A Modified Analytical Method for Total Antioxidant Potential Assay Using RP-HPLC with Electrochemical Detection and Its Application for Pro- and Antioxidative Properties of Dopamine Measurement

Bronislaw K. Glód[1,*], Kinga I. Stanczak[2], and **Anna Wozniak[2]**

[1]Meat and Fat Research Institute, Jubilerska 4, 04-190 and [2]Agriculture University, Nowoursynowska 166, 02-852 Warsaw, Poland

Abstract

Total antioxidant potential (TAP) is usually measured using photometric or fluorometric assays. Preliminary results of a reversed-phase high-performance liquid chromatography–electrochemical detection assay are given. The method is based on the generation of hydroxyl radicals in a Fenton reaction and analysis of the product of their interaction with *p*-hydroxybenzoic acid (3,4-dihydroxybenzoic acid). The method is applied to estimate the TAP of dopamine. As a result, depending on the concentration, dopamine is pro- or antioxidant. The results are compared with TAP measurements using a standard photometric method.

Introduction

Free radicals (FRs) are implicated in the pathogenesis of many diseases, including Parkinson's disease (PD) (1). Among FRs, the most reactive is hydroxyl radical (2–4), which is able to interact with a number of organic compounds through addition, FR substitution, or electron transfer. Because of its high reactivity, it is short-lived and difficult to detect directly. However, FRs can be chromatographically analyzed after spin trapping (5). OH radical attacks the benzene ring of an aromatic compound (spin trap), and hydroxylated products of this reaction are separated and detected. For instance, *p*-hydroxybenzoic acid (*p*-HBA) can be used as a spin trap (5). The reaction product, 3,4-dihydroxybenzoic acid (3,4-DHBA), may be separated using reversed-phase (RP) high-performance liquid chromatography (HPLC) with photometry or, for more sensitivity, electrochemical detection (6).

PD is mainly caused by decreased efficiency of the extrapyramidal movement system, where catecholamines play a crucial role in its pathomechanism (7). Injury of substantia nigra by FRs decreases concentration of the main neurotransmitter dopamine (8–10). Patients are usually treated substitutionally with L-dopa (and inhibitors of its decarboxylase), which is easily absorbed in the brain because it is a precursor of dopamine.

In the literature, some studies indicated that catecholamines (including dopamine and L-dopa) may be pro-oxidants as well as antioxidants (11). Therefore, changes in FR concentration and, indirectly, total antioxidant potential (TAP) of blood plasma of PD patients should be expected. Additionally, uptake of L-dopa decreases the characteristic PD tremor, which leads to hypermetabolism and increased FR generation.

To assess and compare the strength of particular antioxidants, in order to investigate their influence on some diseases or select those with the highest potential for further development as drugs, a method is required. Frequently, more information (e.g., synergetic effects) is obtained by the measurement of TAP of biological samples than by the concentration of particular, separate antioxidants (12).

Presented are the preliminary results of the application of RP-HPLC to the estimation of TAP after hydroxyl radical are generated in a Fenton reaction and spin-trapped with *p*-HBA. Hydroxyl radicals are the most reactive and dangerous species; therefore, this method of TAP measurements should give more useful and practical results, as opposed to the photometric methods described in the literature. As an example of practical application, the method was applied to the measurement of pro- and antioxidant dopamine properties.

Experimental

Instrumentation

Chromatographic measurements were performed using a chromatograph consisting of an interface box, K-5004 4-channel degasser, K-1500 solvent organizer, dynamic mixing chamber,

* Author to whom correspondence should be addressed: email bkg@cmdik.pan.pl.

K-1001 HPLC pump, K-2600 fast-scanning UV detector, Eurochrom 2000 chromatographic data acquisition and analysis software (all from Knauer GmbH, Berlin, Germany), Basic+ marathon autosampler (Spark Holland B.V., Emmen, the Netherlands), Jet-Stream plus column thermostat (Industrial Electronics, Langenzersdorf, Austria), and L-3500A LaChrom amperometric detector (Merck, Darmstadt, Germany). Samples were separated on a Hibar RP-18 column (250- × 4-mm i.d., 5 µm) (E. Merck). Photometric measurements were performed using a DigiScan photometer (AsysHitech, Eugendorf, Austria).

