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Short communication

Simultaneous determination of L-dopa and 3-O-methyldopa in human platelets and plasma using high-performance liquid chromatography with electrochemical detection

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Abstract

Various high-performance liquid chromatographic (HPLC) methods for the determination of plasma levels of L-dopa and of its metabolite, 3-O-methyldopa (3-OMD), have been previously described. In this study, we report a modification of these methods, that enables the assay of these two compounds in platelets and plasma obtained from the same sample of whole blood. Reversed-phase (RP) HPLC with electrochemical (coulometric) detection was used. The within-run and between-run coefficients of variations, for the two compounds, were less than 10%, in both platelets and plasma; the detection limits for platelet levels of L-dopa and 3-OMD were 2 and 6 ng/10^9 platelets, respectively. In plasma, the detection limits for L-dopa and 3-OMD were 1 and 3 ng/ml, respectively. The method is rapid and simple. When applied to a population of patients with Parkinson's disease under treatment with L-dopa, this method revealed detectable levels of L-dopa and 3-OMD in the platelets of all patients. The application of this technique may provide new insights into the pharmacokinetics of L-dopa in patients with Parkinson's disease. © 1997 Elsevier Science B.V.

Keywords: L-Dopa; 3-O-Methyldopa

1. Introduction

Treatment with L-dopa, the direct precursor of dopamine, is the most effective therapy for Parkinson's disease (PD). However, after 3–5 years of therapy most patients begin to experience "fluctuations" in the ability of L-dopa to control PD motor symptoms ("wearing off" and "on–off" phenomena). The mechanisms responsible for these oscillations are not fully understood, although central and/or peripheral L-dopa pharmacokinetics are certainly involved [1–5]. Determination of plasma levels of L-dopa, and of its long-lasting catabolite 3-O-methyldopa (3-OMD), has been proposed, to explore L-dopa pharmacokinetics in PD patients and various high-performance liquid chromatographic (HPLC) methods have been developed for this purpose [6–10]. However, plasma levels of L-dopa and motor responses of patients are not systematically correlated [11,12]. As a consequence, information provided by the assay of blood levels of L-dopa and 3-OMD rarely have a real impact on the management of PD therapy.

Blood platelets share many characteristics with

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monoaminergic neurons, such as the presence of serotonergic and adrenergic receptors on the cell membrane, a specific transport mechanism for serotonin, and mitochondrial monoamine-oxidase B activity [13,14]. Platelets are also able to take up norepinephrine and epinephrine from the blood and to store them, at stable levels, for relatively long periods of time (4–7 days) [15–18]. It has been demonstrated that platelets posses an active transport system for dopamine [19,20], but, to our knowledge, no information about the ability of platelets to incorporate its direct precursor, L-dopa, has ever been provided. The aim of this study was to modify existing HPLC methods, to obtain a rapid and simple technique that allows the determination of L-dopa and 3-OMD in platelets and plasma obtained from the same sample of whole blood.

2. Experimental

2.1. Chemicals

L-Dopa, 3-OMD, KH_2PO_4, $NaHCO_3$, NaCl, KCl, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), NaH_2PO_4, EDTA, $MgCl_2$ and dextrose were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was purchased from Bio-Rad (Richmond, CA, USA), acetonitrile, HPLC-grade, from Carlo Erba (Milan, Italy).

2.2. Sample preparation

Venous blood (4.5 ml) was collected from the antecubital vein, without a tourniquet, in vacuum tubes where it was mixed 9:1 with 0.129 *M* sodium citrate (Terumo Medical, Elkton, MD, USA). Blood was then centrifuged at 200 *g* for 15 min to obtain platelet rich plasma (PRP). This speed of centrifugation was chosen because it allowed one to combine the highest number of platelets with the absence of contamination from red blood cells or leukocytes in the PRP sample (data not shown). PRP was separated and platelets were counted with an automated cell analyzer (Technicon H1, Technicon Instruments, Tarrytown, NY, USA), which also verified the absence of contamination from other blood cells. PRP was then centrifuged at 2000 *g* for 15 min to obtain platelet pellets; platelet poor plasma (PPP) obtained with this procedure was immediately stored at −80°C. Platelet pellets were washed by resuspension in a volume (equal to the original PRP volume) of modified Tyrode's solution (7 m*M* $NaHCO_3$, 150 m*M* NaCl, 2.7 m*M* KCl, 5 m*M* HEPES, 0.55 m*M* NaH_2PO_4, 1 m*M* EDTA, 0.5 m*M* $MgCl_2$, 5.6 m*M* dextrose), then reconstituted by means of a final centrifugation at 2000 *g* for 15 min. Supernatant were discarded and pellets were stored at −80°C until the assays were performed (always within a week). All centrifugations were performed at room temperature.

