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Determination of phenolic derivatives of antipyrine in plasma with solid-phase extraction and high-performance liquid chromatography–atmospheric-pressure chemical ionization mass spectrometry

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Abstract

This manuscript describes a method to determine antipyrine and its phenolic derivatives in plasma by reversed-phase high-performance liquid chromatography–mass spectrometry (RP-HPLC–MS). The sample pretreatment consisted of a C_{18} solid-phase extraction (SPE), to remove the salts and proteins. The retention behavior of antipyrine and its phenolic derivatives in the SPE procedure was estimated by the k values determined on a C_{18} HPLC column at different pH values and with different buffer compositions. Recoveries of antipyrine and its phenolic products were 90% in water and 100% in plasma. Atmospheric pressure chemical ionization (APCI) was used to introduce the components into the mass spectrometer. The mass spectrometer was operated in the single ion monitoring mode (SIM mode) as well as in the selective reaction (SR) mode. The SR mode or tandem MS resulted in the best signal-to-noise ratio, with a detection limit for antipyrine of 6 pg in 20 μl. For the different phenolic antipyrines, different target ions were used and conditions were optimized for each.
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1. Introduction

In the research field of free radicals in biological samples, determining the degree of free radical damage is still a major problem. One of the most aggressive radicals is the hydroxyl radical. Consequently, a good analytical method is necessary to determine the extent of free radical damage caused by hydroxyl radicals in vivo.

Several methods exist to quantify the damaging effects of these radicals. Most of the methods use endogenous markers to determine this damage, e.g. the determination of pentane and butane in human breath [1–3], the state of lipid peroxidation [4–7] and the formation of DNA adducts (e.g. 8-hydroxy-deoxyguanosine) [8–10]. For a review about this topic, see Hageman et al. [11]. The most commonly used method determines the amount of malondial-

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dehyde formed during oxidative stress [12,13]. However, Draper et al. [14] and Cherif et al. [15] showed that the extent of oxidative damage can be misinterpreted if malondialdehyde is used as a marker. The major disadvantage of using endogenous markers in vivo is the possibility that they are not only formed by free radicals but also by other pathways, e.g., enzymatically.

Another approach is the measurement of free-radical damage via radical trapping by exogenous markers. A method that is often used in free radical research is the measurement of phenolic free radical products of salicylic acid. Several researchers used the absolute amount of 2,3-dihydroxybenzoic acid (2,3-DHB) and 2,5-dihydroxybenzoic acid (2,5-DHB) to measure the degree of free-radical damage [8,16,17].

In this manuscript, an optimized analytical method is described to determine the amount of antipyrine and its phenolic derivatives. We have proposed the use of antipyrine as an alternative exogenous marker for free-radical damage, using the ratio of phenolic and native antipyrine. Antipyrine has good properties that are required for an exogenous marker: it reacts quickly with hydroxyl radicals [18], its metabolic breakdown is independent of blood flow to the liver, it can be administered in a high dose and its metabolic pathway is well known. When a solution of antipyrine in water is exposed to a high concentration of free radicals, resulting from γ-irradiation, three phenolic derivatives of antipyrine are formed (see Fig. 1) [19]. The *meta*-phenolic and *ortho*-phenolic derivatives of antipyrine are known not to be naturally formed metabolites. At a moderate level of oxidative stress, the concentration of these phenolic derivatives will be low in plasma. Therefore, a selective and sensitive analytical method is needed. From the literature, it is known that antipyrine and its phenolic derivatives can be separated by means of reversed-phase high-performance chromatography (RP-HPLC) with UV detection [19].

However, the expected concentrations in vivo will be lower then the detection limit of the LC–UV system. Therefore, a more sensitive detector is necessary. The sensitivity of the HPLC method increases by a factor of 100–1000 if a mass spectrometer is used as a detector instead of an UV spectrophotometer.

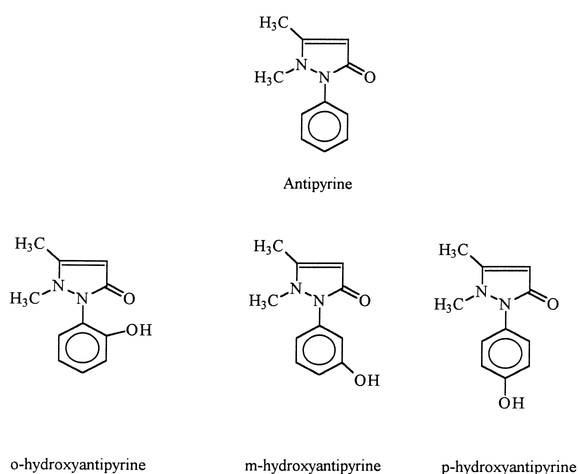

Fig. 1. Antipyrine and its free-radical phenolic derivatives.

