, Netherlands). Acetonitrile (HPLC grade), KH_2PO_4 (>99%), sodium tetraborate·$10H_2O$ (>99.5%), boric acid (analytical-reagent grade), HCl and Tris (>99%) were obtained from Merck (Darmstadt, Germany).

2.3. Separation of antipyrine and hydroxylated antipyrine radiation products

2.3.1. Capillary zone electrophoresis

CZE experiments were performed, in order to determine the pK_a values of the radiation products of antipyrine. A P/ACE 2200 capillary electrophoresis system (Beckman, Fullerton, CA, USA) was used for all CZE experiments. The capillary was an untreated fused-silica capillary from J&W (Fulsom, CA, USA), 570 mm (l_c)×50 μm I.D., with an effective length of 500 mm (l_d). The capillary was rinsed for 1 min with 0.1 *M* NaOH and for 2 min with the running buffer prior to analysis. The applied voltage was +20 kV, the injection time was 5 s, the injection pressure was 0.5 p.s.i. ($3.3 \cdot 10^3$ Pa). The UV detector was operated at 214 nm. The running buffer consisted of 25 m*M* HCl adjusted to pH with Tris. The pH range used was 8.3–9.3.

2.3.2. Micellar electrokinetic capillary chromatography

For the MECC experiments, the same experimental setup as described in Section 2.3.1 was used. The UV detector was operated at 254 nm. A modified method of Brunner et al. [34] was used. The running buffer consisted of 50 m*M* SDS, 10 m*M* sodium tetraborate and was adjusted to pH 8.2 with boric acid.

2.3.3. High-performance liquid chromatography

A reversed-phase Bischoff Supersphere 100 RP18 Endcapped column (Biscchoff Chromatography, Leonberg, Germany), 150×3 mm I.D., d_p=4 μm was attached to a Philips P4 HPLC system (Unicam Analytical Systems, Eindhoven, Netherlands). The chromatographic system consisted of a Philips P4 4100 solvent delivery pump, a Philips P4 UV–Vis detector (operated at 254 nm) and a Marathon autosampler (Spark Holland, Emmen, Netherlands). A Merck–Hitachi D-2500 chromatointegrator (Hitachi, Tokyo, Japan) was used for data acquisition and peak analysis. The isocratic separation was performed with a solvent that consisted of 90% 20 m*M* KH_2PO_4 dissolved in water (pH 7.4) and 10% acetonitrile. The chromatographic separation was carried out at a flow-rate of 0.5 ml/min and 20 μl injection.

2.4. Isolation of the ^{60}Co γ-radiation products of antipyrine

2.4.1. Preparative high-performance liquid chromatography

In order to characterize the different radiation products, preparative HPLC was used to separate and

collect the different fractions of the radiation derivatives of antipyrine. One liter of 1 m*M* antipyrine solution in water, exposed to 4 kGy, was evaporated to dryness under vacuum at 45°C. The residue was dissolved in 50 ml demineralized water. A reversed-phase LiChrosorb RP18 Merck column, 100×16 mm I.D., d_p=5 μm, ε=60 Å was attached to an LKB HPLC system (Pharmacia, Uppsala, Sweden), comprising of two Model 2248 solvent delivery pumps connected to a high-pressure mixer, a Model 2252 LC controller, a Model 2211 Superfrac fraction collector, a Model 2510 uvicord SD detector (operated at 254 nm) and an autosampler (Spark Marathon, Emmen, Netherlands). Data acquisition was performed with the software package Caesar 4.0 for windows (van Mierlo Consultancy, Eindhoven, Netherlands). The separation was performed with a gradient using two different solvents: (A) 100% acetonitrile, (B) 20 m*M* NaH_2PO_4 solution in wwater. The separation was carried out at a flow-rate of 5 ml/min and 150 μl injection. The solvent program started at 10% acetonitrile and was kept constant for 5 min. A linear gradient increased acetonitrile to 14% within 5 min and was kept constant for 5 min before returning to initial conditions in 5 min.

2.5. Characterization of hydroxylated antipyrine radiation products

2.5.1. Mass spectrometry

Pneumatically assisted electrospray (ion spray) ionization mass spectrometry was conducted with an API 300 triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada) in the positive ionscan mode. The collected fractions were directly injected with a Harvard Apparatus syringe pump Model 11 (South Natick, USA). Scans were taken from *m/z* 160 to 600 with a scan duration of 4.5 s, using a step size of 0.1 u and a 1 ms dwell time per step. The mass spectrometer was set to the following parameters: ion spray voltage 5.2 kV and orifice voltage 10 kV. The nebulizer gas (air) and curtain gas (nitrogen) were adjusted to 1.5 l/min and 0.8 l/min, respectively. Ten spectra were averaged for one measurement.

