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DETERMINATION OF CATECHOLAMINES AND THE TOTAL ANTIOXIDANT POTENTIAL OF BLOOD PLASMA BY USE OF AN IMPROVED RPHPLC–ED ASSAY

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ABSTRACT

Preliminary results are presented from application of reversed-phase HPLC with electrochemical detection to analysis of L-dopa and total antioxidant potential. It was possible to perform both analyses by using the same chromatographic conditions. The method was used to estimate their daily profiles in the blood plasma of patients treated with different types of drug.

INTRODUCTION

Parkinson's disease (PD) usually results from reduction of the efficiency of the extrapyramidal movement system. Catecholamines play a crucial role in the pathomechanism of Parkinson's disease [1]. Injury of the *substantia nigra* by free radicals reduces the concentration of its main neurotransmitter, dopamine [2,3]. Patients are usually treated substitutionally with L-dopa (and inhibitors of dopa decarboxylase), a precursor of dopamine readily absorbed in the brain. Analysis of L-dopa in plasma enables diagnosis of PD, individualization of L-dopa dose, and avoidance of side effects (fluctuations and dyskinesias).

Changes in the concentration of free radicals and, indirectly, the total antioxidant potential (TAP) of blood plasma occur for two main reasons. First, patients are treated with L-dopa and other catecholamines which are antioxidants [4] and the hypermetabolism caused by characteristic PD

tremors increases the concentration of free radicals. Free radicals adversely modify biologically active molecules and whole cells and are implicated in a variety of degenerative diseases and ageing [5–7]. Processes mediated by free radicals have been implicated in the pathogenesis of several diseases, for example PD [8]. It is widely believed that these modifications can be prevented by use of exogenous antioxidants. There is a need for a method to assess and compare the strength of particular antioxidants to enable selection of those with the highest potential for further development as drugs. It has, however, been found that more information (e.g. synergetic effects) is frequently obtained by measuring the total antioxidant potential of biological samples than by measuring the concentrations of particular antioxidants separately.

This paper reports preliminary results from use of reversed-phase (RP) HPLC to estimate total antioxidant potential, after generation of hydroxyl radicals by the Fenton reaction and their spin trapping with hydroxybenzoate. The method was used to determine the hydroxyl radical-trapping potential of some catecholamines and of the plasma of PD patients treated with L-dopa. The paper shows it is possible to analyse L-dopa levels in plasma and the total antioxidant potential of plasma using exactly the same chromatographic conditions.

EXPERIMENTAL

Instrumentation

HPLC was performed with a chromatograph comprising an interface box, K-5004 four-channel degasser, K-1500 solvent organizer, dynamic mixing chamber, K-1001 HPLC pump, K-2600 fast-scanning UV detector, and Eurochrom 2000 chromatographic data acquisition and analysis software (all from Knauer, Berlin, Germany), Basic^{+} marathon autosampler (Spark Holland, Emmen, The Netherlands), Jet-Stream Plus column thermostat (Industrial Electronics, Langenzersdorf, Austria), and LaChrom L-3500A amperometric detector (Merck, Darmstadt, Germany). Compounds were separated on a 250 mm × 4 mm i.d., 5-μm particle, Hibar RP-18 column (Merck, Darmstadt, Germany).

Reagents

All reagents (Sigma, St Louis, USA; Fluka, Buchs, Switzerland; and POCh, Gliwice, Poland) were of analytical-reagent grade and were used

without further purification. Water was passed through Millipore (Bedford, USA) Milli-RO4 and Milli-Q water purification systems. Mobile phases were filtered through a 0.22-μm membrane filter (Millipore, Bedford, USA).

Procedures

Acetate–citrate buffer (pH 4.3) containing 0.125 mmol L^{-1} EDTA and 5% methanol was used as mobile phase at a flow rate of 1 mL min^{-1}. Before chromatographic measurements the column was stabilized at 30°C for 1 h with passage of mobile phase.

Stock solutions (10 mmol L^{-1}) of the compounds analysed were prepared in Milli-Q water and diluted to the required concentration before use. Samples (20 μL) were injected by use of an autosampler. Output signals from a photometric detector working simultaneously at 210, 254, and 280 nm, and an amperometric detector working at +0.8 V relative to Ag/AgCl, were continuously displayed on the computer. Every sample was injected six times and averages of results were computed for further elaboration.

Hydroxyl radicals were generated by the Fenton reaction [9,10], by incubating 0.5 mmol L^{-1} Fe^{2+}, 2 mmol L^{-1} ADP, and 2 mmol L^{-1} H_2O_2 in 50 mmol L^{-1} phosphate buffer (pH 7.4) for 1 min at 37°C with 1 mmol L^{-1} *p*-hydroxybenzoic acid and the sample under investigation. The reaction was stopped by addition of 2 mmol L^{-1} DMSO and 0.1 mg mL^{-1} Desferal; the reaction mixture was then analysed immediately by HPLC.