In previous experiments, LC was combined with thermospray mass spectrometry (MS) for the characterization of ^{60}Co γ-irradiation-induced products of antipyrine [19]. HPLC–MS experiments require that all salts and proteins are removed from plasma. Therefore, a solid-phase extraction (SPE) sample pretreatment step was optimized. Capillary zone electrophoresis (CZE) was used to verify that salts and proteins were removed by the SPE procedure. The HPLC conditions were optimized for the HPLC–MS procedure. Methanol was used as an organic modifier, combined with a volatile buffer in different volume ratios.

The amount of methanol influences the dissociation of phenolic compounds, which leads to different retention behaviors. The pH and the composition of the eluent were optimized. Both modifications resulted in a better signal-to-noise ratio. Electrospray ionization (ESI) was first used as the ionization method in conjunction with MS detection. A disadvantage of ESI is the low flow that is needed. As a consequence, the eluent flow has to be split, which is a disadvantage for the determination of low concentrations as it results in a higher detection limit. Experiments were also carried out using an atmospheric pressure chemical ionization (APCI) chamber as the interface between the LC and the MS systems. APCI has the advantage that it can deal with the high flow-rates of the eluent, which result from the high evaporation rate obtained under atmospheric pres-

sure. APCI can handle flows of 1 ml/min, which are commonly used in conventional HPLC. Such a mass spectrometer is also easy to connect to a HPLC system.

In the SIM mode, the mass spectrometer is a detector that measures only specific pre-selected masses. In our experiments, the single ion mode was used at *m/z* 189 for antipyrine and *m/z* 205 for the phenolic derivatives of antipyrine.

Higher selectivities and sensitivities were achieved using the tandem MS mode. In the tandem MS mode, the selected ions are fragmented in the second quad of the MS. The fragments that are formed are detected in the third quad. This fragmentation process is called collision-activated dissociation (CAD). Mostly, the fragment with the highest intensity is chosen. The HPLC–APCI was optimized in the tandem MS mode for antipyrine at *m/z* 189/104 (precursor/product ion) and for the phenolic derivatives at *m/z* 205/120 (precursor/product) and *m/z* 205/98 (precursor/product).

2. Experimental

2.1. Materials

Antipyrine (99%) was obtained from Janssen (Geel, Belgium), methanol (HPLC grade) was obtained from Biosolve (Valkenswaard, The Netherlands), and ammonium acetate (>99%), acetic acid (p.a.), boric acid (p.a.), imidazole (>99%) and sodium hydroxide (>99%) were obtained from Merck (Darmstadt, Germany). De-ionized water was obtained from a Millipore apparatus (Bedford, MA, USA).

2.2.1. High-performance liquid chromatography

For the HPLC–UV experiments, a reversed-phase Bischoff Supersphere RP18 endcapped column (Bisschof Chromatography, Leonsberg, Germany), 150×3 mm I.D., d_p 4 μm, was attached to an LC system comprising a Bischoff 2200 HPLC pump connected to a Bischoff Lamda 1000 UV spectrophotometer.

For the HPLC–MS experiments, the same column was attached to a liquid chromatographic system comprising a Shimadzu LC-10AT pump (Shimadzu, Kyoto, Japan), an Applied Biosystems 785 UV detector (Applied Biosystems, San Jose, CA, USA) and a Triathlon autosampler (Spark Holland, Emmen, The Netherlands). The HPLC system was connected to a PE Sciex API-300 LC–MS–MS system (Perkin Elmer Sciex Instruments, Thornhill, Canada) with a heated nebulizer–atmospheric pressure chemical ionization (HN–APCI) interface.

2.2.2. Determination of the k value of antipyrine and its phenolic derivatives

The same column as described in the Section 2.2.1 for the HPLC–MS experiments was attached to a liquid chromatographic system consisting of a Bischoff 2200 LC pump and a Bischoff Lambda 1000 UV detector. The UV detector was connected to a multilab interface (Eindhoven University of Technology, Eindhoven, The Netherlands). Data acquisition was performed using Caesar 4.61 for DOS.

2.2.3. Capillary zone electrophoresis

All CZE experiments were performed on a P/ACE 2200 capillary electrophoresis system (Beckman, Fullerton, CA, USA). The capillary was an untreated fused-silica capillary (J&W, Fulsom, CA, USA), total length 270 mm, length to detector 200 mm, 75 μm I.D. Data acquisition was performed with P/ACE 3.0 software, and peak integration was performed using Caesar 4.1 for windows software.

2.2.4. Preparation of the standard sample solution

For all experiments, a $1 \cdot 10^{-3}$ *M* antipyrine solution in demineralized water was used. This solution was exposed to γ-irradiation under normal atmospheric conditions at the Interfaculty Reactor Institute at the Delft University of Technology (IRI, Delft, The Netherlands). ^{60}Co was used as a gamma irradiation source. The dose was 4 kGy, with a dose rate of 1.5 kGy/h. The temperature during the radiation was ca. 20°C.

2.2.5. High-performance liquid chromatography

Isocratic separation was performed with a solvent that consisted of water and methanol (75:25, v/v). The chromatographic separation was carried out at a flow-rate of 0.5 ml/min, with an injection volume of 20 μl. For the HPLC–UV measurements, the UV detector was operated at a wavelength of 254 nm.