2.5.2. Nuclear magnetic resonance spectrometry

The fractions, collected with preparative HPLC, with an *m/z* of 205 were evaporated to dryness under vacuum at 45°C and dissolved in ca. 3 ml ^2H_2O. ^1H NMR spectra of these fractions were recorded at 300.08 MHz on a Varian Gemini-300 spectrometer. About 64 scans were collected for a good signal-to-noise ratio for the hydroxylated products. All spectra were acquired with a set of 32 *k* data points. Chemical shifts are given in ppm downfield from tetramethylsilane and were referenced to the residual HDO resonance (δ=4.68 ppm).

3. Results and discussion

3.1. General discussion

During oxidative stress, a higher level of hydroxyl radicals are present in the human body. In order to see what radical products might be formed, we performed in vitro experiments with antipyrine as an exogenous marker. Due to its aromatic ring, antipyrine has a high reaction rate constant (10^{10} l mol^{-1} s^{-1} [33]) with hydroxyl radicals. A solution of antipyrine was exposed to ^{60}Co γ-radiation, causing the radiolysis of water. This method produces several oxidizing and reducing species, however it is chemically clean, has good reproducibility and has several advantages over other biochemical or chemical sources of free radicals. An important advantage of using a γ-^{60}Co free radical source is that only reactions of antipyrine with free radicals occur. There are no other chemicals that can scavenge the formed radicals. Also no chemicals are present that react with antipyrine to form different derivatives than the free radicals products of antipyrine.

We were interested in the different hydroxylated radiolysis products of antipyrine. In order to characterize these products, the different hydroxylated derivatives needed to be isolated. This section will first discuss the influence of the radiation dose on the conversion of antipyrine and the formation of its radiolysis derivatives. Secondly this section will discuss the analytical methods and their performances. Finally, the characterization of *ortho*-, *meta*- and *para*-hydroxyantipyrine will be discussed.

3.2. Influence of the radiation dose on the conversion of antipyrine and the formation of its radiolysis derivatives

In order to be able to make an estimation of the total breakdown by the γ-radiation, the influence of the radiation dose, on the conversion of antipyrine has been examined (Fig. 2). The conversion antipyrine was calculated from the normalized areas of the antipyrine peak, determined by means of MECC and HPLC, and plotted against the radiation dose. The conversion antipyrine can be calculated according to Eq. (3):

$$X_{AP} = \frac{[AP]_0 - [AP]_t}{[AP]_0} = 1 - \frac{[AP]_t}{[AP]_0} \quad (1)$$

where X is the conversion, [AP] is the concentration antipyrine.

$$AP + OH^{\cdot} \xrightarrow{K} APOH^{\cdot} \rightarrow \text{Reaction products}$$

where K is the reaction rate constant.

If the reaction between antipyrine and $OH^{\cdot}$ occurs according to pseudo first order kinetics, the concentration antipyrine is given by Eq. (2):

$$[AP]_t = [AP]_0 \exp(-K_{ps} t) \quad (2)$$

where $K_{ps} = K[OH^{\cdot}]$, t is the time (s).

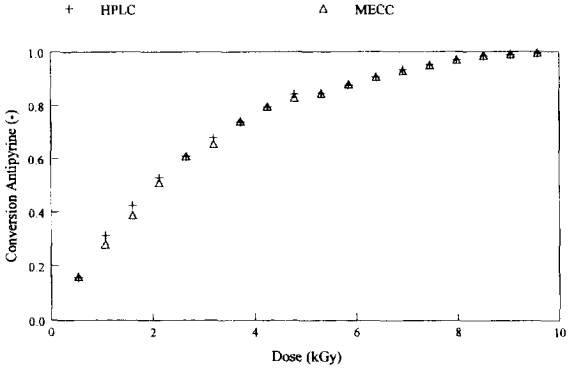

Fig. 2. Conversion of a 1 m*M* antipyrine solution in water after exposure to varying doses of γ-radiation (dose 0–10 kGy, dose rate 1.5 kGy/h). Analyses were carried out according to the MECC (△) and HPLC (+) procedure (see Sections 2.3.2 and 2.3.3).

