APPLICATION OF RP-HPLC–ED ASSAY
TO ANALYSIS OF THE BLOOD
OF PARKINSON'S DISEASE PATIENTS

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SUMMARY

Results from application of reversed-phase HPLC with electrochemical detection to analysis of catecholamines and total antioxidant potential are presented. The assay was used to estimate their daily profiles in blood serum of patients treated with anti-Parkinson drugs. It was found that increases in the concentration of hydroxy radicals in blood serum, and of the total antioxidant potential related to the hydroxy radical, was inversely correlated with the concentration of catecholamines in the serum. This suggests that catecholamines are pro-oxidants of hydroxy radicals. The method also enabled us to study changes in the concentrations of catecholamines during their storage at room temperature. It was found that change of drug color was caused by the oxidation of benseraside.

INTRODUCTION

Free radicals are implicated in the pathogenesis of many diseases including, e.g., cancer, Alzheimer's disease, rheumatoid arthritis, and Parkinson's disease [1]. For this reason the literature contains descriptions of many methods of analysis of free radicals [2–6]. Because of the interaction between free radicals and antioxidants, in pathology changes of antioxidant concentration are observed. Antioxidant concentrations can also be measured [7–10]. It has been found that an estimate of total antioxidant potential (TAP) is frequently much more useful then separate analysis of all the antioxidants [11–17]. Cooperation between different antioxidants sometime results in better protection than separate compounds. Examples of this synergism are glutathione, which regenerates ascorbate [18], and ascorbic acid, which regenerates α-tocopherol [19]. Both these quantities, i.e. oxidative

stress and total antioxidant potential, are correlated with each other and even at times identified [20,21].

TAP has been introduced for evaluating, mainly, the antioxidant capacity of complex biological samples, such as serum. These tests exploit different free-radical generators (usually thermolabile diazo compounds generating peroxy radical) and oxidation of the samples analyzed. Because of competition, the sample inhibits interaction between the detector and radical. Results are calculated from the delay time during which antioxidants are consumed. The basic requirement of analyzed sample is that its oxidation rate constant is much higher then that of the compound used as a 'detector'. One literature method is based on generation of peroxy radicals and then fluorimetric or chemiluminescence kinetic measurement of the product of their reaction with the so-called detector, i.e. a compound which is easy to detect. A derivative of fluorescein is usually used as detector for fluorimetric [15] or photometric [12,21] measurement; luminol is used for the chemiluminescence method [22].

Parkinson's disease (PD) usually results from a reduction in the efficiency of the extrapyramidal movement system. Dopamine (DA) plays a crucial role in the pathomechanism of PD [23]. Injury of the *substantia nigra* by free radicals reduces the concentration of dopamine, its main neurotransmitter [23,24]. Patients are usually treated substitutionally with L-dopa (and inhibitors of L-dopa decarboxylase), a precursor of dopamine readily absorbed in the brain. Analysis of L-dopa in the serum enables diagnosis of PD and enables individualization of dose selection, and thus avoidance of side-effects (fluctuations and dyskinesis).

Catecholamines (among them L-dopa) can be analyzed by many methods, for example capillary electrophoresis [25], gas chromatography with mass spectrometric detection (GC–MS) [26,27], radioimmunoenzymatic methods [25,28] and thin-layer chromatography [26,27]. Most frequently, however, they are analyzed by HPLC, usually in the reversed-phase mode, with amperometric detection [29,30]. In this technique sample retention is directly proportional to its hydrophobicity, which means that ionized (dissociated) compounds are usually not retained on the column and are eluted in the dead column volume. Retention of acidic catecholamines therefore increases with decreasing pH whereas that of basic catecholamines decreases.

In the blood serum of PD patients changes of free radical concentrations and, indirectly, total antioxidant potential (TAP) are expected for two main reasons. Patients are treated with huge dose of L-dopa and other

catecholamines, which are strong antioxidants [31,32]. Also, hypermetabolism caused by the characteristic PD tremor increases free radical concentrations.

In this paper we present results from application of RP-HPLC to the estimation of TAP. TAP was measured by generation of hydroxy radicals in the Fenton reaction and their spin trapping with hydroxybenzoate in the presence of the sample. The daily profile of L-dopa in PD patients will be correlated with TAP values.

