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Detection of levodopa, dopamine and its metabolites in rat striatum dialysates following peripheral administration of L-DOPA prodrugs by mean of HPLC–EC

G. Cannazza[a,*], A. Di Stefano[b], B. Mosciatti[a], D. Braghiroli[b], M. Baraldi[a], F. Pinnen[b], P. Sozio[b], C. Benatti[a], C. Parenti[a]

[a] *Dipartimento di Scienze Farmaceutiche, Università degli studi di Modena e Reggio Emilia, Via Campi 183, 41100 Modena, Italy*
[b] *Dipartimento di Scienze del Farmaco, Università degli Studi "G.D'Annunzio", Via dei Vestini, 66013 Chieti, Italy*

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Abstract

A high performance liquid chromatography (HPLC) method was developed to detected simultaneously L-dihydroxyphenylalanine (L-DOPA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in rat striatum dilaysates following oral administration of L-DOPA or its prodrugs. The chromatographic system uses a reversed-phase C_{18} column with electrochemical detection at +0.30 V. Mobile phase consisted of 0.05 M citric acid, sodium EDTA 50 μM, sodium octylsulphonate 0.4 nM at pH of 2.9 and 8% methanol (v/v) at a flow rate of 1 ml/min. The calibration curves were linear over the concentration range of 10 nm to 100 μM and the lower limits of detections were 125 fmol for L-DOPA, 50 fmol for DOPAC, 250 fmol for DA and 150 fmol for HVA at signal noise to ratio of 3. The repeatability (or intra-day precision), expressed by the relative standard deviation, were better than 4%. The construction of microdialysis probes has been described. The in vitro relative recoveries of each microdialysis probe were evaluated and the results show that they are similar and reproducible for all the analytes with CVs from 1 to 4%. The HPLC–EC method was applied to detect the extracellular levels of L-DOPA, DA, DOPAC and HVA in the striatum dialysates of freely moving rats after oral administration of six new potential L-DOPA prodrugs.

Keywords: L-DOPA; L-DOPA prodrugs; Liquid chromatography; Electrochemical detector; Microdialysis

1. Introduction

Parkinson's disease is characterized by selective loss of neurons in the substantia nigra pars compacta and significant reduction of neostriatal content of dopamine (DA) and its major acidic metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); consequently, the functioning of the nigrostriatal dopaminergic system is impaired. Parkinsonian symptoms are relieved by administration of L-dihydroxyphenylalanine (L-DOPA), which is converted by neuronal aromatic L-amino acid decarboxylase (AADC) into DA, hence restoring DA levels in surviving neurons [1].

Despite a good initial response, complications are associated with long-term therapy; these include motor fluctuations, dyskinesias, mental changes and loss of efficacy [2]. The main disadvantages of L-DOPA is low water solubility, its sensitivity to chemical oxidation and peripheral decarboxylation. Only 1% of the administered dose reaches the brain after oral administration [3]. In order to improve the bioavailability and

* Corresponding author. Tel.: +39 059 2055136; fax: +39 059 371590.
E-mail address: pippi@unimo.it (G. Cannazza).

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to minimize unfavorable side effects there has been interest in developing prodrug derivatives of L-DOPA, which would be well absorbed, penetrates rapidly into the brain and are slowly converted to L-DOPA in vivo. A number of prodrugs of L-DOPA have been prepared but the biological activity of these compounds has not been greater than that of L-DOPA itself [4,5].

With the aim to prolong the pharmacological activity enhancing absorption and providing a protection against metabolism we studied new dimeric L-DOPA derivatives **(+)1**, **(+)2** and **(+)3a–d** (Fig. 1) as potential prodrugs. All the new compounds showed chemical stability at acidic pH and also at the physiological pH; a relatively slow release of L-DOPA in rat and human plasma was observed [7]. The present microdialysis study has been performed in order to assess whether these new prodrugs are able to be absorbed after oral administration, to reach the brain and to enhance the extracellular levels of L-DOPA, DA and its metabolites in the striatum of freely moving rats [3,6]. Indeed with the microdialysis technique it has became possible to actually measure as well as to influence the concentration of compounds in the interstitial space of an organ or tissue [8]. Then microdialysis can provide useful informations about the neurochemical effects in the rat striatum of oral administration of the prodrugs **(+)1**, **(+)2** and **(+)3a–d**.

