Modified Analytical Method for Hydroxyl Radicals Using Spin Trapping and Ion Exclusion Chromatography**

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Ion exclusion chromatography (IEC) is the predestinated chromatographic technique for weak acids separation and analysis. Preliminary results are presented concerning the application of IEC to the determination of hydroxyl free radicals with the use of hydroxybenzoate spin trapping. Products of the reaction, dihydroxycarboxylic acids, are retained due to the mixed ion exclusion and π-electron interaction retention mechanism. The method was applied to the determination of hydroxyl radical trapping potential of some organic solvents, phenylacetates, and biogenic polyamines. The later were found to scavenge efficiently hydroxyl radicals, which property may contribute to their cyto- and neuroprotective properties.

Free radicals (FR) are implicated in the pathogenesis of many diseases including, e.g., cancer, Alzheimer’s and Parkinson’s diseases and rheumatoid arthritis. They may also significantly contribute to cellular degenerative changes of aging. FR are present in every living cell, but in pathological conditions, because of their increased production and/or decreased capacity of antioxidant systems, redox homeostasis can be severed, leading to cell degeneration and ultimately death [1–4]. Cell constituents susceptible to free radical-induced damage include polyunsaturated lipids, proteins and nucleic acids. Of cellular organelles the most prone to the FR attack are mitochondria and cell membranes [5].

Among FR to which cells are exposed the most reactive is the hydroxyl radical [6], which is able to interact with a number of organic compounds through addition, free radical substitution or electron transfer. One of the most damaging effects of hydroxyl radicals is the chain peroxidation of polyunsaturated lipids located in cellular and intracellular membranes. It is estimated that a single OH radical may damage up to 700 lipid molecules.

Due to high reactivity hydroxyl radicals are short-lived and difficult to detect directly. However, they can be chromatographically analyzed after spin trapping. Benzene ring of an aromatic compound (spin trap) is attacked by OH radical, and hydroxylated products of this reaction are separated and detected. As spin traps, endogenous phenylalanine, or exogenous aspirin can be used [7]. The aspirin-based assay is the most popular. In the biological environment aspirin (o-acetylsalicylic acid) is quickly hydrolyzed to salicylic acid which reacts with hydroxyl radicals giving three main products: 2,3- and 2,5-dihydroxybenzoic acids (DHBA) and o-catechol (with the yields of 49, 40 and 11%, respectively). These derivatives may be separated using reversed phase HPLC with photometric detection. Electrochemical detection is more sensitive, but is applicable only to DHBA [7,8].

Salicylic acid is not the optimal spin trap for hydroxyl radicals. Detection limit is increased because two main products are formed, and aspirin and its derivatives present in food may contaminate biological samples. To avoid these problems Ste-Marie at al. have proposed the use of p-hydroxybenzoic (PHB) acid which upon the reaction with OH radicals is converted to one main product, 3,4-dihydroxybenzoic acid [9].

To improve the analytical properties of the aspirin- and PHB-based spin trap assays of hydroxyl radicals, we have applied ion exclusion chromatography for the analysis of the reaction products. Ion exclusion chromatography (IEC) is widely used for separation of mixtures of ionic and nonionic compounds, and of weak acids or bases [10]. The characteristic feature of this technique is that analyzed ionic compounds and dissociated functional groups of the ion-exchange resin have the same electric charge. It follows that samples of negatively charged ions, e.g., dissociated acidic compounds, are separated on cation exchange resins with anionic functional groups (usually sulfonic acid groups). Similarly, samples containing positively charged spe-
cies (bases) are separated on the anion exchange resin containing cationic functional groups (usually tetraalkylammonium groups). The same columns can be used in IEC and in ion exchange chromatography, although in the first case true ion-exchange reaction is not involved.