Reagents

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA); 2,2'-diazobis(2-amidinopropane) dihydrochloride (AAPH); ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); and PBS tablets were obtained from Sigma (St. Louis, MO). All other reagents (Sigma; Merck; Fluka, Buchs, Switzerland; and POCh, Gliwice, Poland) were of highest available reagent grade and were used without further purification. Water was passed through Milli-RO4 and Milli-Q water purification systems (Millipore, Bedford, MA). Mobile phases were filtered through a 0.22-µm membrane filter (Millipore).

Procedures

Chromatographic experiments were performed with a flow rate of 1 mL/min. The column was stabilized at 30°C by the passage of a mobile phase for 1 h prior to the chromatographic measurements. The mobile phase consisted of 20mM citric acid, 50mM sodium acetate, and 0.125mM EDTA in Milli-Q water, which was buffered with glacial acetic acid to pH 4.3, after which 5% (v/v) HPLC-grade methanol was added (6).

A 10mM stock solution of the analyzed compounds was added to the Milli-Q water and diluted to the required concentration before use. Samples (20 µL) were injected using the autosampler. The output signal from the photometric detector working simultaneously at 210, 254, and 280 nm, as well as amperometric detector working at +0.8 V versus Ag/AgCl, were continuously displayed on the computer. Every sample was injected six times and the average was taken for further elaboration.

Hydroxyl radicals were generated through the Fenton reaction (5,13) with a 1-min incubation of 0.5mM Fe^{2+}, 2mM adenosine 5'-diphosphate (ADP), and 2mM H_2O_2 in 50mM phosphate buffer (pH 7.4) and in the presence of 1mM *p*-HBA. The sample was analyzed at 30°C. The reaction was completed using 2mM dimethyl sulfoxide (DMSO), and the reaction mixture was analyzed immediately using HPLC.

Photometric TAP measurements are based on a modified Valkonen and Kuusi method (14). Peroxyl radicals were obtained from thermal decomposition of AAPH (final concentration, 56mM). In the first step, carbon radicals are formed in pairs that react rapidly with oxygen molecules to give peroxyl radicals. Their concentration was monitored photometrically at 504 nm, measuring the conversion of DCFH-DA to dichlorofluorescein (DCF). The reaction was performed in a 50mM phosphate-buffered saline (PBS) solution at 30°C. DCFH-DA was first dissolved in DMSO, followed by the same amount of water to obtain its final concentration, 14µM. Analyzed sample (final concentration, 0.1mM) shifts resulted in an S-shape kinetic curve. Results were the delay time (measured at half of the time of reaction) of competition kinetics, during which the antioxidant was consumed. This parameter measures TAP and is defined as the sum, over all the antioxidant present in the sample, of the product of reaction rate constant and concentration. The measurements were repeated three times for each sample, and the results were averaged and expressed relative to the average result for the control samples (containing no samples). Results were recalculated to trolox equivalent antioxidant capacity (TEAC) (mM).

Data analysis

Intrasubject comparisons were performed using the Student's *t*-test for dependent variables. Significance was set at $p < 0.05$.

Results and Discussion

As aromatic compounds, catecholamines can be monitored using photometric detection. However, amperometric detection is much more sensitive (~ 3×) (5). Hydrodynamic woltamograms obtained for catecholamines, 3,4-DHBA, and *p*-HBA are presented in Figure 1. For further experiments, the potential of

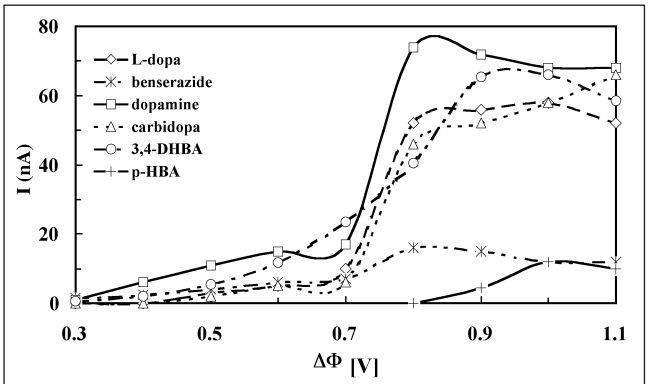

Figure 1. Influence of potential ΔΦ (mV) (vs. Ag–AgCl) on the peak height of L-dopa, dopamine, benserazide, carbidopa, 3,4-DHBA, and *p*-HBA. Chromatographic conditions: column with precolumn, Hibar RP-18 (250- × 4-mm i.d., 5 µm) (Knauer); temperature, 30°C; flow rate, 1 mL/min; and mobile phase, 0.125mM EDTA (95:5, v/v) in acetate–citrate buffer (pH 4.3) with 5% methanol.