EXPERIMENTAL

Instrumentation

Measurements were performed by means of a chromatograph comprising interface box, K-5004 four-channel degasser, K-1500 solvent organizer, dynamic mixing chamber, K-1001 HPLC pump, K-2600 fast scanning UV detector, 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 (E. Merck, Darmstadt, Germany). Samples were separated on a 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column (Merck).

Reagents

p-Hydroxybenzoic acid (pHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), and phosphate buffered-saline (PBS) tablets were obtained from Sigma (St Louis, MO, USA). All other reagents (Sigma; 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).

Procedures

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

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

Hydroxy radicals were generated by means of the Fenton reaction [33], by incubation, at 37 ºC for 1 min, of 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) in the presence of 1 mmol L^{-1} p-hydroxybenzoic acid and the analyzed sample. The product of reaction of pHBA with hydroxy radicals, i.e. 3,4-DHBA was detected amperometrically. Addition of sample (serum or dopamine) to the reaction mixture reduced its peak, because of competitive reaction with the radicals. The reaction was stopped by addition of 2 mmol L^{-1} DMSO and 0.1 mg mL^{-1} Desferal, and the reaction mixture was immediately analyzed by HPLC.

Subjects

Blood plasma was obtained from sixteen patients with idiopathic PD (12 men and 4 women, age from 53 to 80 years, average 64 ± 7 years, duration of disease 5–15 years). Informed written consent was obtained from every patient. Permission for the study was obtained from the Bio-Ethnic Commission of the Military Medical Chamber. Patients were hospitalized for two weeks in the Rehabilitation Clinic of the Military Medical Institute in Warsaw. They received chronically an oral dose of 250 mg of L-dopa four times daily (Madopar; F. Hoffmann–La Roche, Bazylea, Switzerland). Each session started at 7 a.m. Blood samples were taken immediately and 1, 2, 3, and 5 h after L-dopa administration. Blood plasma was deproteinized by use of saturated uranyl acetate, excess of which was removed by use of pH 4 phosphate buffer.

Data Analysis

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

RESULTS AND DISCUSSION

Separation of Catecholamines

The aim of our measurements was to separate L-dopa from the other amines and from other reducing agents present in the serum. We also wanted to use the same conditions for estimation of TAP. This seemed possible because TAP measurements are based on analysis of a catechol group-containing compound, 3,4-dihydroxybenzoic acid.

For mobile phases we tested buffers of different pH and containing different concentrations of methanol. It was found that reducing the pH improved the separation of L-dopa. Addition of methanol, at concentrations as low as 5%, to the mobile phase improved peak symmetry and reduced retention of the amines up to 20 min. Further increasing the methanol concentration reduced retention so much that the amines were not separated from compounds used for estimation of TAP.
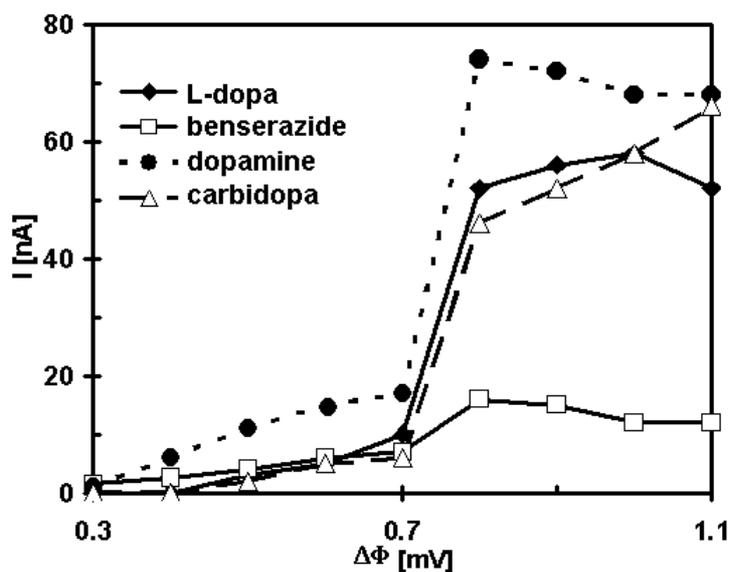