The samples were analyzed in positive single ion mode (SIM mode) using APCI at *m/z* 189 for antipyrene and at *m/z* 205 for the phenolic derivatives of antipyrine. In the SIM mode, the dwell time for antipyrine and its phenolic derivatives was 200 ms and the pause time was 5 ms. In addition, the selective reaction (SR) mode of the MS was optimized. In the SR mode, the dwell time for the phenolic antipyrines was 750 ms and the pause time was 50 ms. Target ions were selected at *m/z* 189/104 for antipyrine and *m/z* 205/120 for the phenolic derivatives of antipyrine. The mass spectrometer was set to the following optimized parameters: needle current, 2 μA; orifice voltage, 30.0 V; focusing ring voltage, 150.0 V; temperature, 500°C; dwell time, 750 ms; nebulizer gas (synthetic air, purity 5.0) and curtain gas (nitrogen, purity 5.0), 13 and 10 l/min, respectively.

2.2.6. Determination of the capacity factor, k, of antipyrine and its phenolic derivatives

The isocratic separation was performed with three different eluents; a 10 mmol/l ammonium acetate buffer adjusted to pH 3.6 or pH 7 with acetic acid combined with an organic modifier. The third eluent consisted of demineralized water with organic modifier. The organic modifier was methanol, at concentrations of 10 to 40%. The chromatographic separation was carried out at a flow-rate of 0.5 ml/min, with an injection volume of 20 μl. The UV detector was operated at a wavelength of 254 nm.

2.2.7. Capillary zone electrophoresis

The injection pressure used was 0.5 p.s.i. (3.3 kPa) for the determination of both sodium and the proteins. The UV detector was operated at 200 nm. The capillary was rinsed for 1 min with 0.1 *M* NaOH and for 1 min with the running buffer prior to analysis. The applied voltage was +5 kV.

The running buffer for the determination of sodium was 10 mmol/l imidazole, adjusted to pH 4.5 with acetic acid. The injection time was 10 s.

The running buffer for the determination of plasma proteins was 150 mmol/l boric acid, adjusted to pH 10.0 with sodium hydroxide. The injection time was 1 s.

2.2.8. Solid-phase extraction

All SPE experiments were carried out using Alltech 200 mg C_{18} 3 ml columns (Alltech Associates, Deerfield, IL, USA). The column was conditioned with 10 ml of methanol, followed by 10 ml of deionized water. Plasma samples were diluted with water (1:1, v/v) and 1 ml of the diluted plasma was loaded on the column. The equilibration time was 5 min. After washing the column twice with 1 ml of 10 mmol/l ammonium acetate buffer, pH 5 (adjusted with acetic acid), in order to remove proteins and sodium chloride, the components were eluted with 3×0.5 ml of methanol. The samples were evaporated to dryness under a gentle stream of helium and dissolved in 1 ml of HPLC eluent.

3. Results and discussion

3.1. Determination of the capacity factor, k, for antipyrine and its phenolic derivatives

In order to determine antipyrine and its phenolic derivatives in plasma, which were formed during oxidative stress, SPE was optimized for plasma. To estimate the retention behavior of antipyrine and its phenolic derivatives on C_{18} columns, the capacity factor, *k*, of the different components was determined on an analytical column (see Section 2.2.6).

It has to be admitted that the packing material of the analytical column was different from that of the columns used in SPE. The reason for this was that no SPE columns were available that had the same packing material as that of the analytical column. The separation was carried out using HPLC with eluents that had different percentages of organic modifier. Fig. 2 shows the *k* value vs. the percentage of methanol in water. The retention behavior was also determined at two other pH values. In the pH range used, there was no significant difference between the *k* values of the components. It can be seen that, in all cases, the *k* value of antipyrine is higher or at least equal to the *k* values of the phenolic derivatives (see Fig. 2). This is as expected since the phenolic derivatives are more hydrophilic than antipyrine. It can also be seen that there is only a small difference in the *k* value between *ortho*-, *meta*- and *para*-hydroxyantipyrine. This is due to