Rearrangement of Eqs. (1) and (2) results in the following equation for the conversion of antipyrine:

$$X = 1 - C_0 \exp(-C_1 t) \quad (3)$$

where C_0 and C_1 are constants.

Experimental data was fitted according to Eq. (3). The correlation coefficient was 0.998. The assumption that antipyrine and $OH^{\cdot}$ react according to pseudo first order kinetics is right for the concentrations used in these experiments.

In order to characterize the different radiolysis products of antipyrine, we needed a γ-radiation dose, that produced these radiolysis derivatives in sufficiently high concentrations to perform NMR spectrometry experiments. Consequently, the relationship between the total formation of the radiolysis derivatives and the radiation dose has been examined (Fig. 3). Fig. 3 shows the formation of three hydroxylated antipyrine products during an increasing dose of the γ-radiation. It can be seen that after a radiation dose of 1 kGy [middle chromatogram (2)], *ortho*-, *meta*- and *para*-hydroxylated antipyrine have been formed. Fig. 3 also shows that some other products are being formed, some smaller peaks in the area until 5 min and a large quantity of a radiation product at approximately 7 min. After an exposure of 2.6 kGy

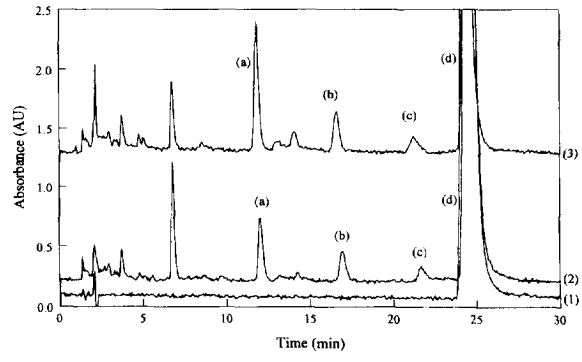

Fig. 3. Formation of radiation products of a 1 m*M* antipyrine solution in water vs. time, during exposure to ^{60}Co γ-radiation (dose rate 1.5 kGy/h). Dose: 0 Gy lower chromatogram (1); 1065 Gy middle chromatogram (2); 2650 Gy upper chromatogram (3). Analysis were performed according to the initial HPLC procedure (see Section 2.3.3). *para*-Hydroxylated antipyrine (a), *meta*-hydroxylated antipyrine (b), *ortho*-hydroxylated antipyrine (c), antipyrine (d).

[upper chromatogram (3)], the amount *ortho*-, *meta*- and *para*-hydroxylated antipyrine has increased compared to lower doses. We can also notice an increase in some of the products from the area until 5 min. We see a slightly decrease of the peak at 7 min. In this study only the hydroxylated products of antipyrine were characterized. The characterization of the other radiolysis products is still under investigation.

3.3. Separation of antipyrine and hydroxylated antipyrine radiation products

3.3.1. Capillary zone electrophoresis

In order to separate the different phenolic antipyrine derivatives CZE was used (Fig. 4A). The separation was not possible because the *ortho*- and *para*-hydroxylated antipyrine have the same absolute mobility and the same pK_a value (see Section 3.4.2). Therefore, at a certain pH both components have the same dissociation degree. Because they are isomers, they also have to same charge-to-mass ratio. Consequently their migration behavior in capillary electrophoresis experiments will be the same, so no separation will take place. In the CZE experiments, the *meta*-hydroxylated compound was baseline separated from the *ortho*- and *para*-hydroxylated. As expected for a neutral compound, antipyrine migrates with the electroosmotic flow (EOF). The total analysis time with CZE as the analytical technique was ca. 5 min.

3.3.2. Micellar electrokinetic capillary chromatography

Because separation of antipyrine and its hydroxylated derivatives was not possible with CZE, a micellar electrokinetic capillary method was developed. MECC analysis (Fig. 4B) showed baseline separation of the *ortho*-, *meta*- and *para*-hydroxylated compounds. Also baseline separation of the phenolic derivatives and antipyrine was achieved. In MECC a pseudo-stationary phase of SDS micelles is present, causing extra selectivity during the separation. The difference in selectivity for *ortho*- and *para*-hydroxylated antipyrine can be explained with the following assumption. *ortho*-Hydroxylated antipyrine can form an intramolecular hydrogen bond between the aromatic hydroxyl group and the carbonyl group of the pyrazolon ring, causing a more