2.3. HPLC

The HPLC system consisted of a pump (1350 Softstart, Bio-Rad, Hercules, CA, USA) equipped with a C_{18}, reversed-phase, 70×4.6 mm I.D., 3 μm column (Ultrasphere XL ODS, Beckman), fitted with a 5×4.6 mm pre-column (Ultrasphere XL ODS, Beckman). The system was connected to an autosampler (AS 100, Bio-Rad, Richmond, CA, USA). The detection device was a three-coulometric–electrode system (Coulochem 5100, ESA, Bedford, MA, USA) consisting of one conditioning cell (with one electrode set at +0.20 V), placed before the column, and one analytical cell with two electrodes set at −0.20 V and +0.30 V, respectively. The signal generated by the third electrode was converted into a chromatographic trace and analyzed by a computing integrator (HP 3396 series II, Hewlett-Packard, Avondale, PA, USA). The mobile phase (pH 2.9) consisted of 50 m*M* KH_2PO_4, 0.7 m*M* SDS and 0.3 m*M* EDTA, mixed with 12% (v/v) acetonitrile.

Prior to injection, pellets were re-suspended in 1 ml of ice-cold 0.4 *M* $HClO_4$, and homogenized by means of ultrasounds (Ultrasonic 2000, Artek, Farmingdale, NY, USA). Homogenates were then centrifuged at 15 000 *g* for 5 min, at room temperature; 10 μl of the supernatant obtained were directly injected into the HPLC system. As for plasma, the procedure followed was the same as previously described [7]. In brief, 100 μl samples were deproteinized by adding 100 μl of 1.2 *M* $HClO_4$. Samples were then diluted 1:5 with distilled water and centrifuged at 15 000 *g* for 5 min at room temperature. Ten microliters of the supernatant obtained were directly injected into the HPLC system.

Samples were eluted isocratically at room temperature, at a flow-rate of 1.2 ml/min. L-Dopa and 3-OMD concentrations in the samples were quantified by comparing peak heights of the two compounds in the samples with the peak heights of a standard mixture containing known amounts of L-dopa and 3-OMD.

2.4. Subjects

Preliminary experiments were carried out on blood samples obtained from five healthy volunteers (two males and three females), ranging in age from 22 to 34 years; after separation, PRPs were pooled and then aliquoted in 2-ml samples. Samples were incubated for increasing periods of time (15, 30, 45, 60, 120 and 180 min) with L-dopa at a concentration (2 μg/ml) which is in the range of concentrations usually found in the plasma of PD patients undergoing regular therapy with the drug. In another experiment, samples obtained from another pool of PRP were incubated, for 30 min, with increasing doses of L-dopa (0.5, 1, 2, 4, 8 and 16 μg/ml). The recovery of the method was evaluated by measuring the concentrations of L-dopa and 3-OMD in PPP samples previously spiked with known amounts of the two compounds. Blood samples were then obtained from fifteen patients suffering from PD (ten males and five females, ranging in age from 44 to 76 years). All patients were taking L-dopa, at different dosages; 75 to 1825 mg/day. Blood samples were drawn in the morning, 3 to 4 h after the administration of the drug. Blood was also obtained from ten healthy volunteers (six males and four females, ranging in age from 48 to 66 years). In four patients, extra blood samples were drawn; PRPs were then pooled and subsequently aliquoted; platelet pellets and PPP were prepared as described above and used to determine the within-run and between-run precision of the method. All experiments were carried out in duplicate.

2.5. Statistics

The existence of correlations between platelet and plasma levels of L-dopa and 3-OMD was explored by calculating the Pearson's correlation coefficient (r).

3. Results and discussion

After incubation of PRP with L-dopa 2 μg/ml, clearly detectable L-dopa peaks were found in platelets, at all the time-points studied. The incubation time did not affect platelet levels of the drug significantly, the concentrations reached after 15 min of incubation being maintained at the subsequent time-points (Fig. 1A). The increase in L-dopa concentration was paralleled by an almost superimposable increase in the levels of platelet L-dopa (Fig. 1B), with platelet levels of L-dopa doubling as the amount of drug added doubled. The detection limits for platelet levels of L-dopa and 3-OMD, at a signal-to-noise ratio of 3, were 2 and 6 ng/10^9 platelets, respectively. Detection limits for plasma L-dopa and 3-OMD were 1 and 3 ng/ml, respectively. As far as the precision of the method was concerned, the within-run and between-run coefficients of variation

