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ORIGINAL ARTICLE

Kinetic measurements of peroxynitrite scavenging properties of hydroxybenzoates

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ABSTRACT

Objective: Hydroxybenzoic acids and their derivatives belongs to phenols, which are characterized by salubrious properties, leading to a reduction of the incidence of chronic diseases. The most significant health related features of hydroxybenzoic acids and their derivatives are their antioxidant properties. Peroxynitrite is a reactive nitrogen compound which can cause lipid peroxidation with subsequent cellular damage. The aim of the present work was to determine the peroxynitrite scavenging property of different hydroxybenzoates, with regard to their chemical structure. **Methods:** In our study, we tested 14 hydroxybenzoic acids and a number of their derivatives in the reaction with peroxynitrite. The compounds were selected to have different number and different position of the hydroxyl group(s) attached to the benzene ring. In this way we ensured the study of different suitable model compounds by testing their antioxidant properties. The peroxynitrite scavenging properties of the model compounds were studied using kinetic spectrophotometric measurements. The reaction rate was calculated based on the decrease in peroxynitrite concentration. **Results:** The most significant peroxynitrite scavenging properties based on the reaction rate were detected in compounds with hydroxyl groups substituted in the positions *meta* and *para*: 3,4-dihydroxybenzoic acid, gallic acid and caffeic acid. Also, increased reaction rates were recorded in the reaction of 2,4-dihydroxybenzoic acid, acetylsalicylic acid and 2-hydroxybenzoic acid with peroxynitrite. **Conclusion:** Our results confirmed the thesis that the free radical scavenging properties are dependent on the position and the number of the hydroxyl group(s) in relation to the carboxylic functional group attached to the benzene ring. Better understanding of the relations between the structure and the action of these antioxidants may be helpful in their proper utilization during prevention and treatment of diseases.

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Key Words: Hydroxybenzoic acid; hydroxyl group; peroxynitrite; spectrophotometry

INTRODUCTION

Hydroxybenzoic acids and their derivatives belongs to phenols, secondary metabolites with a large structural diversity, widely distributed in nature. In the scientific literature, most attention is devoted to the natural dietary polyphenols. The claimed health effects are primarily attributed to antioxidant properties. They are effective scavengers of free radicals, reactive oxygen species as well as reactive nitrogen species [1]. It is generally accepted, that a balanced diet rich in polyphenols could contribute to the reduction of risk and to the prevention of diabetes, coronary heart disease, cancer, Alzheimer's diseases, and cataract, and can be used in cancer chemoprevention [2, 3]. Currently, only a few of natural polyphenols are approved as antioxidant food additive; however, due to safety limitations of synthetic antioxidants, natural antioxidants have been of increasing interest, to ensure the health benefits for consumers [4, 5].

The present work is focusing on the antioxidant activity of hydroxybenzoic acids and their derivatives to scavenge peroxynitrite ($ONOO^-$) *in vitro*. Peroxynitrite is a powerful oxidant, produced in the extremely fast reaction ($\sim 1\times10^{10}$/M/s) of nitric oxide (NO•) with superoxide ($O_2^{\bullet -}$), by neutrophils, macrophages and lymphocytes [6]. However, both NO• and $O_2^{\bullet -}$ belongs to radicals, due to their effective system for elimination, they are not toxic *in vivo* [7]. Under physiological conditions, NO• is eliminated by oxyhemoglobin to nitrate, thus preventing the accumulation of it. The major pathway of superoxide elimination is provided by superoxide dismutases (SOD). The biological function for NO• was described for the first time in the 1990s, as an intracellular messenger in white blood cells, blood vessels and as a neuronal messenger [8]. Nitric oxide plays an important role in modulating blood flow, thrombosis and neural activity, and it can act in nonspecific host defense.

The contrasting roles of NO• in physiology and pathology is explained exactly by its resultant peroxynitrite formation [7]. While the direct effect of NO• is beneficial, its oxidation product, peroxynitrite, promotes atherogenesis [6]. Peroxynitrite mediates one or two electron oxidation of biological molecules, particularly those with transition metal centers and thiols [9]. As a result of structural protein nitration by peroxynitrite, filament assembly disruption with major pathological consequences will occur. The formed nitrotyrosine is detectable in vivo and can serve as a footprint of peroxynitrite in various pathological conditions, such as atherosclerosis, myocardial ischemia, septic and distressed lung, inflammatory bowel disease and amyotrophic lateral sclerosis [10]. Strategies aimed at elimination of peroxynitrite are focused on the inhibition of the reaction substrates as well as on degradation of the formed peroxynitrite. The latter mentioned mechanism may be mediated by naturally occurring phenols, like hydroxybenzoates, allowing them to act as a powerful therapeutic tool of the future.