Fig. 1

Effect of potential $\Delta\Phi$ [mV] (relative to Ag/AgCl) on the peak height of catecholamines: 1. L-dopa, 2. dopamine, 3. benserazide, 4. carbidopa. Chromatographic conditions: 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column with precolumn (Knauer); temperature 30°C; mobile phase acetate–citrate buffer, pH 3.5–methanol, 95:5 (*v/v*), flow rate 1 mL min^{-1}

Detection Conditions

Catecholamines, because they are aromatic compounds, can be monitored by use of a photometric detector. Amperometric detection is, however, much more sensitive (approximately three orders of magnitude) [30]. Some of the catecholamine peaks were above the range of the amperometric detector and were, therefore, analyzed by use of serially connected photometric detector.

Hydrodynamic voltammograms obtained from different catecholamines are presented in Fig. 1. In subsequent experiments a potential of +0.8 V relative to the AgCl/Ag electrode was selected as the optimum compromise between the peak height and noise level. It was found that for the catecholamines investigated linear calibration plots were obtained (detection limit approximately 1 nM, $R^2 = 0.97$; linear dynamic range four orders of magnitude and RSD = 5%).

It was found that, in contrast with photometric measurements [12, 30], chromatographic measurements of TAP are not characterized by a linear calibration plot (Fig. 2). This is because we measure the decrease of
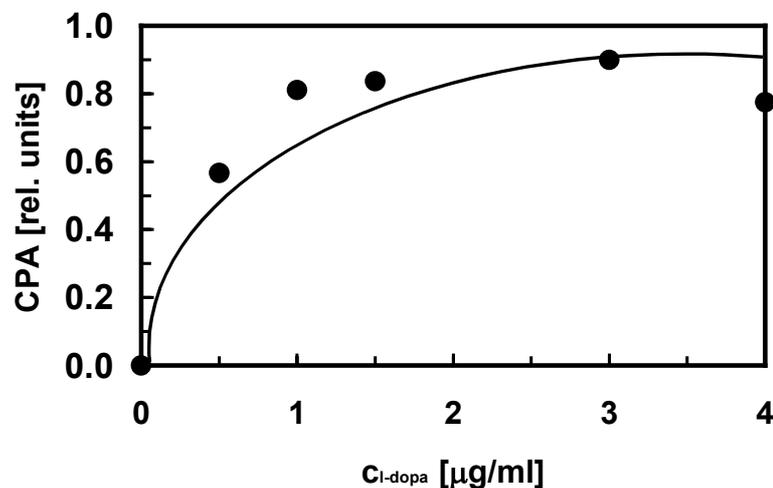

Fig. 2

Effect of L-dopa concentration on TAP of the hydroxy radical (measured as relative changes of 3,4-DHBA peak height). Chromatographic conditions: 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column with precolumn (Knauer); temperature 30°C; mobile phase acetate–citrate buffer, pH 3.5–methanol, 95:5 (v/v), flow rate 1 mL min^{-1}; volume injected 20 μL; amperometric detection at +0.8 V relative to Ag/AgCl

the chromatographic peak. When it completely disappears further increasing the sample concentration has no effect. It is, therefore, convenient to present results on a scale between 0 (lack of the interaction between radical and sample) and 1 (complete scavenging of free radicals by the sample), as is presented in Fig. 2.