The slow perfusion rate with microdialysis (2 μl/min) means, however, that the amount of dialysate, which can be collected in a reasonable time is very small, thus, leading to a need for a high sensitive assay method. Several methods have been reported for the determination of cathecolamines and among these high performance liquid chromatography (HPLC) with electrochemical detection (EC) is considered to be one of the most sensitive [9–13].

In this work, we developed a sensitive and selective HPLC–EC method in which L-DOPA, DOPAC, DA and HVA in microdialysate samples are simultaneously measured without derivatization or an extraction step.

We also report here the construction of microdialysis probes and the evaluation of recovery experiments using the new HPLC–EC method.

2. Experimental

2.1. Chemicals and reagents

L-DOPA, DOPAC, DA, HVA, citric acid, sodium octylsulphonate, EDTA and HPLC grade methanol were obtained from Sigma-Aldrich (Milan, Italy).

L-DOPA prodrugs [**(+)1**, **(+)2**, **(+)3a–d**] were synthesized by us as previously described [7].

All other chemicals were reagent grade or better and were used as received.

Deionized water (Milli Q-Plus system, Millipore) was used for the preparation of all the solutions.

Ringer's solution consisted of 147 mM NaCl, 4 mM of KCl, 2.2 mM of $CaCl_2$ (pH = 7.4).

Stock solutions (10^{-3} M) of the L-DOPA, DOPAC, DA and HVA were prepared in an anti-oxidant mixture (0.1 M NaCl, 0.01 M HCl, 5 mM $Na_2S_2O_5$). Standard solutions were obtained by dilution of the stock solution to the desired concentrations with Ringer's solution immediately prior to use.

2.2. Microdialysis probe construction

The concentric microdialysis probes employed in this work were fabricated using the procedures described below.

A length of dialysis membrane (250 mm), made of cuprammonium Rayon (M.Wt. cut-off point 20,000 Da), was cut and a piece of tungsten wire threaded into the lumen to act as support during the initial preparation of the probe. The dialysis tubing was then inserted into a steel cannula (30G) and secured with epoxy resin. After the epoxy resin was allowed to dry, the tungsten wire was removed and replaced with a fine fused silica–glass capillary tubing (TSP 075150). A small hole was then made in polythene tubing (0.58 mm i.d., 0.96 mm o.d.) using a 25G needle. The tubing was pushed over the end of the steel cannula without the dialysis membrane in such a way that the fused silica capillary passed through the hole. The silica capillary was then insert in a smaller polythene tubing (0.28 mm i.d., 0.61 mm o.d.); the end of the dialysis membrane was cut to the desired length (3 mm), sealed with epoxy resin and left to dry

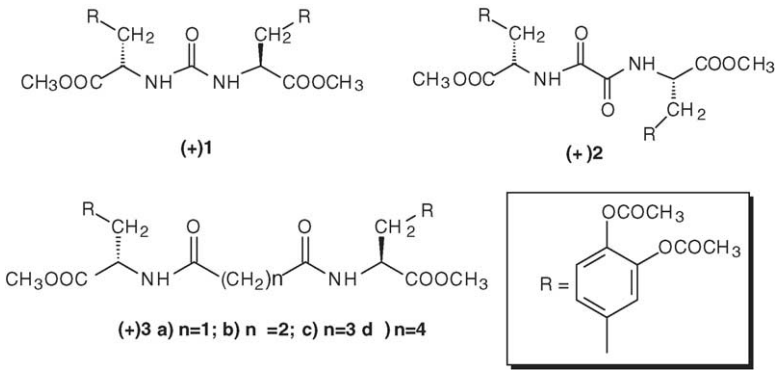

Fig. 1. Chemical structures of prodrugs **(+)1**, **(+)2** and **(+)3a–d**.

for 4 h, as was the junction between the polythene tubing [14].