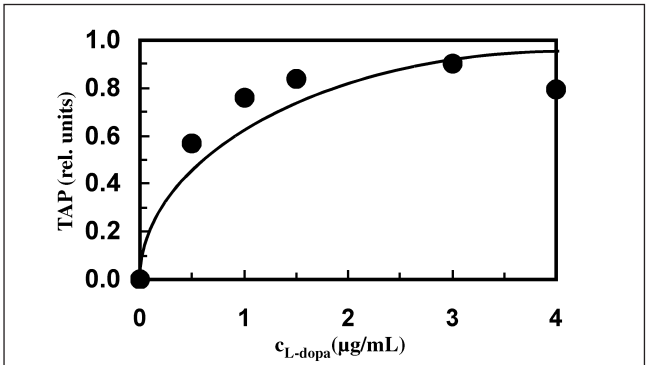

Figure 2. Influence of L-dopa concentration on TAP of the hydroxyl radical (measured as the relative changes of the 3,4-DHBA peak height). Other chromatographic conditions were as in Figure 1.

working electrode was estimated to be the equivalent to 0.8 V versus Ag–AgCl, which gives the optimal compromise between the peak height and the noise level.

It was found that, contrary to the photometric measurements (14,15), chromatographic measurements of TAP are not characterized by the linear calibration curve (Figure 2). It is caused by the fact that, in this case, the decrease of the chromatographic peak was measured. When it completely disappear further increase of the sample concentration gives no effect. Therefore, it is convenient to present results on the scale between 0 (lack of the interaction between radical and sample) and 1 (the sample completely scavenges the FRs), as it is used on Figure 2.

Hydroxyl radicals are very reactive and, therefore, are usually analyzed indirectly, using a spin-trapping agent, by transforming them into a more stable species. The byproduct of this reaction can be used in the procedure of any analytical method. Previously, ion-exclusion chromatography has been used, followed by electrochemical detection (5). In this paper, the possible application of RP-HPLC for the measurement of a product of hydroxyl radicals reaction is shown. In this case, hydroxyl radicals are specially generated in the Fenton reaction. *p*-HBA is used as a spin-trap agent, and the product of the reaction (3,4-DHBA) is amperometrically detected as presented on Figure 3A. The conditions of the separation (chromatographic column and the mobile phase) are the same as those used previously for the analysis catecholamines in the blood plasma (16). Different buffer pH values, as well as methanol concentrations, were tested with the mobile phases. It turned out that the decrease of pH improved the separation of L-dopa. The addition of methanol (at concentration as small as 5%) to the mobile phase improved peak symmetry and decreased

