DETERMINATION OF CATECHOLAMINES AND TOTAL ANTIOXIDANT POTENTIAL OF BLOOD PLASMA USING IMPROVED RP-HPLC-ED ASSAY

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ABSTRACT

Preliminary results are presented concerning the application of reversed phase HPLC with electrochemical detection to the analysis of L-dopa and total antioxidant potential. The assay was applied to the estimation of their daily profiles in blood plasma of patients treated with different types of drugs. It turned out that using the same chromatographic conditions it was possible to analyse both values.

INTRODUCTION

Parkinson disease (PD) is mainly caused by decreased efficiency of extrapyramidal movement system. Catecholamines play crucial role in the pathomechanism of the Parkinson Disease [1]. Injury of substantia nigra by free radicals decreases concentration of its main neurotransmitter, namely dopamine [2, 3]. Patients are usually treated substitutionally with L-dopa (and inhibitors of dopa decarboxylase) - its easily absorbed in brain precursor of dopamine. Analysis of L-dopa in the plasma permits PD diagnose, enables individualization of doses selection and avoid side effects (fluctuations and dyskinesias).

It should be expected changes of free radicals concentration and, indirectly, total antioxidant potential (TAP) of blood plasma because of two main reasons. First of all patients are treated with L-dopa and other catecholamines which are antioxidants [4]. From the other side
hypometabolism caused by characteristic in PD tremor increases free radicals concentration.

Free radicals adversely modify biologically active molecules as well as whole cells and are implicated in various degenerative diseases and aging [7-9]. They mediated process have been implicated in the pathogenesis of several diseases, for example PD [10]. It is widely believed that these modifications are preventable by exogenous antioxidants. There is a need for a method to assess and compare strength of particular antioxidants in order to select these of the highest potential for further development as drugs. However, it turned out that frequently more information (e.g. synergetic effects) is obtained measuring total antioxidant potential of biological samples than concentration of particular antioxidants separately.

Preliminary results are presented concerning the application of RP-HPLC to the estimation of total antioxidant potential after hydroxyl radicals generation in the Fenton reaction and their spin trapping with hydroxybenzoate. The method was applied to the determination of hydroxyl radical trapping potential of some catecholamines and plasma of PD patients treated with L-dopa. The aim of paper was to was shown that it is possible to analyze in plasma L-dopa as well as total antioxidant potential using exactly the same chromatographic conditions.

EXPERIMENTAL

Instrumentation

Measurements were performed by means of a chromatograph comprising a Interface Box, 4 channel Degasser K-5004, Solvent Organizer K-1500, Dynamic Mixing Chamber, HPLC Pump K-1001, Fast Scanning UV Detector K-2600, Eurochrom 2000 chromatographic data acquisition and analysis software (all from Knaue GmbH, Berlin, Germany), Basic + marathon Autosampler (Spark Holland B.V., Emmen, The Netherlands), Jet-Stream Plus Column Thermostat (Industrial Electronics, Langenzersdorf, Austria) and LaChrom Amperometric Detector L-3500A (Merck, Darmstadt, Germany). Samples were separated on a Hibar RP-18 5 μm, 250x4 mm I.D. (E. Merck, Darmstadt, Germany) column.
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

Chromatographic experiments were performed with a flow rate 1 ml/min. Column was stabilized at 30 °C by passage of mobile phase for 1 h prior to the chromatographic measurements. Acetate-citrate buffer (pH 4.3) with 0.125 mmol L⁻¹ EDTA and 5% methanol was used as mobile phase.

10 mmol L⁻¹ stock solutions of the analyzed compounds were prepared in Milli-Q water and diluted to the required concentration before use. 20 μL samples were injected using autosampler. Output signal from the photometric detector working simultaneously at 210, 254 and 280 nm as well as amperometric detector working at +0.8 V vs 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 Fenton reaction [5, 6] 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 mg/mL Desferal, and the reaction mixture was immediately analyzed by HPLC.

Blood plasma was deproteinized using saturated uranyl acetate which excess was removed using phosphate buffer pH 4.

