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Short communication

Modified high-performance liquid chromatography with electrochemical detection method for plasma measurement of levodopa, 3-*O*-methyldopa, dopamine, carbidopa and 3,4-dihydroxyphenyl acetic acid

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Abstract

Plasma measurements of levodopa and its major metabolites including dopamine and 3-*O*-methyldopa have been limited by cumbersome methods and poor sensitivity within relatively narrow ranges of plasma levels. We now report a modification of an HPLC method that permits concomitant measurements of a wide range of concentrations of levodopa, dopamine (DA), carbidopa, 3-*O*-methyldopa (3-OMD) and 3,4-dihydroxyphenyl acetic acid (DOPAC) from one HPLC injection. The recoveries ranged from 77 to 107% with an intra-day precision around 5% (CV) and inter-day CV's about 10–20%. This validated method will simplify pharmacokinetic studies of levodopa and its metabolites for mechanistic studies or therapeutic clinical monitoring which play a crucial role in development of strategies to prolong motor benefits from individual doses and reduce involuntary movements called dykinesias.

Keywords: Levodopa; Plasma measurements; HPLC; Carbidopa; 3-*O*-Methyldopa; Dopamine; DOPAC (3,4-dihydroxyphenyl acetic acid)

1. Introduction

Abnormalities of cerebral dopaminergic pathways play an important role in the pathophysiology or treatment of several neuropsychiatric diseases including Parkinson disease (PD). Degeneration of nigrostriatal neurons with subsequent loss of striatal dopamine produces many of the clinical manifestations of PD [1] and pharmacotherapy with levodopa, the immediate precursor of dopamine, provides substantial clinical benefit [2]. Concomitant administration of a peripheral decarboxylase inhibitor like carbidopa that does not cross the blood brain barrier reduces systemic production of dopamine and reduces untoward effects such as nausea [3]. However, as the disease progresses and treatment continues many patients with PD develop other drug related side effects such as short duration of response to individual doses and involuntary movements called dyskinesias [4]. Inhibitors of catechol-*O*-methyl transferase (COMT) reduce *O*-methylation of levodopa and dopamine thereby potentially prolong motor benefit from individual doses of levodopa [5]. Measurements of levodopa and its metabolites in blood have been crucial for development of these strategies by clarifying the role of systemic pharmacokinetics in the clinical responses [6].

Plasma measurements of levodopa, 3-*O*-methyldopa (3-OMD), dopamine (DA) and carbidopa, however, have been technically difficult in people with PD treated with pharmacologic doses of levodopa in the presence of a decarboxylase inihibitor since levodopa and 3-*O*-methyldopa levels tend to be high, at least 1000-fold higher than plasma dopamine [7]. Measurements of one of the metabolites of dopamine 3,4-dihydroxyphenyl acetic acid (DOPAC) also is useful since DOPAC may be involved in the pathogenesis of PD. Recent animal studies found that DOPAC reacts with nitric oxide in mitochondria to produce compounds that inhibit O_2 uptake that could lead to cell death [8]. Rondelli et al. [9] developed a high performance

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liquid chromatography (HPLC)-based method for levodopa, 3-*O*-methyldopa dopamine and levodopa methyl ester. We now report a modification of this method that permits simultaneous measurements of carbidopa and DOPAC in addition to other analytes.

2. Methods and materials

2.1. Subjects

We took blood samples from eight patients (seven males) with clinical diagnoses of PD. Their age ranged from 55 to 83 years with an average of 70. They had a weight range of 45–93 kg with an average of 76.5 kg. Four of them had been treated chronically with levodopa but refrained from taking medication overnight prior to these studies. Each subject was given carbidopa 200 mg orally at least 1 h prior to oral intake of levodopa/carbidopa 150 mg/37.5 mg. Three milliliter samples were drawn from the intravenous line for 3 h after levodopa. These studies were approved by the Human Studies Committee of Washington University in St. Louis, and all subjects gave written, informed consent.

2.2. Materials and reagents

L-DOPA, DOPAC, dopamine, and 4-dihydroxybenzylamine (DHBA internal standard) were purchased from RBI-Sigma, USP grade. Carbidopa (purity >98%), 3-OMD (purity >99%), heparin, EGTA, glutathione, sodium metabisulfite and perchloric acid were obtained from Sigma–Aldrich, St. Louis, MO. Acetonitrile and methanol were of HPLC grade and obtained from Fisher Scientific. CAT-A-PHASE HPLC buffer, designed for analysis of catecholamines in plasma, was from ESA (catalog #45-0180, Chelmsford, MA). The buffer contains methanol, phosphate buffer and a patented ion pairing agent. We modified the CAT-A-PHASE HPLC buffer by addition of 0.3% acetonitrile and adjusted pH to 3.20 + 0.03 with 2 N sodium hydroxide to facilitate optimal separation of all compounds. The pH adjustment was checked before every use, as small pH changes produce large shifts in retention time for levodopa, 3-*O*-methyldopa and carbidopa.