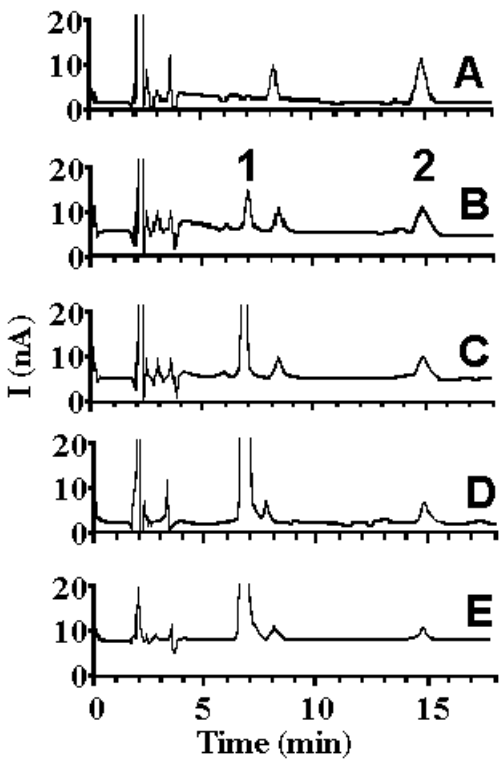

Figure 3. RP-HPLC chromatograms generation in the Fenton reaction (A). As a detector, *p*-HBA was used, which reacts with hydroxyl radicals to generate 3,4-DHBA acid (peak 2). Dopamine (peak 1) added at an increased concentration (3.5, 17, 100, and 200µM) decreased the peak of 3,4-DHBA (B–E). The mobile phase was acetate–citrate buffer (pH 4.3) with 0.125mM EDTA and 5% (v/v) methanol. The flow rate was 1 mL/min; injection volume was 20 µL; and the detector was amperometric, working at +0.8 V versus Ag–AgCl.

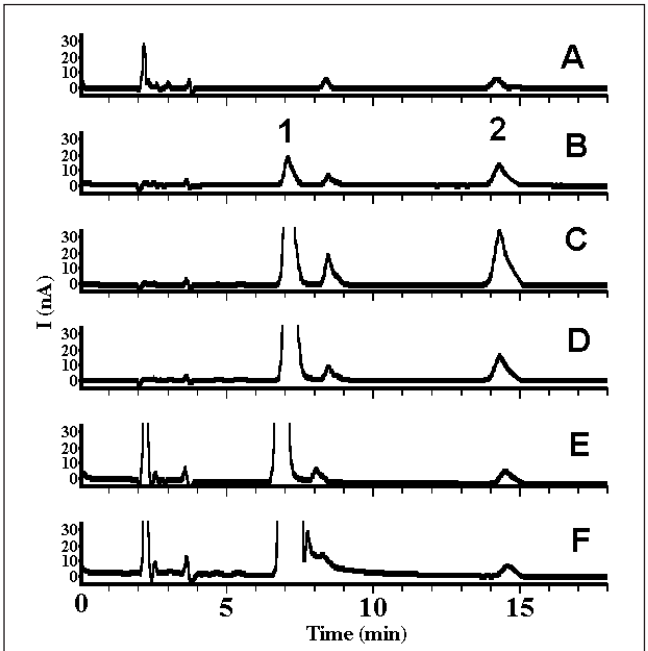

Figure 4. RP-HPLC chromatograms of the reaction mixture of hydroxyl radicals without ADP, containing different dopamine concentrations (A, 0; B, 3.5; C, 17; E, 100; and F, 200µM). Other conditions were as in Figure 3.

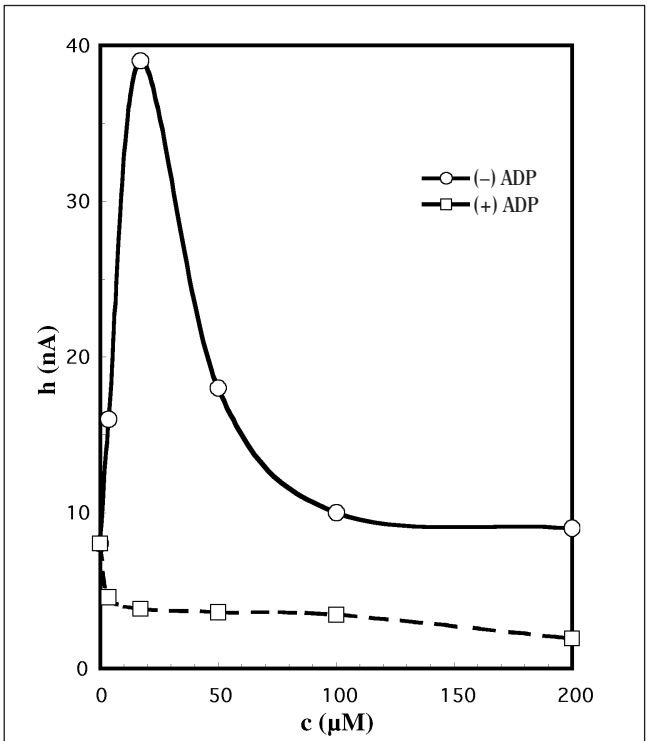

Figure 5. Influence of the dopamine concentration on the height of 3,4-DHBA peak. Other conditions were as in Figure 3.

the retention time of analyzed amines to 20 min. A further increase of methanol concentration decreased retention so significantly that the peak of 3,4-DHBA overlapped with the amines.