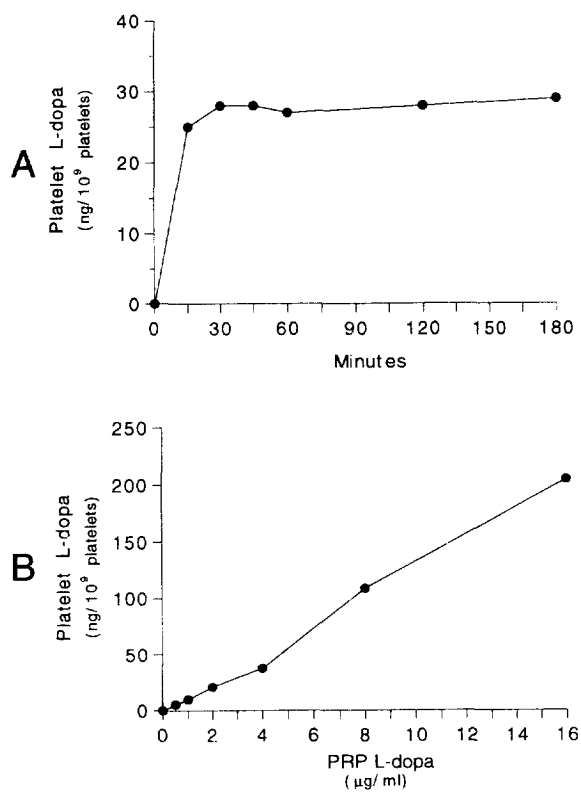

Fig. 1. (A) Platelet levels of L-dopa after incubation of PRP with L-dopa (2 mg/ml) at different time points. (B) Platelet levels of L-dopa after incubation (45 min) of PRP with increasing concentrations of L-dopa.

were 8.7 and 9.1% for platelet L-dopa, 9.2 and 9.6% for platelet 3-OMD. As for plasma, the within-run and between-run variations were 4.7 and 5.9% for L-dopa, 4.9 and 6.3% for 3-OMD. The recovery of the method was 96% for L-dopa and 94% for 3-OMD.

L-Dopa and 3-OMD were detected in the platelets of all patients studied. Fig. 2 shows typical chromatograms of L-dopa and 3-OMD in platelets and plasma. A direct correlation was found between levels of both L-dopa and 3-OMD in the two compartments (Fig. 3). No traces of L-dopa or 3-OMD, either in platelets or plasma, were found in control subjects.

The method described in this study proved rapid and simple, the only steps required prior to injection being sonication of platelet pellets, deproteinization of homogenates (and plasma samples) and centrifugation of samples. It is also characterized by high sensitivity and precision. Incubation of PRP with increasing concentrations of L-dopa, or with the same concentration of drug for different periods of time, showed that L-dopa enters platelets rapidly and that intra-platelet levels of L-dopa are directly related to