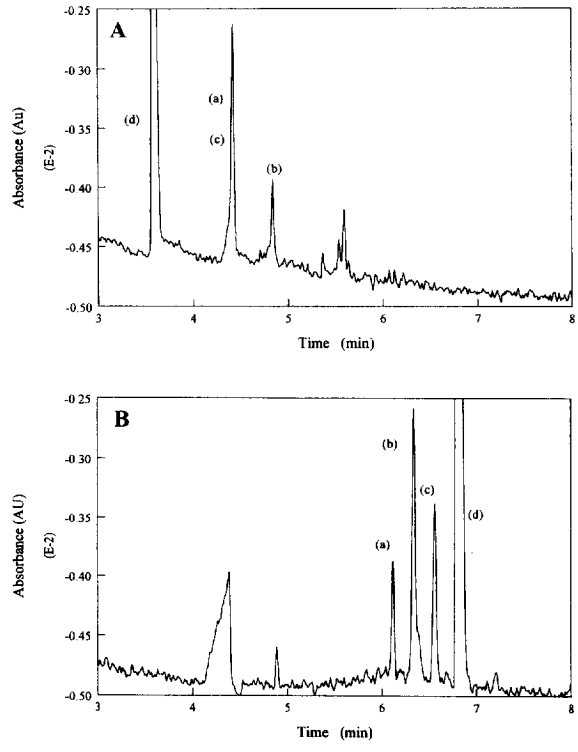

Fig. 4. (A) Capillary zone electropherogram of 1 m*M* antipyrine solution in water exposed to ^{60}Co γ-radiation (dose 4 kGy, dose rate 1.5 kGy/h). Separation conditions: $V = +20$ kV, $l_c/l_d = 570/500$ mm × 50 μm I.D., UV at 214 nm, injection time 5 s, (0.5 p.s.i.), temperature 20°C. Buffer: HCl (25 m*M*)–Tris pH 9.5: *para*-hydroxylated antipyrine (a), *meta*-hydroxylated antipyrine (b), *ortho*-hydroxylated antipyrine (c), antipyrine (d). (B) Micellar electrokinetic capillary chromatogram of 1 m*M* antipyrine solution in water exposed to ^{60}Co γ-radiation (dose 4 kGy, dose rate 1.5 kGy/h). Separation conditions: $V = +20$ kV, $l_c/l_d = 570/500$ mm × 50 μm I.D., UV at 254 nm, injection time 5 s, (0.5 p.s.i.), temperature 20°C, injection time 5 s (0.5 p.s.i.). Buffer: 10 m*M* sodium tetraborate, 50 m*M* SDS acidified to pH 8.2 with boric acid: *para*-hydroxylated antipyrine (a), *meta*-hydroxylated antipyrine (b), *ortho*-hydroxylated antipyrine (c), antipyrine (d).

hydrophobic character, thus increasing the affinity of the molecule with the hydrophobic tails of the micelles. Antipyrine, a neutral compound under these experimental conditions, will also have an interaction with the SDS micelles which migrate in the upstream mode. Therefore, antipyrine has a lower velocity in MECC compared to CZE. The migration times of *para*-, *meta*- and *ortho*-hydroxylated antipyrine were respectively 6.1, 6.3 and 6.6 min. The total analysis time was ca. 7 min.

3.3.3. High-performance liquid chromatography

Since HPLC is the most generally used analytical technique to separate antipyrine and its metabolites [25–30], we compared it with our method (MECC). An isocratic HPLC system was used. As expected, the retention time of the hydroxylated antipyrine derivatives is shorter than the retention time of antipyrine (Fig. 5). Antipyrine is more hydrophobic compared to its phenolic derivatives, resulting in a greater affinity of antipyrine for the hydrophobic stationary phase of the HPLC column. Baseline separation between *ortho*-, *meta*-, *para*-hydroxylated antipyrine and antipyrine was achieved. The retention times of the *ortho*-, *meta*- and *para*-hydroxylated antipyrines were respectively, 21.7, 12.0 and 6.8 min. The total analysis time was approximately 30 min.