2.3. Microdialysis probe calibration

Microdialysis probes were calibrated in vitro by placing them in a standard solution of 100 nM.

L-DOPA, DOPAC, DA and HVA in Ringer's solution maintained at 37 °C. The probe was perfused at 2 μl/min with Ringer's solution and dialysate samples were collected over 30 min intervals. Dialysate was collected directly into 200 ml polypropylene vials. The same procedure was used for in vivo experiments.

The extraction efficiency (recovery) of the microdialysis probes was calculated as:

$$EE_R = 100 \times \frac{C_d}{C_s}$$

where C_d is the concentration in the dialysate and C_s is the concentration in the standard solution [15].

2.4. Surgical procedures

Experiments were carried out on male Sprague–Dawley rats (250–300 g) purchased from Harlan Italy (Udine, Italy). They were housed in a climatized room with free access to food and water and kept on a 12 h light:12 h dark cycle.

All procedures involving animals were performed in accordance with the guidelines of the National (D.L. No. 116/1992) and European legislation (ECC No. 86/609) and of the National Institute of Health on the use and care of laboratory animals.

The rats were anaesthetized with Equithensin (3.5 ml/kg) and placed in a stereotaxic frame. The skull was exposed and the microdialysis probes were implanted in the centre of the right striatum with the tip located at AP 0.3 mm, ML −3.5 mm, DV 5.5 mm from bregma and dura surface, respectively. The coordinates are calculated according to the atlas of Paxinos and Watson [16]. The probe was fixed to the skull of the rat with dental cement and anchor screws.

Experiments were carried out 24 h after probe implantation. The rat was placed in a CMA/120 System for freely-moving animals and the probe was connected with a peristaltic micropump (CMA 100, Carnegie Medicine AB, Stockholm) delivering Ringer's solution at a flow rate of 2 μl/min.

Every 30 min, 60 μl dialysate samples were collected manually and kept at −80 °C until analysis by HPLC with electrochemical detection [10,13]. Blanks were collected for 90 min and dialysis samples were collected for 8−9 h after dosing.

2.5. Drug administration

All animals were pretreated with the peripheral AADC inhibitor benserazide HCl (50 mg/kg, i.p.) [16,17] 30 min prior to oral administration of prodrugs **(+)1**, **(+)2** and **(+)3a–d** (0.25 mmol/kg). In the control experiments L-DOPA was administered at the dose of 0.5 mmol/kg [18].

2.6. Analytical procedure

The HPLC system consisted of an L-6200A Intelligent pump (Merck-Hitachi), a Rheodyne 7295 injector with a 50 μl loop and an ESA 5100A coulometric detector. Separation was achieved on a Lichrosphere RP-C_{18} column (4.6 mm × 250 mm, 5 μm).

The mobile phase consisted of 0.05 M citric acid, 50 μM sodium EDTA and 0.4 nM sodium octylsulphonate. The pH of the mobile phase was adjusted to 2.9 by 1 M potassium hydroxide. Methanol was added to give a final concentration of 8% methanol (v/v). The mobile phase was filtered and degassed by vacuum. A flow rate of 1 ml/min was used in all experiments.

The electrochemical detection system included a model 5011 high sensitivity dual detector analytical cell: detector 1 set at +0.30 V; detector 2 set at −0.20 V.

The signal was recorded using the response from detector 1.