When dopamine was added to the reaction mixture, a decrease of the 3,4-DHBA peak was observed (Figure 3B–3E). This finding indicates that *p*-HBA and dopamine compete between themselves in reaction with hydroxyl radicals. In the other words, dopamine scavenges hydroxyl radical. Similar results were obtained with L-dopa and other catecholamines.

In the next step, hydroxyl radicals were generated without reducer (i.e., ADP). It turned out that increasing dopamine concentration initially increased chromatographic peak high of 3,4-DHBA (Figure 4 A–C). However, this tendency shows changes for concentrations greater than 17µM (Figure 4C–4F). Thus, depending on the concentration, dopamine can be a pro- or antioxidant (Figure 5) (17). The mechanism of this dualism can be explained by the reductive properties of the catechol group. On one hand, it indicates that dopamine is an antioxidant (Figure 6), which additionally favors the formation of intermolecular hydrogen bonds between hydroxyl groups in catechol (18). On the other hand, this reduces Fe(III) to Fe(II) and then, indirectly, increases hydroxyl radical generation in the Fenton reaction. Additionally, dopamine shows chelating properties to Fe(III) [K = 8.36×10^{17} (19)], preventing a Fenton reaction. Altogether, this causes nonlinear dependencies between peak height and dopamine concentration, (as presented in Figure 5).

Afterwards, the described method has been compared with the used photometric assay in our laboratory, based on the generation of peroxyl radicals (15). In this case the linear dependence between TAP (expressed as a time of inhibition) and dopamine concentration was obtained. Figure 7 presents TEAC values of some catecholamines used in Parkinson's disease patient treatment, dopamine, its precursor L-dopa, and two inhibitors (carbidopa and benserazide) of the dopa decarboxylase (20). It turned out that all of them are strong antioxidants. However, using chromatographic assay, it has been obtained that dopamine can be pro- or antioxidant, depending on its concentration and the presence of the other reducers (antioxidants) in the sample. It is well known that hydroxyl radicals are the most reactive species among all FRs, and they are responsible for biological cell damage (3,11). Therefore, the chromatographic assay gives more practical results. For example, it explains why dopamine might be harmful to neurons.

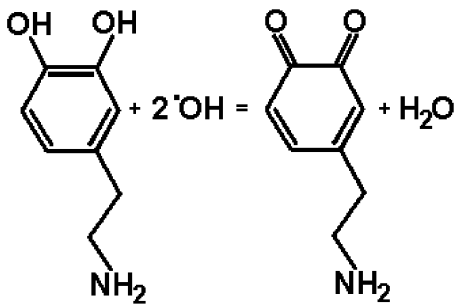

Figure 6. Proposed reaction between dopamine and hydroxyl radical.

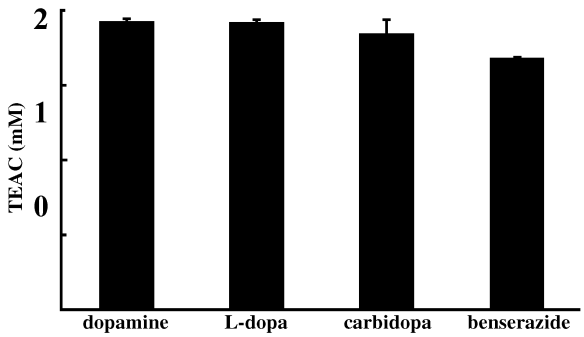

Figure 7. TAP of selected catecholamines (dopamine, L-dopa, carbidopa, and benserazide). Peroxyl radicals were generated by thermal decomposition of AAPH and trapped with DCFH-DA in the presence of the 1mM solutes. The reaction product (DCF) was assayed using a spectrophotometer at 504 nm. The control sample contained no analytes. The results are expressed as TEAC (mM). Data is presented as mean ± standard deviation.

Conclusion

It was found that RP-HPLC with amperometric detection can be used for TAP measurement. The method has been applied to investigate the dopamine influence on TAP. As a result, catecholamines are antioxidants related to the peroxyl radicals. However, using chromatographic assay, it has been obtained that dopamine can be pro- or antioxidant.

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Manuscript received December 1, 2003;
revision received November 19, 2004.