2.3. Standard solutions

Standards were prepared freshly for each sample run using 1 mg/ml stock solutions stored frozen at −80 °C (0.1 M perchloric acid (PCA+) containing 0.1% sodium metabisulfite). A standard mixture of 250 μg levodopa, 500 μg 3-*O*-methyldopa, 200 μg carbidopa, 25 μg dopamine, and 25 μg DOPAC in 1 ml was prepared using aliquots of the stock standards and serially diluted as above to give concentrations that ranged across two orders of magnitude in a total of six dilutions. Each final standard sample contained 0.5 ml aliquot of plasma from a normal not treated with levodopa, 10 μl (0.5 μg) internal standard (1 mg/ml DHBA diluted to 50 μg/ml in PCA+) and 10 μl aliquot of one of the dilutions to yield six standard samples. The standard curve was obtained by calculating the peak–height ratios of each compound to the internal standard plotted against the known concentration of each substance.

2.4. HPLC

We used a Coulochem II HPLC system (ESA, Inc.) with an ESA Model 580 Solvent Delivery Module, a Rheodyne 9125 Sample Injector, and an ESA catecholamine HR-80 Column (8 cm × 4.6 mm I.D. packed with 3-μm C_{18} stationary phase) protected by a Newguard Cartridge (15 mm × 3.2 mm I.D. packed with 7-μm spherical RP-18 Aquapore, a Brownlee product). The conditioning cell was ESA Model 5021 and the analytical cell was ESA model 5011. The system operated in redox mode. The optimal selection of the applied electrode potentials were determined by generating current–voltage curves for each compound. The conditioning cell was set at 450 mV. The first electrode in the analytical cell was set at 50 mV, and the second one at −400 mV. Plasma levels of levodopa and frequently 3-OMD (especially in chronically treated patients) are 100–1000 higher than DOPAC, dopamine and carbidopa. We optimized detector settings depending on the compound with a full-scale gain varying from 100 nA to 2 mA during the run. The change to the more sensitive gain was done before or after the internal standard peak. Output from the second analytical cell was recorded on a ChromJet integrator, Model SP4400 (Thermoseparation Products, Fremont CA, USA). A flow rate of 0.7 ml/min was used, and separations were run at room temperature (about 21 °C). The mobile phase was not recycled during the day of analysis. The length of analysis was 20 min. Up to 20 runs including standards and samples were performed over 8–10 h.

2.5. Sample preparation

Blood samples were collected in a syringe and immediately transferred to 6 ml polypropylene tubes containing 5.7 mg EGTA, 3.6 mg reduced glutathione, and 50 units heparin in 60 μl on ice to maintain stability of the plasma catecholamines. This solution was prepared fresh every 14 days with the pH adjusted to 6.5–7.0. The blood samples remained on ice less than 30 min until centrifuged. Whole blood was spun at 1250 × *g* for 30 min in a CRU-5000 centrifuge IEC at 4 °C. The plasma was transferred to 6 ml polypropylene tubes, snap frozen on dry ice, and stored in a −80 °C freezer until analyzed. Samples were thawed, extracted, and analyzed on the same day. Plasma (0.5 ml) obtained from a normal subject not exposed to levodopa used as a blank, and plasma samples from patients were spiked with 10 μl (0.5 μg) internal standard (1 mg/ml DHBA diluted to 50 μg/ml in PCA+). To precipitate proteins perchloric acid (1.2 M) 0.3 ml was added to the plasma. Tubes were mixed, stored on ice for 10 min and spun for 7 min at 1250 × *g* at 4 °C; 300 μl of the supernatant were added to 200 μl of 2 M potassium citrate buffer (pH 3.8) to precipitate the perchlorate. Each tube was vortexed for 1 min, left on ice for 10 min, then centrifuged as above. Three hundred microliters of the clear supernatant were filtered through 0.22 mm syringe filters and 20 μl of the supernatant were injected onto the HPLC for analysis. Recov-

eries were calculated for two different sets of samples. The first set of samples was prepared as described above with internal standards and the compound added prior to protein extraction. The second set had internal standard and compound added after the extraction procedure and just before HPLC. HPLC was run on the day of sample preparation since re-analysis after freezing for a day or two did not yield reproducible results for carbidopa measurements.