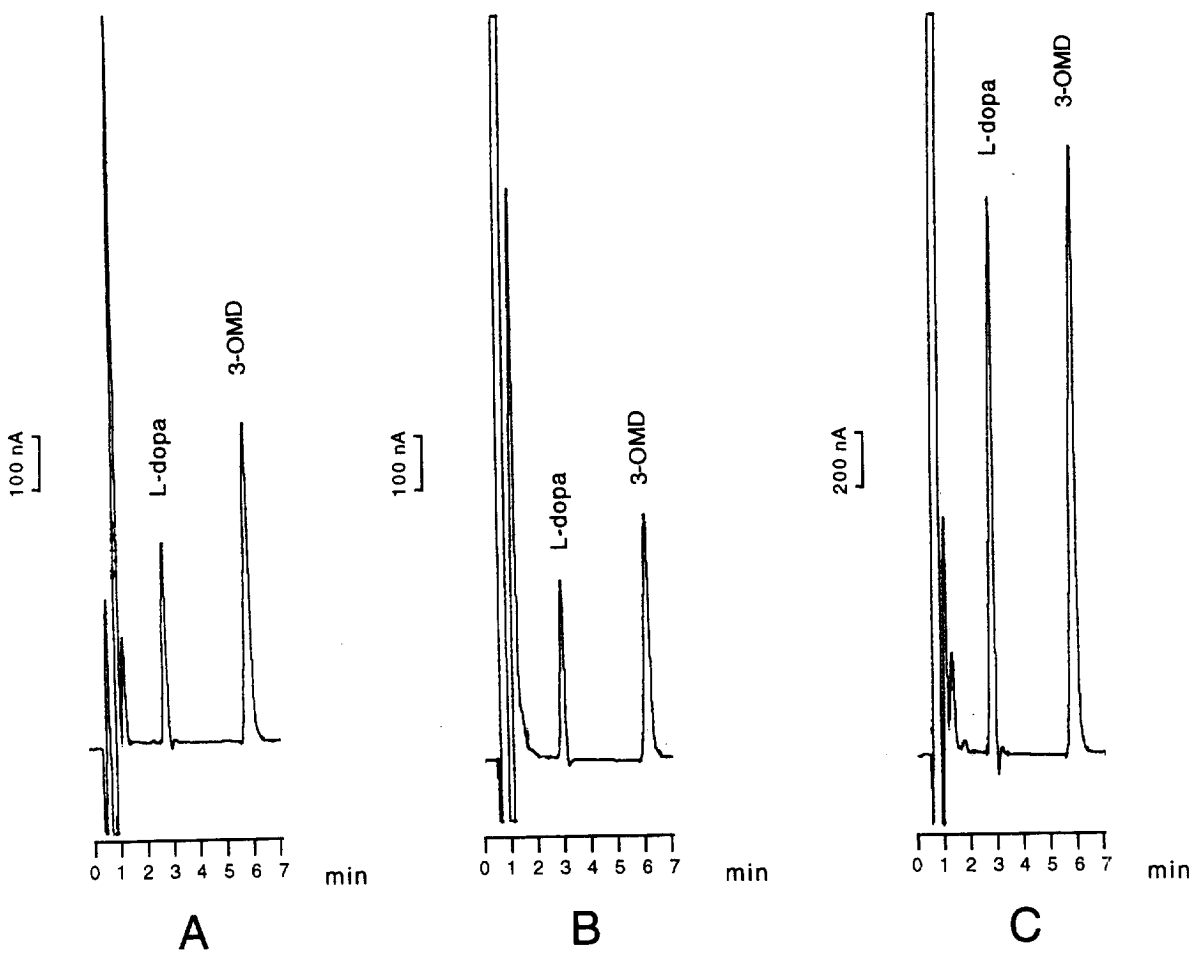

Fig. 2. (A) Chromatogram of a standard solution containing L-dopa (50 ng/ml) and 3-OMD (200 ng/ml). (B) Peaks of platelet L-dopa and 3-OMD (L-dopa: 45 ng/10^9 platelets; 3-OMD: 154 ng/10^9 platelets). (C) Peaks of plasma L-dopa and 3-OMD (L-dopa: 1.37 μg/ml; 3-OMD: 3.84 μg/ml).

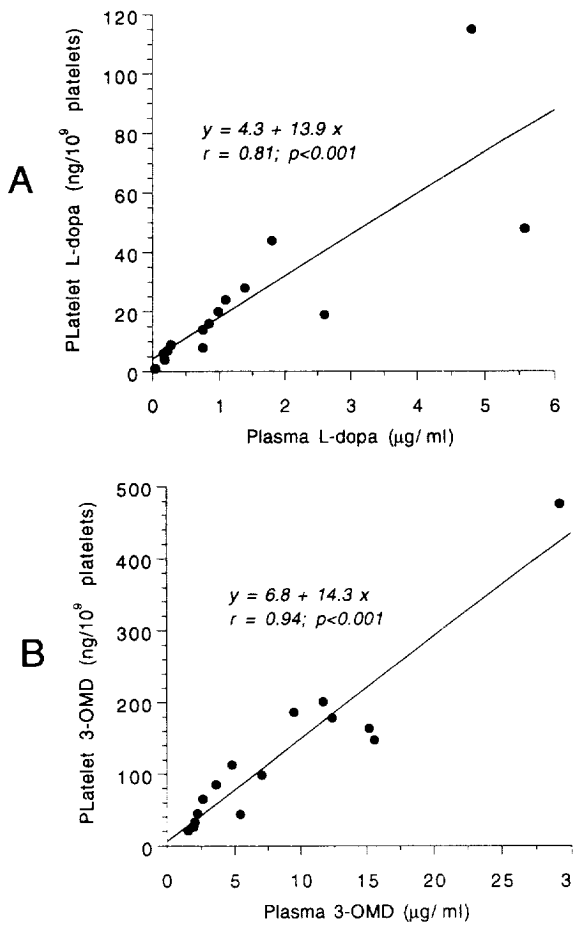

Fig. 3. Correlation between plasma and platelet levels of L-dopa (A) and 3-OMD (B) in patients with Parkinson's disease.

its plasma levels. L-Dopa and 3-OMD were always detectable in platelets of PD patients, even in those patients taking low doses of the drug and, also in PD patients, plasma and platelet levels of L-dopa and 3-OMD were linked by a direct correlation.

In conclusion, we described a modification of existing HPLC methods, that allows the evaluation of L-dopa and its methylated catabolite 3-OMD in platelets and plasma obtained from the same sample of whole blood. The method requires no additional blood sampling, compared to methods that measure only plasma L-dopa and 3-OMD, and might provide a valid tool to explore the real utilization of L-dopa in PD patients.

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