2.7. Statistical analysis

Absolute values of L-DOPA, DOPAC, DA and HVA concentration in dialysate are given in ng/ml. All calculations were performed with the data expressed as percentages of mean value from basal levels. Basal concentrations were determined as the mean of at least three measurements with ≤5% variation obtained at the beginning of the experiment. One-way ANOVA was used to evaluate the effect of procedures on each group of animals. If a general effect was determined by ANOVA, post-hoc analysis was performed with the Scheffe or Students' *t*-test with $P \leq 0.05$ used as the level of significance.

3. Results and discussion

3.1. Microdialysis probe calibration

The microdialysis probes were characterized in vitro prior to implantation and used in our experiments. We have determined relative recoveries of L-DOPA, DOPAC, DA and HVA in triplicate and the results show that relative recoveries of all compounds are similar and reproducible. The values of the in vitro recovery were L-DOPA (48 ± 3%), DOPAC (43 ± 2%), DA (31 ± 4%) and HVA (45 ± 1%). The inter-probe variability is quite small: on average 3 ± 2%. In vitro testing of 3 mm microdialysis probes after 8–10 h of use in the striatum showed no decrease in recovery of all compounds.

3.2. Liquid chromatography

Typical chromatograms of dialysate obtained by in vivo sampling are shown in Fig. 2.