Blood plasma was deproteinated by addition of saturated uranyl acetate, excess of which was removed by use of phosphate buffer at pH 4.

RESULTS AND DISCUSSION

HPLC Measurement of Total Antioxidant Potential

Because of their extreme reactivity, hydroxyl radicals are primarily analysed by use of radical-trapping agents then HPLC determination of the reaction products. This radical trapping (or ‘spin-trapping’) process enables free radicals to be investigated by transforming them into more stable species. When this method is used for biological analysis the radical-trapping agent should be selected extremely carefully to avoid toxicity problems. Examples of suitable radical-trapping reagents are phenyl-alanine (with which hydroxyl free radicals react to produce tyrosines) [11]

or derivatives of aspirin (*o*-acetylsalicylic acid) [12]. These derivatives can be separated by reversed-phase HPLC [12] and detected photometrically or, more sensitively, by electrochemical detection using a glassy carbon electrode at 0.8 V relative to Ag/AgCl [9]. Ion-exclusion chromatography, with *p*-hydroxybenzoic acid as the radical-trapping agent, has previously been used for analysis of hydroxyl radicals and total antioxidant potential [10,13]. In this paper we report the use of reversed-phase HPLC to estimate TAP.

Hydroxyl radicals can be analysed after spin trapping with, for example, *p*-HBA. This is apparent from the chromatogram in Fig. 1A, for

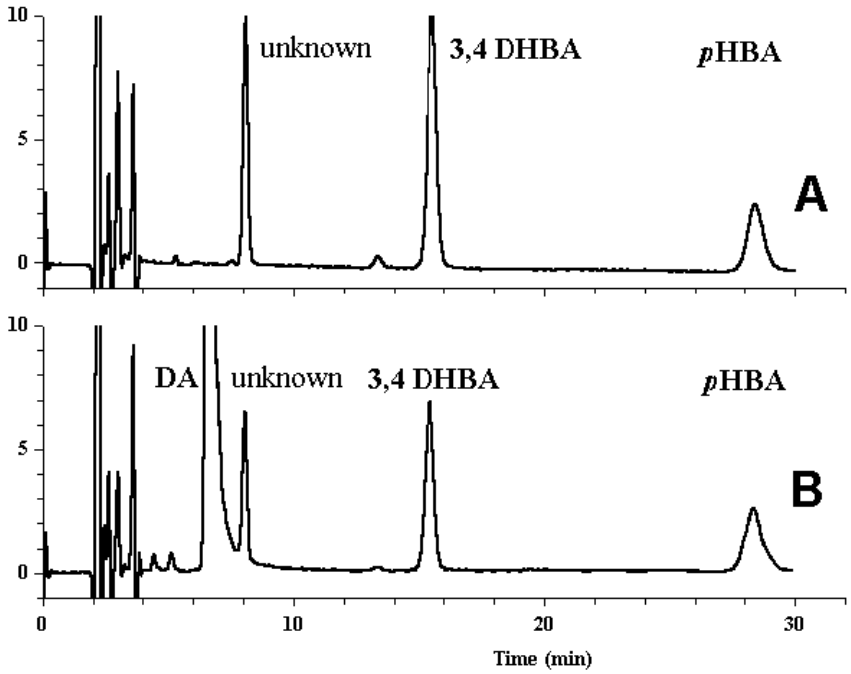

Fig. 1

RP-HPLC chromatograms illustrating generation of hydroxyl radicals by the Fenton reaction (A). *p*-Hydroxybenzoic acid (*p*-HBA) was used as detector; on reaction with hydroxyl radicals *p*-HBA generates 3,4 dihydroxybenzoic acid (3,4 DHBA). Addition of dopamine (DA) reduced the size of the 3,4 DHBA peak (B). The mobile phase was acetate–citrate buffer (pH 4.3) containing 0.125 mmol L^{-1} EDTA and 5% methanol, the flow rate was 1 mL min^{-1}, and the volume injected was 20 µL. The amperometric detector was operated at +0.8 V relative to Ag/AgCl

which hydroxyl radicals were generated by the Fenton reaction [9]. The amount of the radicals is directly proportional to the concentration of the product of the trapping reaction, 3,4-DHBA, and hence to the height of the chromatographic peak. This system can be used to estimate the antioxidant potential. Fig. 1B shows a similar chromatogram obtained when dopamine was added to the reaction mixture. The decrease in the size of the 3,4-DHBA peak is indicative of competition between *p*-HBA and dopamine in the reaction with hydroxyl radicals. In other words, dopamine is a scavenger of hydroxyl radicals. Similar results were obtained with L-dopa. This means that both compounds important in treatment of Parkinson's disease are antioxidants. This system can also be used for analysis of the pro-oxidative properties of the compound investigated [14].

