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ESTIMATION OF ANTIOXIDATIVE PROPERTIES OF PHENYLACETIC ACIDS USING ION-EXCLUSION CHROMATOGRAPHY

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SUMMARY

Cellular oxidative stress has been implicated as a key mechanism of cell differentiation provoked by several compounds (doxorubicins, fibrades, edelfosine). Phenylacetate and its derivatives in submillimolar to millimolar concentrations induce cell differentiation. Ion-exclusion chromatography is widely used for separation and analysis of weak acids. We applied it to the analysis of phenylacetic acids and to the determination of the hydroxyl radical-trapping potential of some of these compounds. Their total antioxidant potential for peroxy radicals was also measured using a photometric method. It was found that phenylacetates are weak antioxidants and in the concentrations which induce cell differentiation effects may substantially reinforce intracellular antioxidative potential.

INTRODUCTION

Compounds containing phenolic rings (e.g., flavonoids, salicylates) frequently have strong free radical-scavenging properties. If such compound is biologically or pharmacologically active through mechanisms involving free radicals, free radical scavenging may modulate its activity.

Phenylacetate (PA) and phenylbutyrate (PB) are phenolic ring-containing cell-differentiating compounds [1,2], which may also induce apoptosis of malignant cells [3]. Their radiosensitizing properties [4,5] and additive or synergistic interactions with other cytotoxic drugs [6] have also been demonstrated. Unlike most anticancer drugs which are active in nanomolar to micromolar concentrations, PA and PB exert in-vitro and in-vivo anticancer activity only at relatively high concentrations (>0.5 mM in cell culture media or plasma). They are, nevertheless, fairly well tolerated; they are, in particular, devoid of bone marrow toxicity [7]. These compounds

are currently under development as monotherapy of refractory malignancies. Their use in combination with other cytotoxic agents or radiotherapy may be considered on the basis of the aforementioned in-vitro data.

Free radicals (FR) are implicated in the pathogenesis of many diseases including, e.g., cancer, Alzheimer's and Parkinson's disease, and rheumatoid arthritis. They may also significantly contribute to cellular degenerative changes of aging [8–11]. Among FR to which cells are exposed the most reactive is the hydroxyl radical [12], which can interact with several organic compounds by addition, free radical substitution, or electron transfer.

Because of their high reactivity hydroxyl radicals are short-lived and difficult to detect directly. They can, however, be analyzed chromatographically after spin trapping. OH radical attacks benzene ring of an aromatic compound (spin trap) and hydroxylated products of this reaction are separated and detected. Endogenous phenylalanine or exogenous aspirin can be used as spin traps [13]. 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. These derivatives can be separated by reversed-phase HPLC with photometric detection. Electrochemical detection is more sensitive but is applicable only to DHBA [13,14]. Salicylic acid is not the optimum 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 et al. have proposed the use of *p*-hydroxybenzoic (PHB) which, on reaction with OH radicals, is converted to one main product, 3,4-dihydroxybenzoic acid [15].

To improve the analytical properties of the PHB-based spin trap assays of hydroxyl radicals we have applied ion-exclusion chromatography (IEC) for analysis of the reaction products. This technique is widely used for separation of mixtures of ionic and nonionic compounds, and of weak acids or bases [16–18]. 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 sign. It follows that samples of negatively charged ions, e.g. dissociated acidic compounds, are separated on cation-exchange resins with anionic functional groups. Usually these are sulfonic acid groups. Similarly, samples containing positively charged species (bases) are separated on anion-exchange resins containing cationic

functional groups (usually tetraalkylammonium groups). The same columns can be used in IEC and in ion-exchange chromatography, although in the first true ion-exchange is not involved.

Total radical trapping antioxidant potential (TRAP) has been introduced for evaluating, mainly, the antioxidant capacity of complex biological samples, for example plasma [19]. These tests use different free radicals generators (usually thermolabile diazo compounds) and oxidation of analyzed samples. Results are calculated from the delay time during which antioxidants are consumed. The basic requirement of an analyzed sample is that its oxidation rate constant is much higher then that of the compound used as a 'detector'. In this paper we have adopted this method for investigation of phenylacetic acids.