3.3.4. Comparison of HPLC and MECC

In order to compare both analytical techniques, a solution of antipyrine in demineralized water was exposed to γ-radiation doses, varying from 0 to 10 kGy. The conversion of antipyrine was calculated from areas of the antipyrine peak before and after radiation. For MECC the normalized areas were used to calculate the conversion of antipyrine. These are the peak areas, corrected for the different migration times of the components. The conversion of antipyrine, determined with HPLC was plotted against the conversion of antipyrine determined with MECC. The correlation coefficient between HPLC and MECC was 0.9984. This is in agreement with earlier findings [35]. The good correlation justifies the use of MECC as a good alternative for HPLC for the separation of antipyrine and its hydroxylated derivatives. If the detection limit (10^{-5} *M*) is sufficient MECC is preferred to be used, due to the shorter analysis time and higher efficiency.

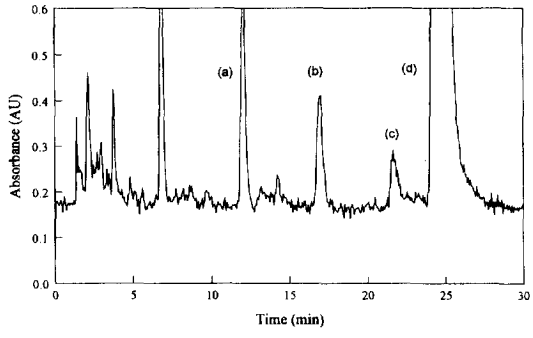

Fig. 5. High-performance liquid chromatogram of 1 m*M* antipyrine solution in water, exposed to ^{60}Co γ-radiation (dose 4 kGy, dose rate 1.5 kGy/h). Column, Supersphere 100 RP18 4 μm endcapped, 150×3 mm I.D., UV at 254 nm, injection volume 20 μl, temperature ca. 20°C. Eluent disodium hydrogenphosphate (20 m*M*, pH 7.4)–acetonitrile (90:10): *para*-hydroxylated antipyrine (a), *meta*-hydroxylated antipyrine (b), *ortho*-hydroxylated antipyrine (c), antipyrine (d).

3.4. Characterization of hydroxylated antipyrine radiation products

In order to characterize the different hydroxylated products, 1 l of a 10^{-3} *M* antipyrine solution in water was exposed to a dose of 4 kGy. The radiation dose resulted in an adequate conversion of antipyrine (ca. 80%). Hence the concentration hydroxylated derivatives of antipyrine was adequate to record NMR spectra, after fraction collection with preparative HPLC.

3.4.1. Mass spectrometry

Mass spectra were recorded from the different fractions, collected with preparative HPLC. The three components with retention times of 6.8, 12 and 21.7 min (Fig. 5) appear to have mass-to-charge ratio of 205, pointing at hydroxylated antipyrine. The components of the other fractions could not be charged with the electrospray interface. As mentioned before, the electrophilic aromatic ring of antipyrine reacts fast with radicals. It was also mentioned that the level of hydroxyl radicals is higher during a situation of oxidative stress. Therefore, we were interested in the hydroxylated derivatives of antipyrine. At this moment the other radiolysis products of antipyrine that are formed during radiolysis of water, are still under investigation.

3.4.2. Determination of the pK_a values of hydroxylated antipyrine radiation products

In order to see if these isolated components are phenolic antipyrines, the pK_a values of these components were determined with CZE. According to the Henderson–Hasselbalch equation:

$$pK_a = pH - \log\left(\frac{\alpha}{1-\alpha}\right) \quad (4)$$

where α is the degree of dissociation of the weak acid.

This equation combined with the relationship of the effective mobility (m_{eff}) with the absolute mobility (m_0) and the degree of dissociation according to Tiselius [36]:

$$m_{eff} = m_0 \alpha \quad (5)$$

Rearrangement of Eqs. (4) and (5) gives:

$$\frac{1}{m_{eff}} = \frac{1}{m_0} + \frac{10^{pK_a}}{m_0} \cdot [H^+] \quad (6)$$

The reciprocal value of m_{eff} was plotted against the concentration H^+ (Fig. 6). The pK_a values of *ortho*-, *meta*- and *para*-hydroxylated antipyrine were respectively, 9, 8.6 and 9, all in the range of the pK_a values of similar phenolic compounds.