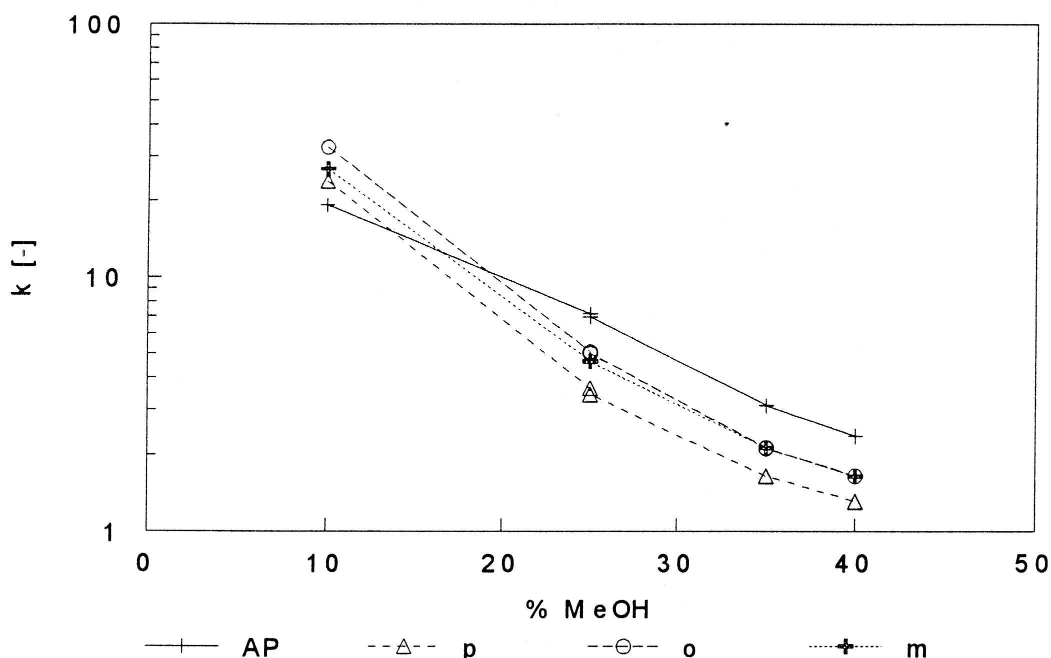

Fig. 2. k values of antipyrine (AP), *para*-hydroxyantipyrine (p), *ortho*-hydroxyantipyrine (o) and *meta*-hydroxyantipyrine (m) vs. the percentage of methanol (MeOH), which was used as the organic modifier. The k values were determined by means of RP-HPLC. For experimental conditions, see the Experimental section. The eluent used consisted of water combined with different percentages of methanol.

their pK_a values. The pK_a values of *o*-, *m*- and *p*-hydroxyantipyrine are 9.0, 8.6 and 9.0, respectively [19]. Since the highest pH of the eluent used was 6.8, the difference in the degree of dissociation would be virtually zero. Fig. 2 also shows that the k value in pure water will probably be sufficiently high to result in good retention behavior on C_{18} columns during SPE experiments.

3.2. Solid-phase extraction

3.2.1. The removal of proteins from plasma using solid-phase extraction

The separation of antipyrine and its phenolic derivatives by HPLC–MS requires the removal of proteins and salts from plasma. In order to achieve this, the solid-phase column was washed with a buffer solution after loading the diluted sample. Fig. 3 shows the CZE analysis of the eluent after washing the SPE column twice with 1 ml of buffer (solid line). An electropherogram of an untreated diluted plasma sample (dashed line) is also shown. If the SPE column was washed more than twice with 1 ml of washing solution, no proteins were found in the waste water. In addition, no proteins were left in the eluent. It can be concluded that washing with 2×1 ml of buffer results in the complete removal of all proteins that were present in plasma.

3.2.2. Removal of sodium salts from plasma by solid-phase extraction

The same washing procedure was used to remove the sodium chloride. If we look at the amount of sodium left in the sample after washing with different amounts of water (determined by CZE), we can see that sodium is almost completely removed after washing with 2 ml of water (see Fig. 4). The eluent also contained no sodium after the washing step.

Water as well as 10 mmol/l ammonium acetate buffer, pH 5, were used as washing solutions. Both methods resulted in the removal of the proteins and sodium chloride present in plasma. Plasma diluted with an irradiated antipyrine solution (see Section 2.2.4) was used to determine the recoveries of antipyrine and its phenolic products. Contrary to expectations regarding the k values, the results show

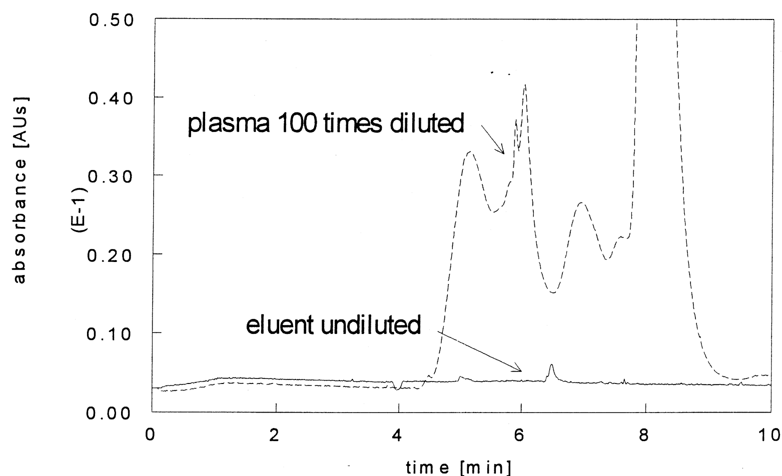

Fig. 3. Electropherogram of diluted plasma (untreated; dashed line) and undiluted eluent after washing with 2×1 ml of buffer using the SPE washing procedure. For the experimental conditions of the separation and the SPE procedure, see the Experimental section.

that *o*-hydroxyantipyrine was not present in the sample after elution if water was used as the washing solution (see Fig. 5).