In this study IEC was used to separate phenylacetic acids and we have attempted to quantitate the hydroxyl and peroxy radical-scavenging efficacy of some phenylacetate derivatives. The issue is important, because free radical-mediated processes such as peroxidation may play a role in chemotherapy-induced apoptotic death of malignant cells [20], and a variety of radical oxidants including peroxy radicals are involved in radiation-induced cell death [21]. Furthermore, recent data implicate free radicals and cellular oxidative stress in the chemically-induced differentiation of several malignant cell types [22–27]. If phenylacetates have significant radical-scavenging properties, these may significantly interfere with susceptibility of cells to their differentiation-inducing and apoptosis-inducing activity, unless the mechanism of phenylacetate-induced differentiation and apoptosis does not involve free radicals.

EXPERIMENTAL

Equipment

Chromatography was performed with a P-580-A-LPG degasser and pump, STH-585 column oven, UVD 170S four-channel photometric detector (Gynkotek, Germering, Germany), 2097 injector (Rheodyne, USA), and a strong cation-exchange column based on PS/DVB, a copolymer of macroporous styrene and divinylbenzene (TSK-GEL SCX(H^+) 5 μm, 300 mm × 7.8 mm i.d., >4.2 meq g^{-1}; TosoHaas, Japan). Detection was achieved with an Amor (Spark Holland, Netherlands) amperometric detector equipped with a V-560 digital multimeter (Meratronix, Warsaw, Poland) and Rex-101 (Pharmacia-LKB, Sweden) chart recorder. The system was

controlled by Chromleon (Gynkotek, Germering, Germany) software installed on an IBM-PC Pentium computer. TRAP measurements were performed by use of a DigiScan photometer (AsysHitech, Eugendorf, Austria).

Materials

Sodium adenosine 5′-diphosphate (ADP), phenylacetic acids, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH DA), 2,2′-diazobis(2-amidinopropane)dihydrochloride (AAPH), and phosphate-buffered saline (PBS) tablets were obtained from Sigma (St Louis, MO, USA), hydrogen peroxide and $FeCl_2$ from Merck (Darmstadt, Germany), and Desferal from Novartis (Basle, Switzerland). Other reagents were of analytical reagent grade and were used without further purification. Milli-Q (Millipore, Bedford, USA) water was used to prepare all solutions. The mobile phases were filtered through a Millipore 0.22 μm membrane filter and degassed in an ultrasonic bath before use.

Procedures

Chromatographic experiments were performed at a flow rate of 0.9 mL min^{-1}. The column was stabilized at 20°C for 1 h before chromatographic measurements. The used mobile phase was 1 mM sulfuric acid, 0.125 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM KCl in 20% acetonitrile.

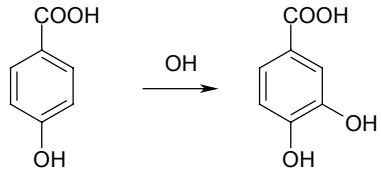
Stock solutions (10 mM) of the analyzed compounds were prepared in Milli-Q water and diluted to the required concentration before use. The samples were injected into the chromatographic system with a 100-μL syringe (Hamilton, Reno, USA) through the injection port. Volumes injected were 20 μL. Output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

Hydroxyl radicals were generated by use of the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

by incubation of 0.5 mM Fe^{2+}, 2 mM ADP, and 2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.4) for 1 min in the presence of 1 mM *p*-hydroxybenzoic acid and the analyzed phenylacetic acid at 37°C. The product of reaction of *p*-hydroxybenzoic acid with hydroxyl radical:

$$p\text{HBA} + OH^{\bullet} \rightarrow \text{3,4-DHBA}$$



3,4-dihydroxybenzoic acid, was analyzed chromatographically. The reaction was stopped by addition of 2 mM dimethyl sulfoxide (DMSO) and 0.1 mg mL^{-1} Desferal, and the reaction mixture was immediately analyzed by IEC.

TRAP measurements were based on the modified method of Valkonen and Kuusi [28]. This property was introduced as a measure of the antioxidant capacity of complex biological samples, such as blood plasma [28–34]. Peroxy radicals were obtained by thermal decomposition of AAPH (final concentration 56 mM). In the first step carbon radicals are formed in pairs (*e* dentes reaction, yielding):

$$\text{R-N=N-R} \rightarrow \text{R}^{\bullet} + \text{N}_2 + \text{R}^{\bullet} \begin{cases} \rightarrow (1\text{-e})\text{R-R} \\ \rightarrow \text{e R}^{\bullet} \end{cases}$$

and react rapidly with oxygen molecules to give peroxy radicals:

$$\text{R}^{\bullet} + \text{O}_2 \rightarrow \text{RO}^{\bullet}_2$$

The concentration of these was monitored photometrically, at 504 nm, by measuring the conversion of DCFH DA to dichlorofluorescein (DCF). The reaction was performed in 50 mM PBS solution at 24°C. DCFH DA was dissolved first in DMSO; the same amount of water was then added to give a final concentration of 14 μM. The analyzed sample (final concentration 0.1 mM) shifts the measured S-shape kinetic curve. Results are the delay time (measured at half time of the reaction) of competition kinetics during which the antioxidant is consumed. This property measures the total antioxidant reactivity and is defined as the sum, over all the antioxidant present in the sample, of the product of reaction rate constant and concentration. Measurements of the antioxidant capacity were repeated three times for each sample and the results were averaged and expressed relative to the average result for control samples containing no sample.

RESULTS

Separation of Phenylacetic Acids on Ion-Exclusion Column

The chromatographic resin commonly used in IEC is a macro-porous PS/DVB copolymer. For some compounds the retention observed is greater than that which can be attributed solely to an ion-exclusion mechanism. This can be explained by hydrophobic and π-electron interaction of the analyte with the resin network and has been confirmed by showing that retention decreases as the concentration of organic modifier in the mobile phase is increased [16]. The dielectric constant of the organic solvent (e.g. 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. The opposite effect has been observed, however, which is suggestive of hydrophobic interactions. The aforementioned mixed retention mechanism makes IEC a predestinated technique for separation and assay of weak aromatic acids. It has been found that these mechanisms in particular enabled us to separate phenylbutyric and bromophenylacetic acids (Fig. 1), which were investigated because of their potential free radical-scavenging property.

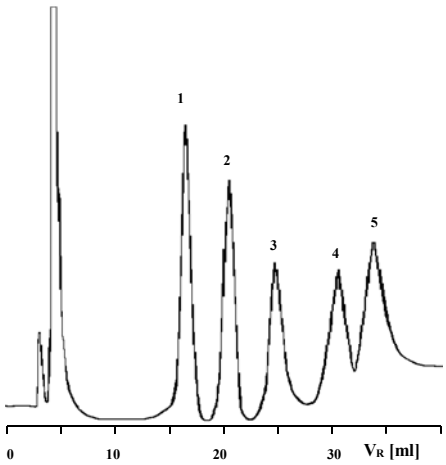


Fig. 1

Ion-exclusion chromatogram of 1 mM *p*-hydroxybenzoic (1), *o*-bromophenylacetic (2), 4-phenylbutyric (3), *m*-bromophenylacetic (4) and *p*-bromophenylacetic (5) acids. Chromatographic conditions: column 300 mm × 7.8 mm i.d. TSK-GEL SCX(H^+) (TosoHaas); mobile phase 1 mM H_2SO_4, 1 mM KCl, 0.125 mM EDTA, 20% ACN; temperature 20°C, flow rate 0.9 mL min^{-1}, detection UV 210 nm

Two chromatographic detectors, amperometric (+0.8 V relative to Ag/AgCl) and photometric (254 nm), were compared. It was found that the lower detection limit for the dihydroxybenzoic acids was obtained with the amperometric detector. For 3,4-dihydroxybenzoic acid it was below 1 pmol for a signal/noise ratio of 3. The limit of detection with the photometric detector was nearly two orders of magnitude higher, although this detector seemed more sensitive for *p*-hydroxybenzoic acid. Phenylacetic acids were detected on the photometric detector only. Connection of both detectors serially enabled efficient detection of both the substrate and the product of the reaction.

Spin Trapping and Determination of Antioxidant Capacity

Products from the spin-trap reaction of hydroxyl radicals with phenylacetic acids were analyzed by chromatographic separation then electrochemical detection. When *p*-hydroxybenzoic acid was used as spin trap the single reaction product 3,4-dihydroxybenzoic acid was completely separated from the spin trap agent [18].

The method described above has been employed for 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, *p*-hydroxybenzoic acid. Hydroxyl radicals are generated by the Fenton reaction and both the detector and the analyte scavenge the radicals. If the analyte 'performs better' than the detector, generation of the dihydroxybenzoic acid is reduced. This assay enables comparison of the OH radical-scavenging performance of different substances, measured as a decrease of the peak height of 3,4-dihydroxybenzoic acid.