Chromatographic resin commonly used in IEC is the macro-porous styrene and divinylbenzene copolymer (PS/DVB). For certain compounds the retention observed is greater than that implied by the ion exclusion mechanism. This can be explained by hydrophobic as well as \( \pi \)-electron interactions of analyte with the resin network [10]. The aforementioned mixed retention mechanism makes IEC a predestinated technique for separation and assay of weak, aromatic acids.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of P–580–A–LPG degasser and pump, STH–585 column oven, UVD 170S four channel photometric detector (Gynkotek, Germering, Germany) and 2097 injector (Rheodyne, USA). The system was controlled under Chromleon (Gynkotek, Germering, Germany) software installed on an IBM–PC Pentium computer. Amor (Spark Holland, Netherlands) amperometric detector equipped with V–560 digital multimeter (Meratronix, Warsaw, Poland) and Rex–101 (Pharmacia–LKB, Sweden) chart recorder were used. Five different columns were tested: Hibar RP–18 5 \( \mu \)m, 250 \( \times \) 4 mm I.D. (E. Merck, Darmstadt, Germany), Separon SGX CN 5 \( \mu \)m, 150 \( \times \) 4 mm I.D. (Tessek, Prague, Czech Republic) and three ion exclusion columns. Two of these were strong cation exchange columns, one based on silica gel (Spherisorb SCX 5 \( \mu \)m, 250 \( \times \) 4 mm I.D. (ISCO, USA)) and the other on PS/DVB (TSK–GEL SCX(H\(^{+}\)) 5 \( \mu \)m, 300 \( \times \) 7.8 mm I.D., >4.2 meq/g. (TosoHaas, Japan). The third was a weak cation exchanger (Na\(^+\)), small ion capacity (>0.1 meq/mL) based on methacrylate polymer (TSK–GEL CM–5PW, 50 \( \times \) 7.8 mm I.D. (TosoHaas, Japan). Incubation was performed using TB–9414 (JWE-electronic, Warsaw, Poland) Thermoblock.

Materials

Adenosino-diphosphoric acid (ADP), polyamines and aromatic acids were obtained from Sigma (St. Louis, MO, USA), hydrogen peroxide and FeCl\(_2\) from Merck (Darmstadt, Germany), Desferal from Novartis (Basle, Switzerland), ascorbic acid from Avocado (Heysham, England). Other reagents were of analytical reagent grade and were used without further purification. The Milli–Q (Millipore, Bedford, USA) water system was used to prepare all solutions. The mobile phases were filtered through a Millipore 0.22 \( \mu \)m membrane filter and degassed in an ultrasonic bath prior to use.

Procedure

Chromatographic experiments were performed with a flow rate 0.9 mL min\(^{-1}\). Column was stabilized at 20°C for 1 h prior to the chromatographic measurements. The following mobile phases were tested: (i) 1 mmol L\(^{-1}\) sulfuric acid, 0.125 mmol L\(^{-1}\) EDTA and 1 mmol L\(^{-1}\) KCl in 20% acetonitrile, (ii) acetate-ci-
trate buffer (pH 4.3) with 0.125 mmol L⁻¹ EDTA, (iii) as the previous one, with the addition of 3 mmol L⁻¹ tetrabutylammonium perchlorate.

10 mmol L⁻¹ stock solutions of the analyzed compounds were prepared in Milli-Q water and diluted to the required concentration before use. The samples were injected into the chromatographic system with a 100 µL syringe (Hamilton, Reno, USA) through the injection port. Volumes injected were 20 µL. Output signal from the photometric detector working simultaneously at 205, 215, 254 and 280 nm as well as amperometric detector working at +0.8 V vs Ag/AgCl were continuously displayed on the chart recorder and computer.

Hydroxyl radicals were generated through Fenton reaction [11] by 1 min incubation of 0.5 mmol L⁻¹ Fe²⁺, 2 mmol L⁻¹ ADP, and 2 mmol L⁻¹ H₂O₂ in 50 mmol L⁻¹ phosphate buffer (pH 7.4) in the presence of 1 mmol L⁻¹ p-hydroxybenzoic acid and analyzed sample at 37°C. The reaction was stopped by 2 mmol L⁻¹ DMSO and 0.1 g mL⁻¹ Desferal, and the reaction mixture was immediately analyzed by HPLC.