MATERIAL AND METHODS

In the present study the following phenolic compounds were tested: 2-hydroxybenzoic acid (2-HBA), 3-hydroxybenzoic acid (3-HBA), 4-hydroxybenzoic acid (4-HBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), 2,6-dihydroxybenzoic acid (2,6-DHBA), 3,5-dihydroxybenzoic acid (3,5-DHBA), acetylsalicylic acid (ASA), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), caffeic acid (CA), gallic acid (GA), 4-hydroxybenzoic acid propyl ester (4-HBAP) and 4-hydroxybenzoic acid methyl ester (4-HBAM), all purchased from Sigma (St. Louis, MO, USA) at the highest purity. Dipotassium phosphate (K_2HPO_4) and monopotassium phosphate (KH_2PO_4) were purchased from ITES (Vranov nad Topl'ou, Slovakia).

The stock solutions of the tested hydroxybenzoic acids were diluted in deionized water (Millipore Simplicity, Billerica, MA, USA) to 0.1% (w/w). Peroxynitrite solution was prepared according to the procedure of Beckman *et al* [11]. Hydrogen peroxide (c = 30% (v/v)) in HCl (c = 0.6 mol/l) with addition of KNO_2 (c = 0.6 mol/l) and NaOH (c = 1.2 mol/l) was allowed to freeze overnight at -20 °C. The real concentration of the $ONOO^-$ in the stock solution was calculated on the next day based on the absorbance measured at 302 nm (A_{302nm}), using the equation $\mathbf{c = A_{302nm} / (d \times \varepsilon)}$ when $\mathbf{\varepsilon = 1670\ mol/cm/l}$. Subsequently, the $ONOO^-$ stock solution was diluted with PBS (c = 0.05 mmol/l, pH = 7.4) to exactly 0.5 mmol/l.

The course of the reaction of peroxynitrite with different hydroxybenzoic acids was studied using UV/Vis spectrophotometer Shimadzu MultiSpec-1501 (Kyoto, Japan) with the following specifications: measurement wavelength range 190 to 800 nm, wavelength accuracy ± 1 nm, wavelength repeatability ± 0.01 nm. The reaction rate was determined after addition of 50 μl of the tested compound (c = 0.1% (w/w)) to 2 ml of $ONOO^-$ stock solution (c = 0.5 mmol/l) in kinetic mode of measurement (wavelength range: 200 - 500 nm, reaction temperature: 37 °C; measurement duration: 60 min). The reaction rate was calculated according to following formula: $\mathbf{v = \Delta c / \Delta t}$, when $\mathbf{\Delta c = (c_{60\ min} - c_{0\ min})}$ and $\mathbf{\Delta t = 60\ min}$.

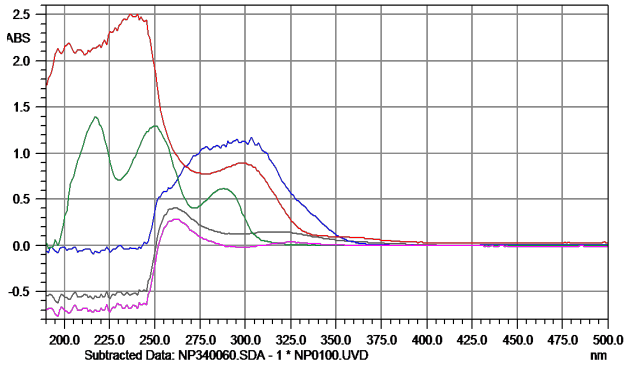


Figure 1. Peroxynitrite scavenging properties of 3,4-dihydroxybenzoic acid (c = 161 μmol/l)
red line, peroxynitrite; **green line**, 3,4-dihydroxybenzoic acid; **blue line**, time course for the beginning of the reaction; **black line**, time course at the 30th minute; **pink line**, time course at the 60th minute

RESULTS

The kinetics of the tested reactions are listed in Table 1. The graphs (Figures 1 to 3) show the reaction of peroxynitrite and the compounds with the best scavenging properties. The red and the green lines corresponds to the spectral characteristics of the stock solution of peroxynitrite and the tested compound, respectively. The blue, black and pink lines indicates the course of the reaction depending on the increase in final products concentration and decrease of substrates concentration in the beginning of the reaction, at the 30th and at the 60th minute, respectively. Absorption spectrum of the final products (marked with black and pink lines) has increased absorbance in the area of 325 - 400 nm compared to the spectrum of the substrates in the beginning of the reaction (marked with blue line). This increase indicates the presence of the formed nitro-derivatives.