Recoveries were determined using 10 mmol/l ammonium acetate as the washing solution and they are shown in Table 1. The recovery was determined for 1:1 (v/v) plasma–irradiated antipyrine (mmol/l range) as well as for 1:1 (v/v) water–irradiated antipyrine (mmol/l range). It can be seen that the recoveries were about 90% for the aqueous solutions and 100% for the plasma solutions. Irradiated solutions of antipyrine in the μmol/l range (range expected in real samples) showed the same recovery. Gravitation was used as the driving force for elution from the SPE columns and the viscosity of the plasma samples was higher. The contact time of the plasma samples with the column may be higher than that of the aqueous solution. This might explain the

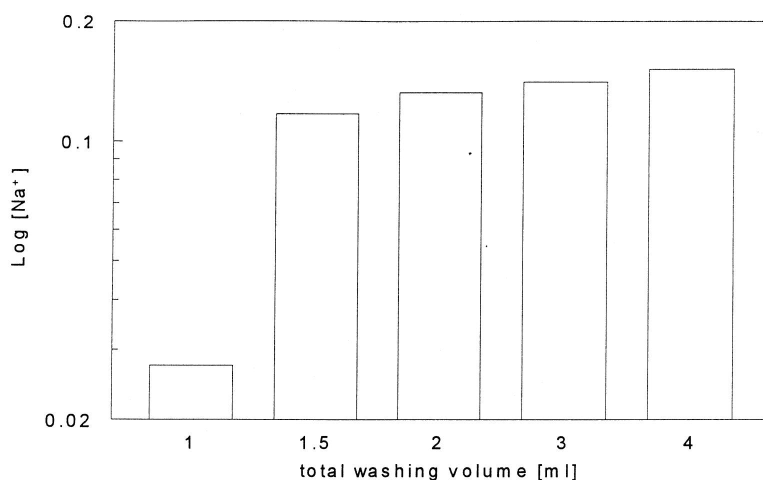

Fig. 4. Logarithm of the cumulated amount of sodium in the waste water after the washing step in the SPE procedure vs. the total volume of washing solution. For conditions used for the separation and the SPE procedure, see the Experimental section.

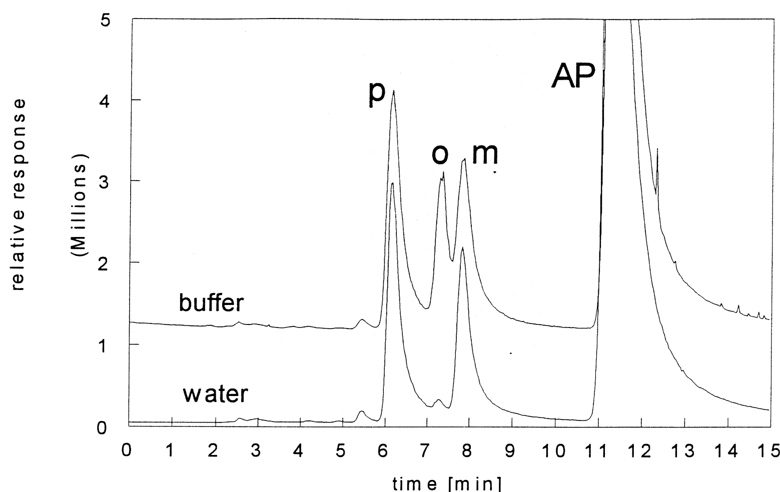

Fig. 5. HPLC–SIM–MS chromatograms of plasma spiked with irradiated antipyrine solution (1:1, v/v) following different washing procedures in the SPE sample pretreatment step using m/z 189 for antipyrine and m/z 205 for the phenolic derivatives. The upper chromatogram shows the results when buffer was used as the washing solution. The lower chromatogram shows the results when water was used as the washing solution. For the abbreviations used, see Fig. 2.

lower recoveries. Recoveries were determined with HPLC–UV and HPLC–MS. As expected, there was good agreement.

3.3. HPLC–MS analyses

3.3.1. Influence of the eluent flow-rate on the peak area

The peak area of all phenolic antipyrine components increases if the flow throughput in the APCI chamber is increased. To compare the influence of different flow-rates on the ionization efficiency of the ESI, the peak area was used as a measure of the sensitivity, since the noise is the same in all experiments. The signal height cannot be used since a lower flow-rate leads to peak-broadening in the column, resulting from diffusion during the separation and leading to lower peak heights. A higher flow-rate leads to a greater number of ions per unit time entering the orifice and, thus, to a higher peak area. This is as expected since the APCI–MS is considered as a mass-flow-sensitive detector. As mentioned previously, the goal of this research is to obtain a detection limit that is as low as possible, meaning the use of the highest flow that enters the mass spectrometer. In this case, there is a pressure limit caused by the dimensions of the column. The optimized (maximum) flow-rate was 0.5 ml/min. The signal-to-noise ratio is dependent on the ionization efficiency as well as on the plate number per meter.