The baseline noise level was determined by injection of buffer. Limit of detection was defined by the injected amount of each compound that resulted in a peak three times as high as baseline noise, while limit of quantitation was defined as the amount giving a signal to noise ratio of 10. The precision of the method was evaluated by inter- and intra-day coefficients of variation (CV) using three concentrations. We also calculated the precision for the concentrations at the limits of quantification. The values over 3 months were used to calculate the inter-day precision while repeated double spiked samples every 3–9 h later were used to determine the intra-day precision. Relative error of the measurements over 3 months was calculated as an estimate of accuracy.

3. Results

We obtained the peaks for levodopa, dopamine, 3-*O*-methyldopa, carbidopa and DOPAC within a run time of 20 min Fig. 1. Calibration curves for these compounds were linear over 100-fold ranges using a global linear least-squares regression line without using a weighting factor. The regression equations were: ($n=8$): $y=0.0033x-0.085$ ($r=0.99$) for levodopa, $y=0.00014x+0.199$ ($r=0.99$) for 3-*O*-methyldopa, $y=0.00097x+0.0034$ ($r=0.99$) for DOPAC, $y=0.0007x+0.0010$ ($r=0.99$) for dopamine, and $y=0.00026x+0.0062$ ($r=0.99$) for carbidopa.

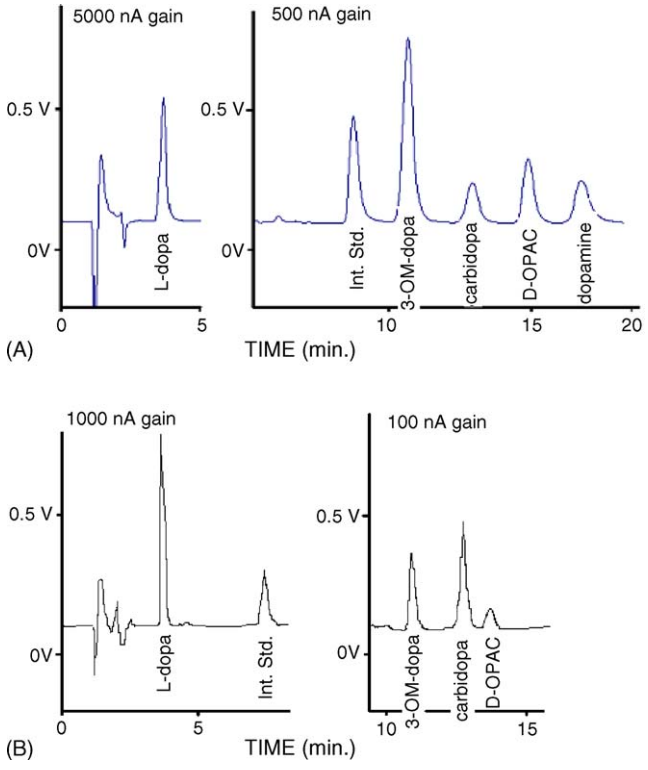

Fig. 1. (A) Typical chromatogram of standards (levodopa 2500 ng/ml, 3-OMD 5000 ng/ml, carbidopa 2000 ng/ml, DOPAC 250 ng/ml, dopamine 250 ng/ml). (B) Typical chromatogram of a patient plasma sample (levodopa 1887 ng/ml, 3-OMD 710 ng/ml, carbidopa 694 ng/ml, DOPAC 40 ng/ml). Note that the change in gain was performed after the internal standard peak was observed.

Table 1
Precision and accuracy of the method. Precision was measured as coefficient of variation (CV%) (100 × mean standard deviation/average%), while accuracy was measured as relative error (RE%) ((true value–measured value) × 100/true value%)

Compound	Concentration (ng/ml)	Inter-day precision CV % ($n=7$)	Intra-day precision CV % ($n=8$)	Accuracy (RE %) ($n=7$)
L-DOPA	125	13.53	3.18	15.15
	500	10.20	4.56	2.80
	2500	10.26	8.15	−0.20
Dopamine	12.5	10.61	8.15	44
	50	10.55	6.35	14.6
	250	8.49	5.02	−11.35
3-*O*-Methyl-DOPA	250	17.31	3.64	4.94
	1000	16.43	5.76	1.06
	5000	14.05	6.18	−0.48
Carbidopa	100	17.39	12.98	−1.87
	400	18.43	5.63	5.12
	2000	14.99	8.45	−0.01
DOPAC	12.5	21.12	5.64	6.19
	50	22.30	5.07	−4.16
	250	17.29	6.18	−0.16

The limits of quantitation were 4 ng/ml for levodopa, 5 ng/ml for 3-*O*-methyldopa, 5 ng/ml for DOPAC (CV = 20%), 5 ng/ml for dopamine (CV = 16%) and 40 ng/ml for carbidopa (CV = 21%). Recoveries ranged from 77% for DOPAC to 107% for dopamine with other components falling in the 85–90% range. Precision and accuracy are listed in Table 1.