RESULTS AND DISCUSSION

Separation

It turned out that on the reversed phase column dihydroxybenzoic acids are eluted near the dead column volume, they are not completely separated and may overlap with catechol- and indoleamines frequently present in biological samples. Similar results were obtained on ion exclusion column based on the sulfonated silica gel. The best separation was obtained using ion exclusion column based on PS/DVB (Fig. 1).

![Figure 1](image-url)
In this case higher retention of aromatic acids can be explained by their \( \pi \)-electron interaction with the resin skeleton [10]. This is confirmed by the decrease of retention with the increased concentration of organic modifier in the mobile phase. Electric permittivity of the organic solvent (acetonitrile) is smaller than that of water. Consequently, the samples are less ionized and this, according to the ion exclusion retention mechanism, should increase retention. However, the opposite effect has been observed, which is suggestive of hydrophobic interactions. Because of its superior performance the PS/DVB column was used for further investigations.

**Detection**

Two chromatographic detectors, amperometric (+0.8 V vs Ag/AgCl) and photometric (254 nm), were compared. It turned out that the lower detection limit for dihydroxybenzoic acids was obtained on amperometric detector. For 3,4-dihydroxybenzoic acid it was below 1 pmol with signal/noise ratio = 3 (Fig. 2).

![Figure 2. Effect of the potential on the detection limit of 3,4-dihydroxybenzoic acid. Chromatographic conditions as on Figure 1](image)

This sensitivity appears sufficient for measurements in biological samples. Detection limit of the photometric detector was nearly two orders of magnitude higher, but this detector appeared more sensitive for \( p \)-hydroxybenzoic acid. Both detectors connected serially enabled efficient detection of both the substrate and the product of the reaction.

Hydrodynamic voltammograms of \( p \)-hydroxybenzoic acid (0.01 and 1 mmol L\(^{-1}\)) and 3,4-dihydroxybenzoic acid (0.01 mmol L\(^{-1}\)) are presented on Figure 3. It was found that the potential equal to +0.8 V vs Ag/AgCl electrode enabled the detection of 3,4-dihydroxybenzoic acid. At this potential \( p \)-hydroxybenzoic acid (as well as salicylic
acid) was detectable only at concentrations above 1 mmol L$^{-1}$. The increase of potential increased peak height (Fig. 3), but at the same time the noise level increased. The minimum of the detection limit was found at +0.8 V (Fig. 2), and this potential was used in further experiments.

![Graph showing hydrodynamic voltammograms](image_url)

**Figure 3.** Hydrodynamic voltammograms of 10 µmol L$^{-1}$ 3,4-dihydroxybenzoic (squares), 10 µmol L$^{-1}$ (triangles) and 1 mmol L$^{-1}$ p-hydroxybenzoic acids (circles). Electrochemical detector +0.8 V vs Ag/AgCl. Other chromatographic conditions as on Figure 1

### Spin trapping

The products of hydroxyl radicals spin trap reaction with aromatic acids were analyzed using chromatographic separation with electrochemical detection. Initially we used the well known method based on salicylic acid [6–8]. As mentioned earlier, when salicylic acid is used, the two main reaction products (2,3- and 2,5-dihydroxybenzoic acids) are not completely separable from each other and from other compounds present in biological matrix. Furthermore, these acids are generated spontaneously during sample preparation. Similar phenomenon has been also reported by the others [12]. It could have been caused by metal-catalyzed hydroxylations, because higher chromatographic peaks of dihydroxybenzoic acids were obtained when syringes with metal pistons were used for sample injection.