Pharmacokinetics of the Catecholamines

Estimation of the daily profile of the L-dopa concentration in the serum of PD patients is very important because of possibility to individualizing therapy for each patient [34]. It was found that the largest concentration of L-dopa in serum was observed 1 h after uptake by the patient (Fig. 3) [35]. Although the concentration of carbidopa also increased during the first hour, it subsequently remained stable (Fig. 4). Changes of concentrations (data not presented) of other amines and other antioxidants present in serum at high concentrations (observation by UV detection) were not statistically significant [36].
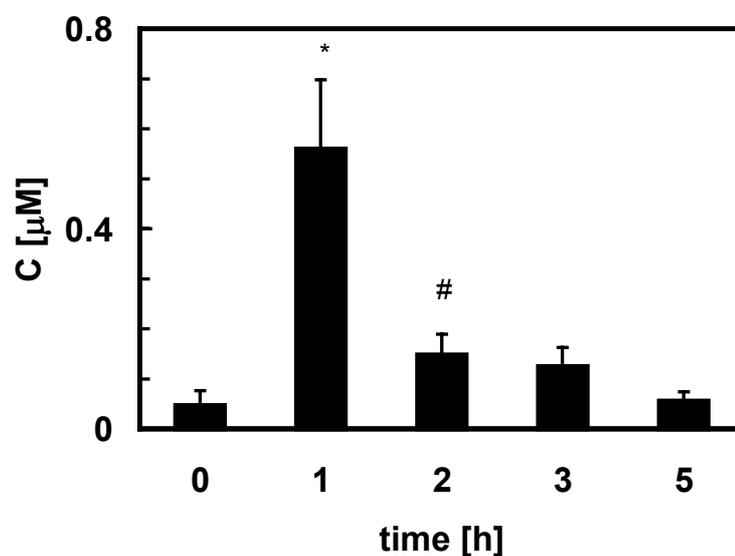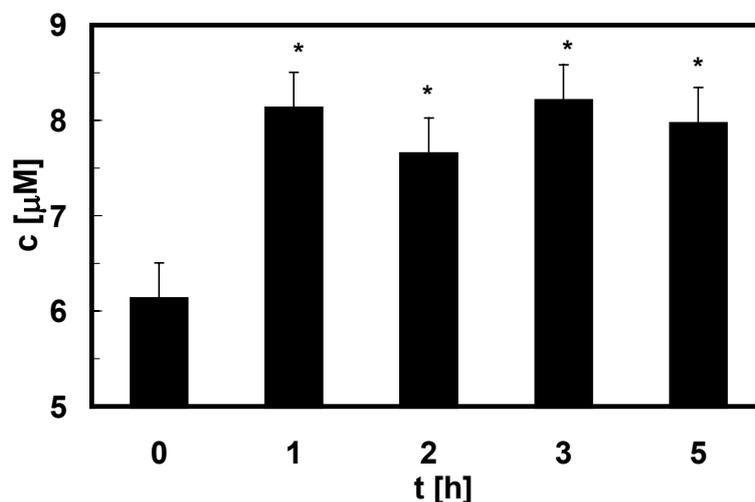
Fig. 3

Changes of L-dopa concentration in the blood serum of Parkinson's disease patients before medicine uptake and after one, two, three, and five hours. $*P < 0.05$ relative to time 0; $^{\#}P < 0.05$ relative to time 1. Chromatographic conditions: 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column with precolumn (Knauer); temperature 30°C; mobile phase acetate–citrate buffer, pH 3.5–methanol, 95:5 (v/v), flow rate 1 mL min^{-1}; volume injected 20 μL; amperometric detection at +0.8 V relative to Ag/AgCl

Fig. 4

Changes of carbidopa concentration in the blood serum of Parkinson's disease patients before medicine uptake and after one, two, three, and five hours. $*P < 0.05$ relative to time 0. Chromatographic conditions: 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column with precolumn (Knauer); temperature 30°C; mobile phase acetate–citrate buffer, pH 3.5–methanol, 95:5 (v/v), flow rate 1 mL min^{-1}; volume injected 20 μL; amperometric detection at +0.8 V relative to Ag/AgCl

Pro- and Antioxidant Properties of Dopamine

In this paper we would like to examine the oxidative properties of DA (related to hydroxy radical) using results obtained by use of this RP-HPLC–ED assay. It was found that addition of DA to the reaction mixture (as described in 'Procedures') reduced the intensity of the 3,4-DHBA peak (compare Figs 5A and 5B). This means that in the presence of the strong antioxidant (ADP) DA scavenges hydroxy radicals. When ADP is absent from the reaction mixture, however, addition of dopamine increases the intensity of the 3,4-DHBA peak (compare Figs 5C and 5D). This means that under these conditions DA is a pro-oxidant, probably because it reduces iron ions, which catalyze the Fenton reaction. Similar results were obtained for L-dopa. This result means that, depending on its concentration and the presence in the solutions of other antioxidants, DA can be a pro- or antioxidant.