Table 1
Recovery (R) determined with HPLC–UV and HPLC–MS and standard deviation (S.D.) (n=10) for the determination of antipyrine (AP) and *para*- (P), *meta*- (M) and *ortho*- (O) hydroxyantipyrine in spiked (mmol/l range) de-ionized water and human plasma (1:1, v/v)

	$R_{\text{HPLC-UV, H}_2\text{O}}$ ±(S.D.) [%]	$R_{\text{HPLC-UV, Plasma}}$ ±(S.D.) [%]	$R_{\text{HPLC-MS, Plasma}}$ ±(S.D.) [%]
O	93 (7)	103 (8)	99 (8)
M	91 (4)	102 (8)	104 (9)
P	91 (4)	98 (3)	100 (4)
AP	94 (1)	96 (6)	–

3.3.2. Influence of the eluent flow-rate on the efficiency

As mentioned in Section 3.3.1, flow-rate has a large influence on the peak area in the separation of antipyrine and its phenolic derivatives. The pressure caused by the flow-rate is not the only restriction that is present. Another parameter that is influenced by the flow is the efficiency. Experiments indicated that the plate number of the three phenolic derivatives of antipyrine decreased with increasing flow-rate, as expected according to classical chromatographic theory. The reason for this is that most of the practical chromatographic separations are carried out at flow-rates that are higher than the optimum. According to the theoretical $H–u$ curve, the plate height will increase with increasing flow and, thus, the number of plates will decrease.

Since sensitivity is the most important parameter in the determination of the phenolic derivatives of antipyrine, a flow-rate of 0.6 ml/min should be used. However, a flow-rate of 0.6 ml/min leads to a high pressure, which will lead to fast degradation of the column. For this reason, all further experiments were carried out at a flow-rate of 0.5 ml/min.

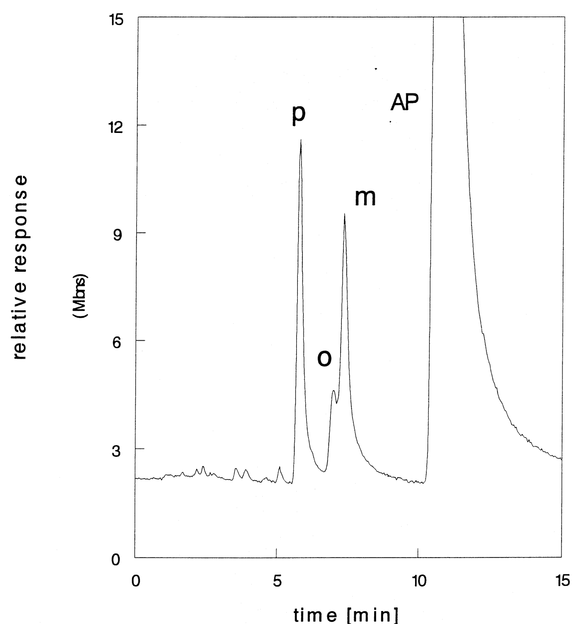

Fig. 6. HPLC–APCI–SIM–MS chromatogram of a 1-mmol/l irradiated antipyrine solution in water. Conditions for the γ-irradiation step were as described in Section 2.2.4. The HPLC–MS conditions were as mentioned in the Experimental section. For abbreviations, see Fig. 2.

3.3.3. HPLC–APCI–MS of antipyrine and its phenolic derivatives in the SIM mode

In order to analyze the phenolic derivatives of antipyrine, which are used as markers for oxidative stress, an APCI method was developed. The interface that was used was an APCI chamber. The advantage of the APCI method is that it is able to handle the high flow-rates of the eluent. This means that, after the column, no splitting of the eluent (leading to loss of signal) is necessary. Another advantage is that the APCI method can handle eluents that consist mainly of water. In this case, the phenolic derivatives of antipyrine were separated using an eluent that contained 25% methanol. Also, since the flow-rate of the eluent was 0.5 ml/min, APCI seemed the appropriate method to use (see Fig. 6). The HPLC–MS experiments with the APCI resulted, under these experimental conditions, in a lower detection limit for antipyrine than found using the HPLC method with ESI.