Fig. 2 demonstrates typical pharmacokinetic plasma curves of the compounds after pretreatment with carbidopa and a single oral dose of levodopa in previously treated and dopa-naïve PD patients. As expected, plasma levodopa peaks at 30–60 min [10]. Further, carbidopa given at least an hour before levodopa maintained nearly constant levels for 90–100 min after levodopa intake. In the previously untreated PD patients, peaks for 3-*O*-methyldopa appeared 15–30 min after administration of levodopa rising steadily for the next 50 min. The 3-*O*-methyldopa level in the first timed blood sample was at least five-fold higher in the chronically treated than the dopa-naïve PD patient.

4. Discussion

Similar to the technique of Rondelli et al. [9] our HPLC method detects clinically relevant minimal amounts of plasma levodopa and its related compounds and yet maintains accuracy across a wider range of concentrations than previous methods [11,12]. However, our approach also permits measurement of DOPAC and carbidopa in the same HPLC run. Six to eight sam-

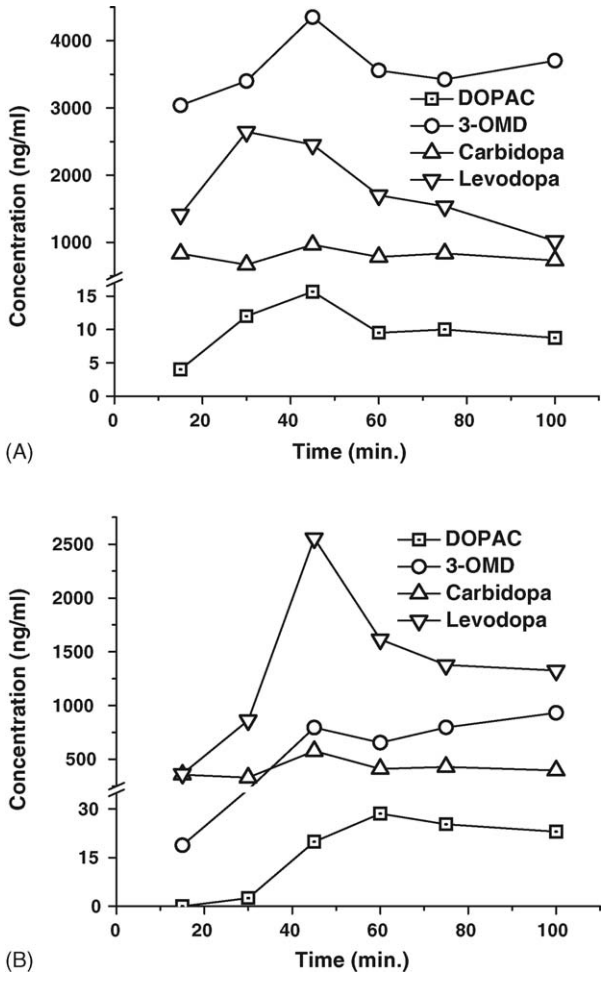

Fig. 2. Averaged plasma measurements of analytes in four PD patients chronically treated with levodopa (A) who withheld medication overnight and plasma measurements of analytes in four dopa-naïve PD patients (B). Both subjects were pretreated with 200 mg carbidopa about 1 h prior to 150 mg oral levodopa intake at time 0.

ples may be analyzed in 1 day along with a set of standards. We achieved these improvements with changes in sample preparation: EGTA and gluthatione were added to stabilize plasma catecholamines and CAT-A-PHASE was used as a buffer to enhance separations. Optimal electrode potentials were determined by analysis of current–voltage curves for each compound. A gain change during the HPLC run permitted identification of DOPAC and other analytes within the same run.

The demonstrated pharmacokinetic plasma curves of dopamine confirm that carbidopa taken orally prior to levodopa blocks any detectable accumulation of plasma dopamine that would reflect peripheral decarboxylation of levodopa. Our findings also reveal high plasma levels of 3-*O*-methyldopa in treated PD patients that likely reflects its long half life [13].

An alternative approach uses liquid chromatography with porous graphitic carbon coupled with mass spectrometry [14]. However, DOPAC cannot be measured simultaneously with the other metabolites, without switching between positive and negative electrospray ionization.

Our validated, relatively simple method provides the means to perform both pharmacokinetic studies of orally or intravenously administered levodopa but does not easily scale to large hundreds of analyses for large studies. Further it can be used for microdialysate measurements in animal studies enabling simultaneous DOPAC estimation, which is gaining increasing relevance in exploring pathophysiology of Parkinson disease [8].

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