The literature contains contradictory information about oxidative properties of dopamine [37–39]. On the one hand dopamine is involved in oxidative stress (because of its catalysis of the Fenton reaction by reduc-
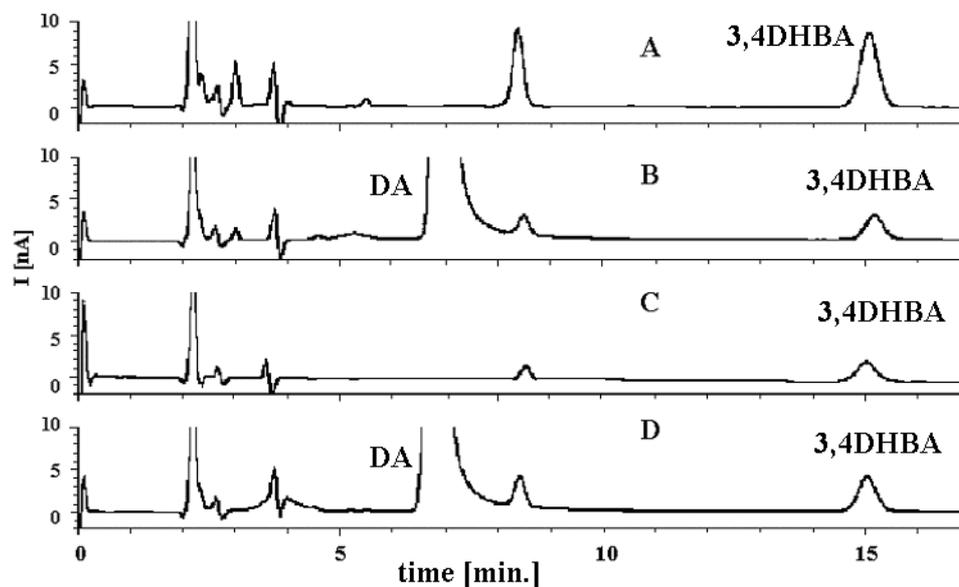
Fig. 5

Pro- and antioxidant properties of dopamine in relation to the hydroxy radical. Description in the text. Chromatographic conditions: 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column with precolumn (Knauer); temperature 30°C; mobile phase acetate–citrate buffer, pH 3.5–methanol, 95:5 (v/v), flow rate 1 mL min^{-1}; volume injected 20 μL; amperometric detection at +0.8 V relative to Ag/AgCl

tion of transition metal ions and generation of semiquinone and superoxide anion radicals during their reduction); on the other hand it acts as an antioxidant (because of its ability to scavenge free radicals, because of its catechol and aromatic groups, and by complexation with transition metal ions).

Enzymatic oxidation of dopamine by monoaminooxidase type B (MAO B) and its auto-oxidation produce hydrogen peroxide (H_2O_2) [37]. In the Fenton reaction (which DA promotes because of its ability to reduce Fe(III) to Fe(II), which catalyzes the reaction) H_2O_2 generates extremely reactive hydroxy radicals [24]. Also, metabolism of DA produces 6-hydroxydopamine and semiquinone radical, which are oxidants. It can, therefore, damage neurons, even causing their death by apoptosis [38].

On the other hand, the catechol group in the DA molecule is characterized by strong antioxidant properties. DA can also complex Fe(II), therefore, preventing the Fenton reaction. We can see that the effect of DA on free radicals is rather complicated and its pro- or antioxidant properties depend on its concentration [39].