Measurements of antioxidant capacity were repeated 4–6 times for each analyte and the results were averaged and expressed relative to the average result for control samples containing no analyte. In Fig. 2 the heights of chromatographic peaks obtained for phenylacetic acids are compared with those for the control (no analyte). It was found that the investigated acids are weaker scavengers of hydroxyl radicals than polyamines or DMSO [18]. Similar results were obtained using the TRAP method (Fig. 3).

DISCUSSION

As mentioned in the introduction, phenylacetates are inducers of cell differentiation or apoptosis, and are under study as potential antican-

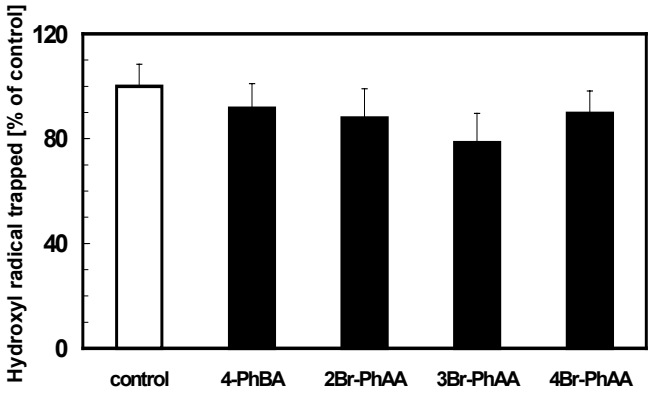


Fig. 2

Antioxidant potentials of 1 mM 4-PhBA (4-phenylbutyric acid), 2Br-PhAA (*o*-bromophenylacetic acid), 3Br-PhAA (*m*-bromophenylacetic acid), and 4Br-PhAA (*p*-bromophenylacetic acid). Hydroxyl radicals were generated using the ADP–Fe(II)–H_2O_2 system and trapped with *p*-hydroxybenzoic acid in the presence of the solvents tested at 0.1% concentration. The reaction product (3,4-dihydroxybenzoic acid) was assayed by ion-exclusion chromatography. The control sample contained no analyte, therefore the height of the reaction product detected was 100%. Chromatographic conditions as in Fig. 1. Data are presented as means ± SD

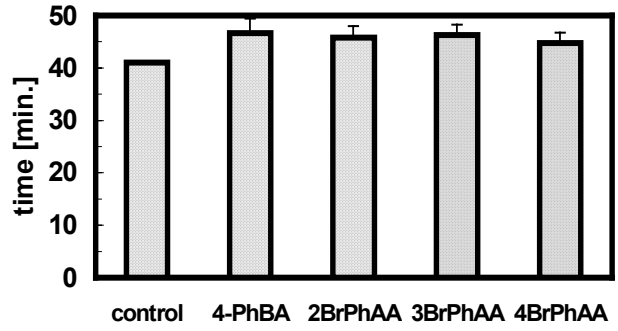


Fig. 3

Spectrophotometrically obtained antioxidant potentials (TRAP) of acids from Fig. 2. Peroxy radicals were formed during thermal decomposition of 2,2′-diazobis(2-amidinopropane)dihydrochloride (AAPH) in buffer solution at pH 7.2, followed by measuring the conversion of 2,7-dichlorodihydrofluorescein diacetate (DCFH DA) to dichlorofluorescein (DCF). DCF formation was measured photometrically at 504 nm. Times of half-waves are presented

cer agents. Recent evidence indicates that other agents which in subcytotoxic doses induce malignant cell differentiation, such as doxorubicin-like drugs [25], fibrates [26] and edelfosine [27] concomitantly induce intracellular oxidative stress. Furthermore, exposing malignant cells (such as hepatoma [24] and neuroblastoma [23]) to free radicals per se induces cell differentiation. On the basis of the aforementioned observations it has been hypothesized that oxidative stress is critically involved in the phenomenon of chemically-induced cell differentiation.

Our present data indicate that phenylacetates, despite being cell differentiation or apoptosis inducers, have the properties of weak free radical scavengers. Considering that these compounds provoke cell differentiation only in relatively high (submillimolar to millimolar) concentrations, one may expect that, at the same time, intracellular antioxidative potential is substantially reinforced. It remains to be shown whether phenylacetates induce intracellular oxidative stress or represent a class of differentiation and apoptosis inducers which act without the involvement of oxidative stress.

CONCLUSIONS

Ion-exclusion chromatography is a suitable technique for analysis of hydroxyl radicals after spin trapping with *p*-hydroxybenzoic acid and for analysis of phenylacetic acids. It was found that phenylacetic acids are scavengers of both hydroxyl and peroxy radicals.

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