One of the reasons for the lower detection limit using APCI compared to ESI was the high flow-rate that is used. A flow-rate of 0.5 ml/min is too high for ESI, which is limited to flow-rates of up to 0.2 ml/min. A reason for this is that the large amount of water in the eluent cannot be evaporated in ESI. The evaporation of the solvent is necessary to create a high concentration of ions that enter the orifice. Consequently, the intensity of the signal decreases dramatically at a flow-rate of 0.5 ml/min when ESI is used. A possible way to increase the sensitivity of the ESI might be to use micro HPLC columns. The SIM mode has a high selectivity since only the target ions (m/z=188.9 for antipyrine and m/z=204.9 for its phenolic derivatives) are measured. However, *m*- and the *o*-hydroxyantipyrine were not baseline separated. An example of a real sample is shown in Fig. 7. In this experiment, a pig was exposed to transportation stress after administration of antipyrine. All three phenolic components were present in the plasma sample that was taken after transportation. The concentrations, estimated from the signal-to-noise ratio, were of the order of μg/l. The plasma sample before administration of antipyrine showed

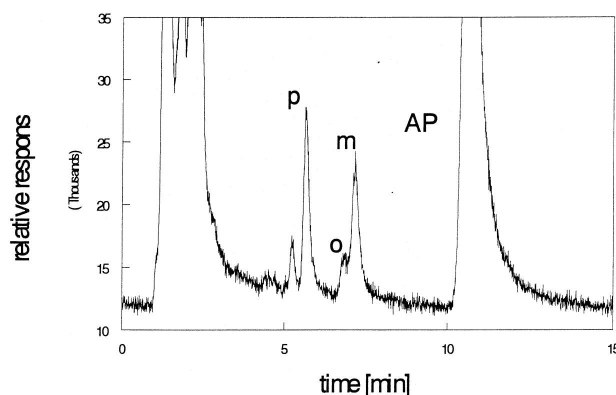

Fig. 7. HPLC–APCI–SIM–MS chromatogram of a plasma sample from a pig that was exposed to transportation stress. Analysis and sample pretreatment were carried out as described in the Experimental section. For abbreviations, see Fig. 2.

no interfering peaks at the retention times of the phenolic compounds.

3.3.4. APCI–HPLC–MS of antipyrine and its phenolic derivatives in the selective reaction mode

In order to improve the sensitivity and selectivity of the HPLC–APCI–SIM–MS method, MS was used in the SR mode. The use of the SR mode can lead to a higher specificity and a higher signal-to-noise ratio. The signal height will decrease since the extra quadrupole leads to a loss of ions. On the other hand, the noise will also decrease as there is a lower background signal. A product ion scan of the fragments of antipyrine (m/z 188.9) and its phenolic derivatives (m/z 204.9) was recorded. The result was a complicated spectrum of fragment ions. Selective target ions were chosen to determine antipyrine and its phenolic derivatives (see Fig. 8). Antipyrine showed a major component at m/z 104 and 131. To distinguish the *m*- and *p*-hydroxyantipyrine from the *o*-hydroxyantipyrine (see Fig. 9), analyses were carried out with m/z 120 and m/z 98. The *m*- and the *p*- adducts showed a major fragmentation adduct of m/z 120. The *ortho* component showed a major fragmentation ion at m/z 98 and a small percentage of m/z 120. The different fragmentation pattern of the *ortho* derivative compared to the *meta*- and *para* derivatives is a consequence of the internal hydrogen bridge that can be formed between the phenolic OH group of *o*-hydroxyantipyrine and the carboxyl group of the pyrazoline group. This is only possible if the OH group is in the *ortho* position because of the short distance between the atoms. This leads to a different diversion and a different fragmentation

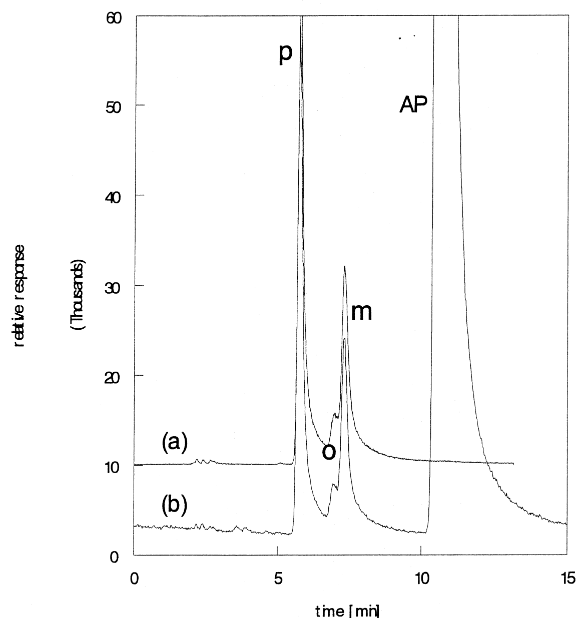

Fig. 8. HPLC–APCI–SR mode–MS chromatogram of a 1-mmol/l irradiated antipyrine solution in water. The fragment ions were m/z 204.9/120.0 and 188.9/104 [lower chromatogram (b)]. To improve the sensitivity, a chromatogram with only the fragment ion m/z 204.9/120.0 was recorded [upper chromatogram (a)]. All other HPLC–MS conditions were as mentioned in the Experimental section. For abbreviations, see Fig. 2.