Changes of TAP in Parkinson's Disease

As was described above, catecholamines can be either strong anti-oxidants or pro-oxidants. PD patients consume them in quite large quantities (up to 1500 mg per day). This reduces the tremor of patients, which increases hypermetabolism and production of free radicals. We therefore wished to check the correlation between their concentration and the TAP value of blood serum. It was found that increasing the concentration of the amine increased the pro-oxidant properties of serum (compare Figs 4–6). It is worth noting that blood serum scavenges peroxy radicals (i.e. it has antioxidant properties) [36]. It is probable that L-dopa metabolism, controlled by the enzyme MAO B, increases the production of superoxide anion radical [37].
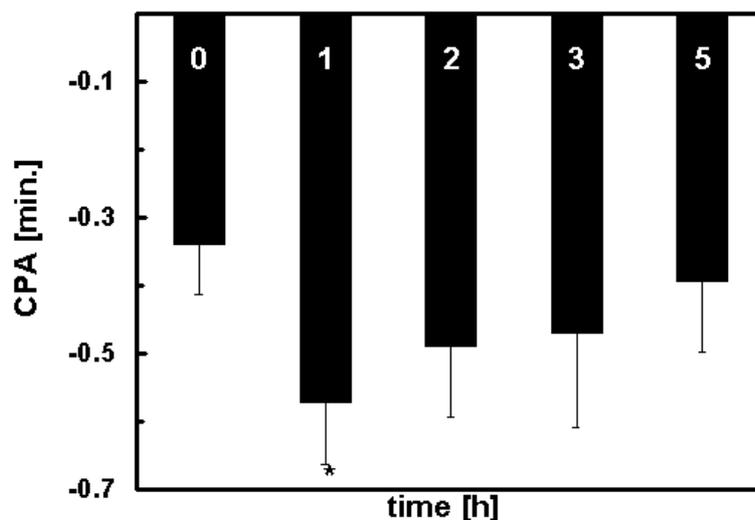

Fig. 6

Changes of TAP in the blood serum of the Parkinson's disease patients before medicine uptake and after one, two, three, and five hours. *$P < 0.05$ relative to time 0. Chromatographic conditions as for Fig. 1. The results are means ± SEM from five independent experiments with triplicate analysis (*$P < 0.05$ compared with control value)

Oxidation of Catecholamines During Storage

We have observed that catecholamine solutions change color during storage. Because they are strong reducing agents, we supposed that they could be oxidized by atmospheric oxygen. Using this method of catechol-

amine analysis we decided to analyze their concentration during storage at 25°C. As the compounds tested we selected catecholamines used as anti-parkinsonian drugs (L-dopa, carbidopa, and benserazide). It was found that the concentration of benseraside deceased the most quickly (Fig. 7) and that this was responsible for the change of color of anti-parkinsonian drugs.
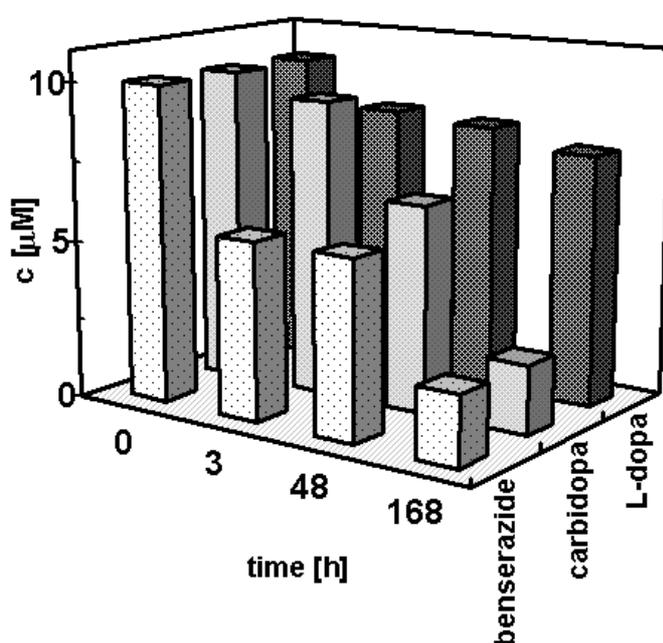

Fig. 7

Changes of concentrations of catecholamines (L-dopa, carbidopa, benseraside) during storage at 25°C. Chromatographic conditions: 250 mm × 4 mm i.d., 5 μm particle, Hibar RP-18 column with precolumn (Knauer); temperature 30°C; mobile phase acetate–citrate buffer, pH 3.5–methanol, 95:5 (v/v), flow rate 1 mL min^{-1}; volume injected 20 μL; ampe-rometric detection at +0.8 V relative to Ag/AgCl

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