The antioxidant properties of the tested substances were considered based on the measured spectral

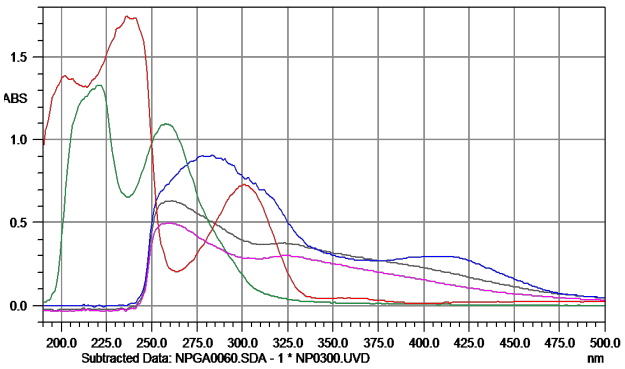


Figure 2. Peroxynitrite scavenging properties of gallic acid (c = 146 μmol/l)
red line: peroxynitrite; **green line:** gallic acid; **blue line:** time course for the beginning of the reaction; **black line:** time course at the 30th minute; **pink line:** time course at the 60th minute

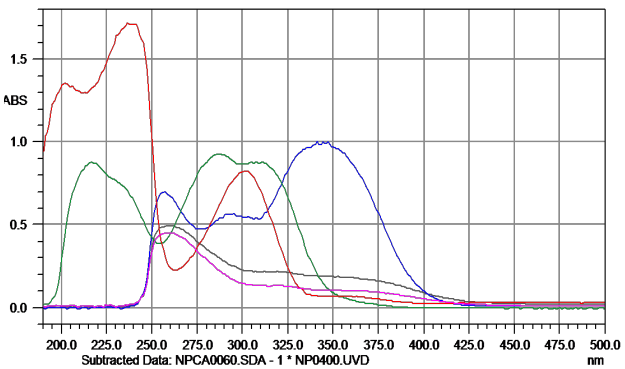
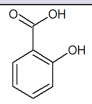
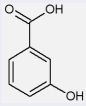
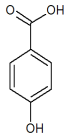
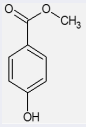
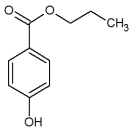
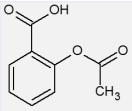
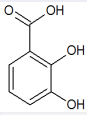
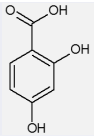
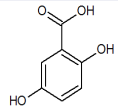
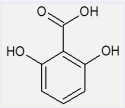
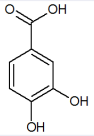
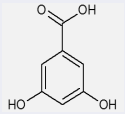
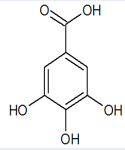
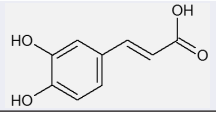


Figure 3. Peroxynitrite scavenging properties of caffeic acid (c = 139 μmol/l)
red line: peroxynitrite; **green line:** caffeic acid; **blue line:** time course for the beginning of the reaction; **black line:** time course at the 30th minute; **pink line:** time course at the 60th minute

Table1. Peroxynitrite scavenging properties of selected hydroxybenzoic acids and their derivatives

Tested compound		Reaction time (min)	Absorbance (A_{302nm})	Peroxynitrite concentration (mmol/l)	Reaction rate (μmol/min)
2-HBA		0	0.429	0.257	1.66
		60	0.263	0.157	
3-HBA		0	0.309	0.185	nd
		60	0.321	0.192	
4-HBA		0	0.579	0.347	0.1
		60	0.569	0.341	
4-HBAM		0	0.987	0.591	0.16
		60	0.971	0.581	
4-HBAP		0	0.765	0.458	0.05
		60	0.76	0.455	
Acetylsalicylic acid		0	0.674	0.404	1.66
		60	0.508	0.304	
2,3-DHBA		0	0.22	0.132	nd
		60	0.237	0.142	
2,4-DHBA		0	0.988	0.592	2.02
		60	0.786	0.472	
2,5-DHBA		0	0.062	0.037	0.08
		60	0.054	0.032	
2,6-DHBA		0	0.384	0.23	0.2
		60	0.364	0.218	
3,4-DHBA		0	1.149	0.688	11.5
		60	0.001	0.001	
3,5-DHBA		0	0.199	0.119	nd
		60	0.238	0.143	
Gallic acid		0	0.772	0.462	4.82
		60	0.289	0.173	
Caffeic acid		0	0.551	0.33	4.08
		60	0.142	0.085	

nd = not determined, the reaction did not follow the presumed mechanism

characteristics first (Figures 1-3) and subsequently on the basis of reaction rates (Table 1). Based on the observed reaction rate, the best ability to scavenge peroxynitrite was observed in 3,4-dihydroxybenzoic acid, gallic acid and caffeic acid (reaction rates 11.5, 4.82 and 4.08 μmol/min, respectively). Even at the reaction of 2,4-DHBA, ASA and 2-HBA with peroxynitrite we observed increased reaction rates with values close to 2 μmol/min.