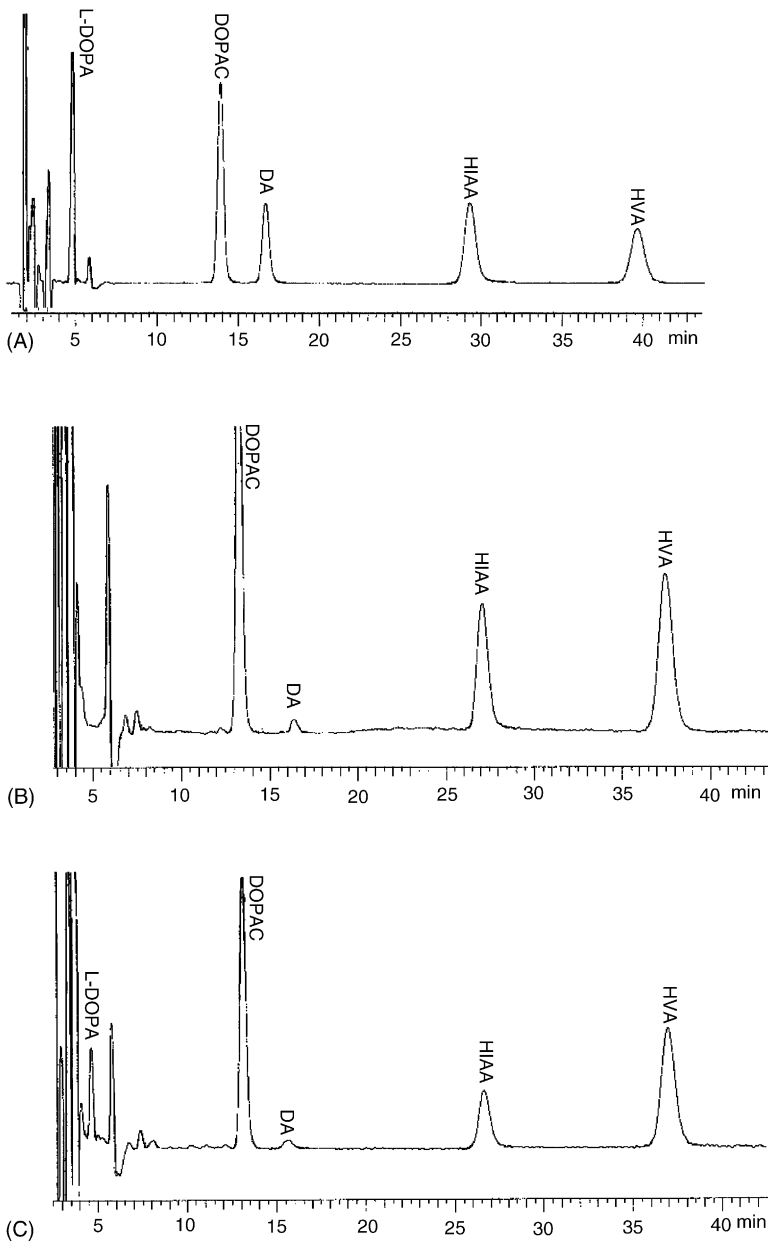

Fig. 2. Typical chromatogram of (A) standard mixture containing L-DOPA (197 ng/ml), DOPAC (168 ng/ml), DA (151 ng/ml), HVA (182 ng/ml), 5-hydroxyindole acetic acid (HIAA) (191 ng/ml) in Ringer's solution. (B) Blank prior to dosing and (C) microdialysis samples after a 0.25 mmol/kg dose of **(+)3c**.

In vivo dialysis samples were directly injected into the HPLC. As can be seen (Fig. 2B), no interferences occured in the blanks obtained prior to dosing of the animal with the drugs and all peaks of interest are clearly separated. The retention time of each peak corresponding to L-DOPA, DOPAC, DA and HVA was identical to that in Fig. 2A, respectively. Basal concentrations (average of 5 rats) in a 60 μl dialysate collected from rat striatum were 21.3 ng/ml for DOPAC, 1.9 ng/ml for DA and 40.8 ng/ml for HVA. The linearity of the method was evaluated over the concentration range of 10 nM to 100 μM. The response was linear for all the compounds over the concentration range tested.

Taking an S/N ratio of **3a** detection limit of 125 fmol for L-DOPA, of 50 fmol for DOPAC, of 250 fmol for DA, of 150 fmol for HVA was achieved.

Standard mixtures of various concentrations were used for determining the analytical accuracy and precision of the assays.

The intra-assay variability was expressed as a percentage of relative standard deviation (R.S.D.). In the standard mixture containing 30 pg to 10 ng of each analytes, all analyses exhibited an acceptable <5% R.S.D.

The inter-assay variability was assessed with the standard mixture containing ~100 pg of each analytes during 5 consecutive working days.