RESULTS AND DISCUSSION

HPLC measurements of the Total Antioxidant Potential

Because of the extreme reactivity of hydroxyl radicals they are primarily analysed using radical trapping agents, followed by HPLC determinations of the reaction products. The radical trapping (or “spin
trapping”)) process allows free radicals to be investigated by transforming them into more stable species. When this method is used for biological analyses the radical trap agent should be selected extremely carefully to avoid toxicity problems. Examples of suitable radical trapping reagents are phenylalanine (with which hydroxyl free radicals react to produce tyrosines) [11] or derivatives of aspirin (o-acetylsalicylic acid) [12]. These derivatives can be separated using reversed phase HPLC [12] and detected photometrically or more sensitively by electrochemical detection using a glassy carbon electrode at 0.8 V vs Ag/AgCl [13]. Previously ion exclusion chromatography and p-hydroxybenzoic acid used as the radical trap agent was applied to the analysis of hydroxyl radicals as well we total antioxidant potential [6, 14]. In this paper we would like to show application of reversed phase HPLC to the estimation of TAP.

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 1.**

RP-HPLC chromatograms presented generation of hydroxyl radicals in Fenton reaction (A). As a detector p-hydroxybenzoic acid (pHBA) was used, which reacting with hydroxyl radicals generates 3,4 dihydroxybenzoic acid (3,4 DHBA). Dopamine (DA) added decreased peak of 3,4 DHBA (B). The mobile phase was acetate-citrate buffer (pH 4.3) with 0.125 mmol L⁻¹ EDTA and 5% methanol, the flow rate 1 mL min⁻¹, and the volume injected 20 μL. Detector – amperometric, working at +0.8 V vs Ag/AgCl.
Hydroxyl radicals can be analyzed after spin trapping with, for example, $p$HBA. It can be observed on chromatograms on Fig. 1A where hydroxyl radicals were generated in the Fenton reaction [5]. Their amount is directly proportional to the concentration (and hence to the height of the chromatographic peak) of the product of reaction – 3,4 DHBA. This system can be applied to the estimation of the antioxidant potential. On Fig. 1B is presented similar chromatogram when dopamine was added to the reaction mixture. Decrease of the 3,4 DHBA peak means competition between $p$HBA and dopamine in reaction with hydroxyl radicals. In the other words it means that dopamine in scavenger of hydroxyl radicals. Similar results were obtained with L-dopa. It means that both compounds important in Parkinson disease are antioxidants. This system can be also applied to the analysis of prooxidative properties of the investigated compound [15].

**Chromatography of catecholamines**

![Chromatogram](image)

**Fig. 2.**
RP-HPLC chromatograms of blood serum of PD patient (A) and presented scavenge by it hydroxyl radicals (B). Other conditions as on Fig. 1.

Catecholamines play crucial role in PD. For example, dopamine is neurotransmitter produced by *substantia nigra*, benserazide and carbidopa
are the decarboxylase inhibitors and homovanillic acid is the final step of dopamine metabolism. Usually they are separated using reversed phase HPLC [16]. We are especially interested in concentration of L-dopa and dopamine in blood plasma of patients treated with medicines containing L-dopa. It was found (Fig. 2A) that their analysis is possible using the same chromatographic conditions as for the total antioxidant potential. It turned out that maximal concentration of L-dopa in plasma is 1 hour after it uptake by patient [17]. It was also possible to estimate TAP of blood plasma, as it is presented on Fig. 2B.

**Detection**

![Graph](image)

**Fig. 3.**
Hydrodynamic voltamograms of L-dopa (▲), dopamine (■) and 3,4 DHBA (●). Other conditions as on Fig. 1.

Catecholamines and 3,4 DHBA (also containing catechol group) can be detected photometrically. However, electrochemical detection based on anodic oxidation of catechol group is more sensitive [6, 14]. Because all catechols react similarly (Fig. 3) they cannot be distinguished in complex bio-medical matrix and separation step is necessary. On Fig. 3 hydrodynamic voltamograms of L-dopa, dopamine and 3,4 DHBA are presented. In all cases limiting current was not obtained. Further measurements were performed at 0.8 V. This potential enabled us sensitive detection (10 – 100 μM) of analyzed compounds at small noise level and long-term stability of the electrode.
CONCLUSIONS

It turned out that it is possible to analyse catecholamines as well as total antioxidant potential using the same chromatographic conditions. It means that the aim of paper was achieved. Preliminary results showed possible application of the elaborated method to the analysis of L-dopa and total antioxidant potential in blood plasma of PD patients.

REFERENCES