3.4.3. NMR spectrometry

In order to characterize the molecular structure of the phenolic compounds (see Sections 3.4.1 and 3.4.2), NMR analysis of the three hydroxylated components revealed the identity of the various hydroxylated antipyrines. The aromatic region of the ^1H NMR spectrum (Fig. 7a) of the component with a retention time 6.8 min (see HPLC, Fig. 5) showed the typical multiplicity of a compound containing two (almost) identical sets of hydrogen nuclei, i.e., two doublets at 7.04 ppm and 7.25 ppm, respectively, of identical intensity. Clearly, this isomer can be identified as *para*-hydroxylated antipyrine. In addition, the component with a retention time 12 min (Fig. 7b) is identifiable as *meta*-hydroxylated antipyrine on the basis of its ^1H NMR spectrum. A triplet at 6.68 ppm with a coupling constant of 1.9 Hz, typical for a long range coupling, indicates an isolated hydrogen nucleus attached to an aromatic ring. Moreover, a triplet at 7.35 ppm with a larger coupling constant of 7.7 Hz can be seen, while two other sets of signals (roughly) comprise double doublets (6.73 ppm and 6.91 ppm), indicative for an aromatic ring containing three adjacent hydrogen nuclei. As a consequence, this leaves the component with a retention time 21.7 min (Fig. 7c) as *ortho*-hydroxylated antipyrine. An exact interpretation of the aromatic region of the ^1H NMR spectrum is not directly feasible, but two triplet-like signals (6.67 ppm and 7.33 ppm) and one doublet-like signal (7.10 ppm), together with a more complicated pattern (ca. 6.8 ppm) confirms this assumption to be correct.

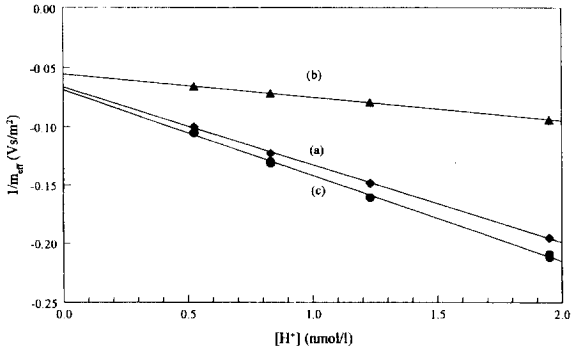

Fig. 6. Reciprocal effective mobility of *ortho*-, *meta*- and *para*-hydroxylated antipyrine, plotted against the concentration H^+. pK_a values were determined from the slope and intercept of the linear curves. pK_a *para*-hydroxylated antipyrine = 9.0 (a), pK_a *meta*-hydroxylated antipyrine = 8.6 (b), pK_a *ortho*-hydroxylated antipyrine = 9.0 (c).

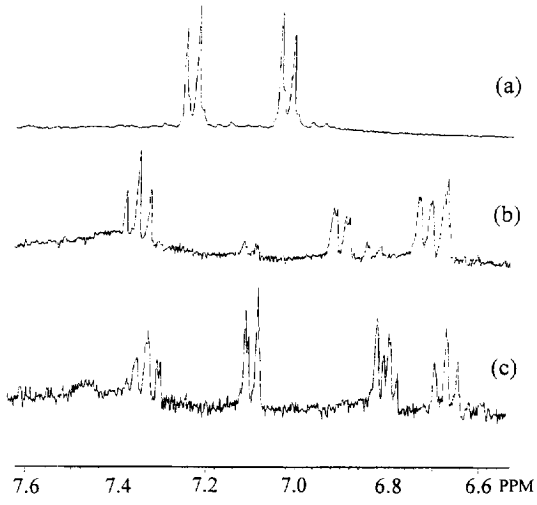

Fig. 7. ^1H NMR spectra of *para*-hydroxylated antipyrine (a), *meta*-hydroxylated antipyrine (b), *ortho*-hydroxylated antipyrine (c), 300.08 MHz Varian Gemini-300 spectrometer. Chemical shifts were referenced to the residual HDO resonance (δ 4.68 ppm).

4. Conclusions

This present study shows that exposure of an antipyrine solution in water to γ-radiation, leads to

the formation of at least three phenolic antipyrine derivatives of which two are not endogenously formed. These products are being formed as a result of the high concentration of hydroxyl radicals that is present during γ-radiation. Under the experimental conditions used in this study, the reaction between hydroxyl radicals and antipyrine occurs according to pseudo first order reaction kinetics. A good separation of these hydroxylated products is achieved with MECC as well as with HPLC. A good correlation between both techniques is shown. MS, NMR spectrometry and the determination of the pK_a values of the isolated fractions showed the formation of *ortho*-, *meta*- and *para*-hydroxylated antipyrine. The pK_a values were respectively, 9, 8.6 and 9.

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