DISCUSSION

Phenolic compounds are secondary plant metabolites widely distributed in nature. Their most important features are anti-inflammatory, antiviral, antibacterial and anti-atherogenic effects. Phenols and their derivatives are efficient in preventing auto-oxidation. They have ability to scavenge free radicals and other ROS and RNS [12]. The free radical scavenging properties of different phenolic compounds are dependent on the position of the hydroxyl group attached to the benzene ring. Peroxynitrite, which is an extracellular, and also intracellular metabolite, exerts various cytotoxic responses [6]. It participates in various pathological conditions, such as disorders of the cardiovascular, respiratory and central nervous systems [10]. Its penetrating capacity across lipid bilayer is about 400 times higher than of NO and superoxide [13]. The adverse effect of peroxynitrite can be inhibited by various hydroxybenzoates that act as free radical terminators [1].

In the present study, the scavenging activity of 14 different hydroxybenzoic acids and their derivatives with different number and position of hydroxyl groups on benzene skeleton was tested. Heijnen et al [14] devoted to the antioxidant properties of phenols pointing to the relationship between structure and the peroxynitrite scavenging activity. They highlighted the importance of the hydroxyl group at the *meta* position [14]. According to our results the best ability to scavenge peroxynitrite was observed in 3,4-DHBA, GA and CA, while all of which has hydroxyl groups substituted in the positions *meta* and *para*. Based on these results we can assume, that similarly to phenols, in hydroxybenzoates the *meta* positioned hydroxyl group is of great importance [15].

The absorption spectrum of 3,4-DHBA, GA and CA with peroxynitrite shows an increased absorbance at wavelengths range between 325 and 400 nm. This increase is caused by the nitration of the tested compounds. Similar increase was observed in the absorption spectrum when testing 2,4-DHBA, ASA and 2-HBA, whilst increase in reaction rate was at a lower rate (2.02, 1.66, and 1.66 μmol/min, respectively). The nitration of the other tested compounds (3-HBA, 4-HBA, 4-HBAM, 4-HBAP, 2,3-DHBA, 2,5-DHBA, 2,6-DHBA and 3,5-DHBA) observed by the increase in absorbance was not significant nor even their reaction rate. We suppose that it is a consequence of the mesomeric effect, manifested on the benzene ring.

Based on the obtained results, we can conclude that the tested hydroxybenzoates with hydroxyl group in *meta* and *para* position are the most effective scavengers of peroxynitrite under laboratory conditions. We recommend further research of hydroxybenzoates *in vivo*, with specific regard to their pro-oxidant as well as antioxidant activity. This research should be important to verify the statement of the Panel on Dietetic Products, Nutrition and Allergies, which criticize the "widely described" claimed health effects of the naturally occurring polyphenols [16].

We believe, that the relation between the structure and the antioxidant properties can be the missing key to explain the beneficial health effects of the polyphenols. Improved comprehension of the complex relationship between the antioxidant properties of substances and their structure is important to understand their proper use in the prevention and treatment of diseases and for the detection of pathological processes. Monitoring and improved understanding of the antioxidant properties of hydroxybenzoic acid derivatives are important due to their frequent use in modern medical nutrition therapies.

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ABBREVIATIONS

ASA; acetylsalicylic acid
CA; caffeic acid
DHBA; dihydroxybenzoic acid
H_2O_2; hydrogen peroxide
HBA; hydroxybenzoic acid
HBAM; hydroxybenzoic acid methyl ester
HBAP; hydroxybenzoic acid propyl ester
HCl; hydrochloric acid
GA; gallic acid
K_2HPO_4; dipotassium phosphate
KH_2PO_4; monopotassium phosphate
KNO_2; potassium nitrite
NaOH; sodium hydroxide
NO; nitric oxide
$O_2^{\bullet -}$; superoxide anion
$ONOO^-$; peroxynitrite
PBS; phosphate buffer solution
RNS; reactive nitrogen species
ROS; reactive oxygen species
SOD; superoxide dismutase

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Conflict of Interest: None declared