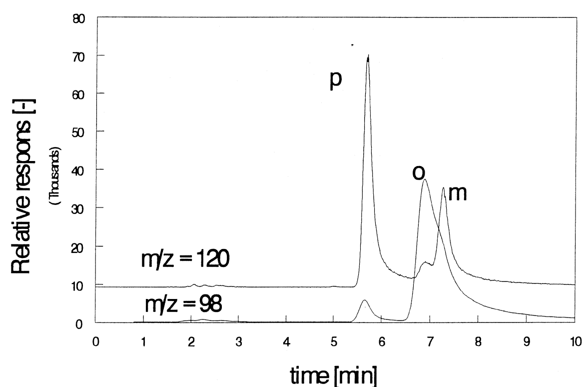

Fig. 9. HPLC–APCI–SR mode–MS chromatogram of a 1-mmol/l irradiated antipyrine solution in water. The fragment ions were *m/z* 204.9/120.0 and 204.9/98. All other HPLC–MS conditions were as mentioned in the Experimental section. For abbreviations, see Fig. 2.

pattern. This is a well known effect in mass spectrometry, called the *ortho* effect.

The SR mode was optimized for the above-mentioned daughter ions. The signal-to-noise ratio of the SR mode had the same order of magnitude as the signal-to-noise ratio of the SIM mode. Since the sensitivity is the most important parameter to optimize in this research, a separation was carried out where only *m/z* 205/120 was determined (see Fig. 8). This increased the signal-to-noise ratio by a factor of ten compared to the measurements with both daughter ions (see Fig. 8). The use of HPLC–APCI–SR mode resulted in a detection limit (α=0.05) of 6 pg for antipyrine. The limit of quantification (LOQ) (α=0.05) was 18 pg, according to the International Union of Pure and Applied chemistry (IUPAC) model. The precision was 5%. The repeatability was 2.8·s=14%. The intermediate precision was also 14%. A calibration curve was determined for antipyrine. The calibration curve showed linearity (R^2=0.9962) in the range 10^{-5}–10^{-9} *M*. The slope of the calibration curve was $5 \cdot 10^6$, the intercept was 1.9. Fig. 10 shows a chromatogram of plasma spiked with a 1-m*M* solution of irradiated antipyrine. The detection limit (α=0.05) was 190 pg, which was a factor of 30 higher than for the standard solutions. It can also be seen that the unspiked plasma does not show any interfering peaks at the retention times of the phenolic derivatives of antipyrine.

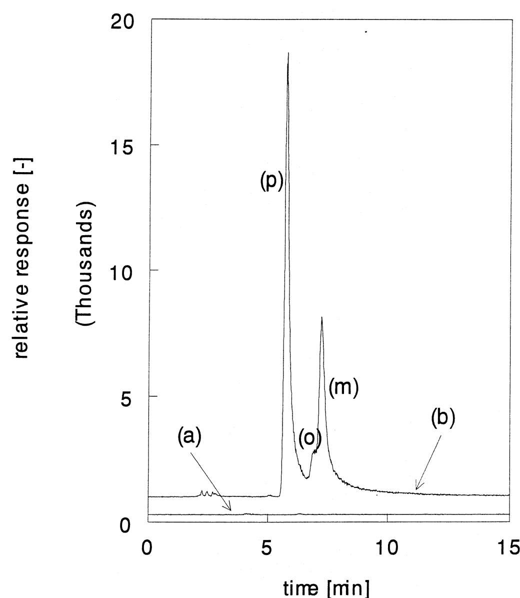

Fig. 10. HPLC–APCI–SR mode–MS chromatogram of a plasma sample (a) and a plasma sample spiked (1:1, v/v) with a 1-mmol/l irradiated antipyrine solution after SPE. The fragment ions were *m/z* 204.9/120.0. The solid-phase conditions and all other HPLC–MS conditions were as mentioned in the Experimental section. For abbreviations, see Fig. 2.

4. Conclusions

This solid-phase extraction study has resulted in a good procedure for the HPLC–MS determination of antipyrine and its phenolic derivatives in plasma. Optimization of the SPE procedure indicated that washing twice with 1 ml of water results in complete removal of all of the proteins and almost all of the sodium salts, which is necessary for the MS experimental conditions. However, washing with water will result in the loss of *o*-hydroxyantipyrine. Therefore, the use of a buffer is necessary under the conditions used in the washing procedure. The *k* values on C_{18} columns, determined against the volume of organic modifier, were high when a buffer was used, resulting in the complete recovery of antipyrine and its phenolic derivatives. Optimization of the HPLC–MS procedure resulted in the use of APCI instead of electrospray as the interface between the LC and the MS detection systems. The use of APCI in combination with MS–MS resulted in higher a signal-to-noise ratio for antipyrine. The

MS–MS mode resulted in different fragment ions for *p*- and *m*-hydroxyantipyrine, and *o*-hydroxyantipyrine. The detection limit for antipyrine, determined using HPLC–APCI–MS in the MS–MS mode, was 6 pg in standard solutions and 190 pg in plasma. The calibration curve was linear over a dynamic range of four decades. These results show the feasibility of using phenolic antipyrine derivatives as an indicator of oxidative stress.

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