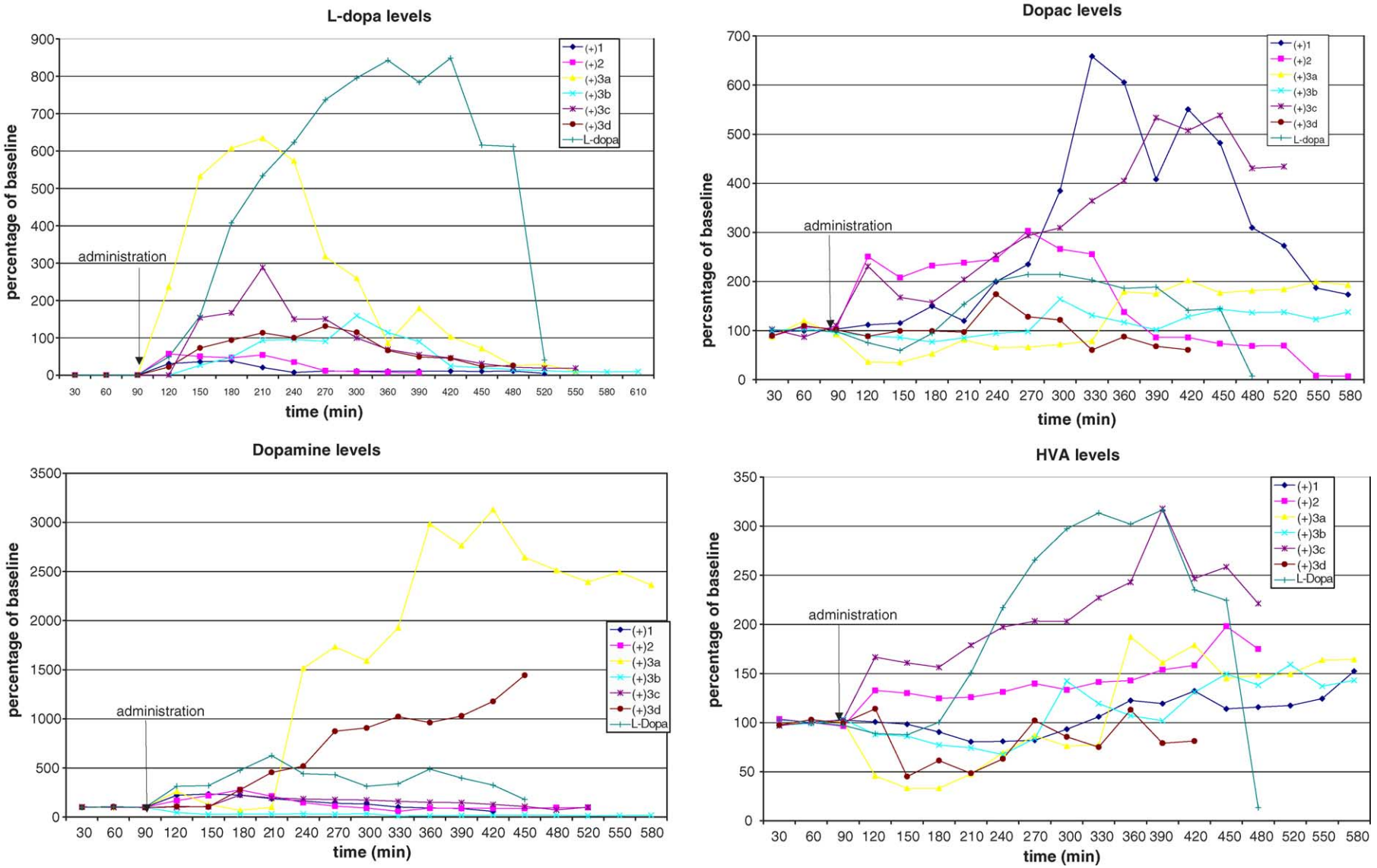

Fig. 3. Effect of oral administration of L-DOPA (0.5 mmol/kg) and L-DOPA prodrugs **(+)1**, **(+)2**, **(+)3a–d** (0.25 mmol/kg) on striatal baseline dialysate of L-DOPA, DA, DOPAC and HVA.

The coefficient of variation values was again satisfactory (R.S.D. < 5–7%).

3.3. *Effect of oral administration of* L-*DOPA and prodrugs* **(+)1**–**(+)3a–d** *on striatal baseline dialysate concentrations of neurochemicals*

The changes in extracellular basal levels of L-DOPA, DA, DOPAC and HVA in rat striatum after oral administration of L-DOPA (0.5 mmol/kg) and prodrugs **(+)1**, **(+)2**, **(+)3a–d** (0.25 mmol/kg) are shown in Fig. 3.

The striatal DA concentration after oral administration of prodrugs **(+)3a** and **(+)3d** (compared with L-DOPA) was markedly improved (6- and 3-fold, respectively). Of particular interest is the finding that the dialysate concentration of DA decreased more slowly after administration of these prodrugs. The results indicate that the **(+)3a** and **(+)3d** could be useful tools for attenuating rapid fluctuations of DA concentration in the striatum and can be effective to increases DA bioavailability in the brain.

4. Conclusion

We developed a sensitive and selective HPLC method with electrochemical detection to measure the concentration of L-DOPA, dopamine, dihydroxyphenyl acetic acid and homovanillic acid in microdialysis samples without derivatization or extraction steps. The method have been utilized to determine the in vivo effects of oral administration of the prodrugs **(+)1**, **(+)2** and **(+)3a–d** upon levels of L-DOPA, dopamine and its metabolites in the striatum of freely moving rats.

Among the studied prodrugs the compounds **(+)3a** and **(+)3d** are of particular interest because they led to a significant increase of DA release that could not be achieved with L-DOPA.

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