Chromatography of Catecholamines

Catecholamines play a crucial role in PD. For example, dopamine is a neurotransmitter produced by the *substantia nigra*, benserazide and carbidopa are decarboxylase inhibitors, and homovanillic acid is the final step of dopamine metabolism. These compounds are usually separated by

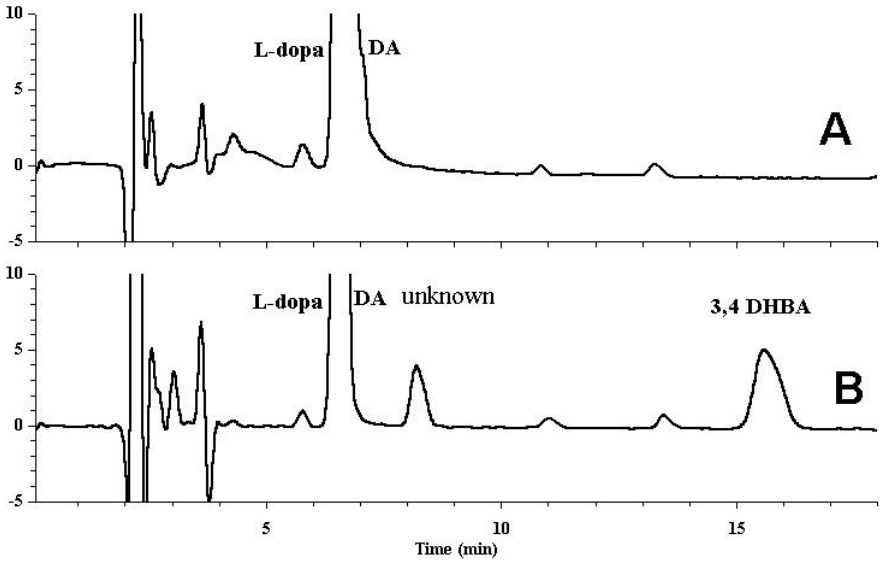

Fig. 2.

RPHPLC chromatograms of blood serum from a PD patient (A) and after scavenging by hydroxyl radicals (B). Other conditions as for Fig. 1

reversed-phase HPLC [15]. We are especially interested in the concentrations of L-dopa and dopamine in the blood plasma of patients treated with medicines containing L-dopa. It was found (Fig. 2A) that analysis of these compounds is possible by use of the same chromatographic conditions as for total antioxidant potential. It was found that the maximum concentration of L-dopa in plasma occurs 1 h after uptake by the patient [16]. It was also possible to estimate the TAP of blood plasma, as is shown in Fig. 2B.

Detection

Although catecholamines and 3,4 DHBA (which also contains a catechol group) can be detected photometrically, electrochemical detection based on anodic oxidation of the catechol group is more sensitive [10,13]. Because all catechols react similarly (Fig. 3), they cannot be distinguished in a complex biomedical matrix and a separation step is necessary. Figure 3 shows hydrodynamic voltammograms obtained from L-dopa, dopamine, and 3,4 DHBA. In all cases limiting current was not obtained. Further measurements were performed at 0.8 V. This potential enabled sensitive detection (10–100 μM) of the compounds with small noise levels and long-term electrode stability.

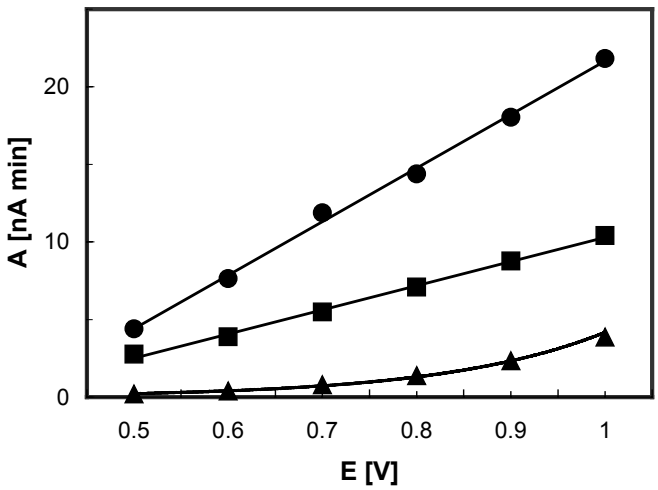

Fig. 3.

Hydrodynamic voltammograms of L-dopa (▲), dopamine (■), and 3,4 DHBA (●). Conditions as for Fig. 1

CONCLUSIONS

It is possible to analyse catecholamines and total antioxidant potential using the same chromatographic conditions, which was the aim of the work described in this paper. Preliminary results showed possible application of the method for analysis of L-dopa and determination of the total antioxidant potential of the blood plasma of PD patients.

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