When p-hydroxybenzoic acid was used as a spin trap, the single reaction product 3,4-dihydroxybenzoic acid is completely separated from spin trap agent (Fig. 1). Furthermore, the improvement of the reproducibility obtained by using syringes with glass pistons and the enrichment of mobile phases with EDTA was less marked. For further investigation the method based on p-hydroxybenzoic acid as a spin trap, ion exclusion column and amperometric detector was used.
Determination of the antioxidant capacity

The method described above has been employed to the determination of the ability to scavenge OH radicals by various substances referred to as their „antioxidant capacity”. In this assay the reaction mixture contains both the substance tested and the „detector” spin trapping agent PHBA. Hydroxyl radicals are generated by Fenton reaction as described elsewhere [11], and the radicals are scavenged by both the detector and the analyte. If the analyte „performs better” than the detector, generation of the dihydroxybenzoic acid is decreased. This assay enables to compare the OH radical scavenging performance of various substances.

The measurements of the antioxidant capacity were repeated 4–6 times for each analyte and the results were averaged and expressed relative to the average result for the control samples containing no analyte.

On Figure 4 the heights of chromatographic peaks obtained for some organic solvents (methanol, ethanol and dimethyl sulfoxide (DMSO)) are presented compared to the control (no analyte).

Figure 4. Antioxidant capacity of methanol, ethanol and dimethyl sulfoxide. Hydroxyl radicals were generated using (ADP/Fe(II)/H₂O₂) system, and trapped with p-hydroxybenzoic acid in the presence of the solvents tested at 0.1% concentration. Reaction product (3,4-dihydroxybenzoic acid) was assayed using ion-exclusion chromatography. The control sample contained no analyte, therefore the height of the reaction product with the detector substance was 100%. Chromatographic conditions as on Figure 3. Data presented as means ±SD.

These solvents are thought to be relatively effective hydroxyl radical scavengers, which property may be responsible for some protective activity of DMSO observed during oxidative stress in brain [13,14]. Our results confirm that all these solvents
possess antioxidant capacity, which is particularly high in the case of DMSO. The decrease in chromatographic peak height of dihydroxybenzoate is proportional to the analyte concentration, but it also is characteristic for each analyte, which probably depends on the rate constant of the reaction with hydroxyl radicals. These rate constants are $8.3 \times 10^8$, $2.2 \times 10^9$ and $7.0 \times 10^9$ mol L$^{-1}$s$^{-1}$ for methanol, ethanol and DMSO, respectively [15,16].

![Graph showing antioxidant capacity of biogenic polyamines]

**Figure 5.** Antioxidant capacity of biogenic polyamines; AGM — agmatine, PUT — putrescine, SPD — spermidine and SPE — spermine. Conditions as on Figure 4

On Figure 5 the heights of chromatographic peaks obtained for biogenic polyamines putrescine, spermidine, spermine and agmatine are presented. These polyamines occur in virtually all living organisms [17]. They are essential in cell growth and replication functions. They also bind antagonists of some common cations like K$^+$, Mg$^{2+}$ or Ca$^{2+}$ and putative epidermal antioxidants [18]. Increases of their concentration are observed during reperfusion after short ischemia [19]. It remains controversial whether their overproduction has neurotoxic or neuroprotective effects [20,21]. Our results indicate that these amines are able to scavenge OH radicals [22]. Similarly to the organic solvents, it appeared that antioxidant capacity of polyamines was roughly proportional to their rate constants with hydroxyl radical, which for putrescine, spermidine and spermine is $1.1 \times 10^8$, $1.2 \times 10^8$ and $1.3 \times 10^8$ mol L$^{-1}$s$^{-1}$, respectively [23].
CONCLUSIONS

Ion exclusion chromatography is a suitable technique for hydroxyl radicals analysis after spin trapping with \( p \)-hydroxybenzoic acid. It allows to separate the substrate and the product of the spin trapping reaction in a complex biological sample (such as brain homogenate). When this method was applied to the estimation of antioxidant capacity of some organic solvents and biogenic polyamines, the proportionality was found between their capability to compete with PHBA for OH radicals and their reaction rate constants.

REFERENCES