High-Performance Liquid Chromatographic Determination of \( L \)-3,4-Dihydroxyphenylalanine (\( L \)-DOPA) and Its Metabolites in the Urine of Patients with Parkinson's Disease, Control Patients and Normal Subjects after Oral Administration of \( L \)-DOPA

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A simple, accurate and reliable method for the simultaneous measurement of \( L \)-3,4-dihydroxyphenylalanine (\( L \)-DOPA) and its metabolites, dopamine (DM), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), excreted in the urine after oral administration of \( L \)-DOPA was developed. The method consists of the addition of internal standards to urine samples, high-performance liquid chromatography for 50 min on a column of sulfonated polystyrene, detection of DOPAC, \( p \)-hydroxyphenylacetic acid (an internal standard) and HVA by UV absorbance measurements and detection of DOPA, 3,4-dihydroxybenzylamine (an internal standard) and DM by measurements of fluorescence generated by the reaction with o-phthaldialdehyde.

\( L \)-DOPA was administered to patients with Parkinson's disease, control patients and normal subjects, and urine samples were collected 0, 2, 4 and 6 hr later. DOPA and metabolites were measured by this method. The excretion patterns in control patients and normal subjects were quite similar to each other and the maximum excretions of these compounds were observed mostly in 0–2 hr urine, whereas in patients with Parkinson's disease the patterns varied and the excretions of all the compounds were delayed in 11 out of 13 patients.

**Keywords**—DOPA; levodopa; Parkinsonism; Parkinson's disease; pharmacokinetics; oral administration; DOPA metabolites; urinary excretion; high-performance liquid chromatography; metabolism

Since the finding of \( L \)-DOPA as a remedy for Parkinson's disease\(^{3}\) there have been many studies on its metabolism\(^{5,6}\) and pharmacokinetics,\(^{5,6}\) including work on improvement of the dosage form of the drug.\(^{5a,6}\) However, there are few comparative studies of \( L \)-DOPA metabolism in patients with Parkinson's disease and in normal subjects and/or control patients.

The main metabolites of \( L \)-DOPA, i.e., DM, DOPAC and HVA, together with unchanged \( L \)-DOPA are excreted in the urine.\(^{5a,6}\) They have been so far determined separately or simultaneously, but by time-consuming and tedious methods.\(^{7}\)

In this work, we developed a simple, accurate and reliable method for the simultaneous determination of \( L \)-DOPA and its metabolites excreted in the urine after the oral administration of \( L \)-DOPA. By use of the method the excretion patterns of these compounds in patients with Parkinson's disease after the administration of the drug were compared with those in control patients and normal subjects.
Materials and Methods

Materials—Deionized distilled water was used throughout the procedures. L-DOPA and DM hydrochloride were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. DOPAC, HVA, HPA, Brij 35 (polyoxyethylene cetylalcohol ether) and 2-mercaptoethanol were obtained from Nakarai Chemical Co., Kyoto, Japan. o-Pthalidialdehyde was from E. Merck, Darmstadt, West Germany. DHBA hydrobromide was synthesized from 4-hydroxy-3-methoxybenzylamine hydrochloride (Aldrich, Milwaukee, Wisc., U.S.A.). Neutral alumina was purchased from Woelm, West Germany, washed with hydrochloric acid and water, and dried. All other chemicals were of reagent grade.

Subjects—The subjects were 13 patients with Parkinson’s disease (28 to 72 years, mean age 52 years), 5 control patients (mainly with hepatic disease, 45 to 73 years, mean age 57) and 9 normal subjects (22 to 56 years, mean age 32). Among the patients with Parkinson’s disease, 6 patients (fasting) had not received L-DOPA, while the others had received it for 4 months to 4 years.

L-DOPA Loading Test—All previous medications were stopped from the last evening dose, and the subjects were obliged to fast after the last evening meal until 1 p.m. The first urine excreted in the morning was discarded and a certain amount of water was taken after every urination. At 9 a.m., 0.5 g of L-DOPA (Larodopa, Hoffmann-La Roche, Basel, Switzerland) was administered orally with a certain amount of water, and urine was collected at 0, 2, 4 and 6 hr later. To each urine sample, 25 mg of EDTA-2Na and 50 mg of ascorbic acid were added as preservatives for catecholamines. After measurement of the volume, aliquots of the urine samples were stored at -20° until analysis.

HPLC of DOPA, DM, DOPAC and HVA—A high-performance amino acid analyzer (Atto Co., Tokyo, Japan) was used with a column of strong cation exchange resin (TSK LS 212, sulfonated polystyrene, 6 μm av. diameter; Toyo Soda Manufacturing Co., Tokyo) packed in a 25 cm × 4 mm I.D. stainless steel tube (Kyowa Seimitsu Co.). Samples were applied to the column with an auto-sampler (model KHP24-2510AS) or a line sample injector (both products of Kyowa Seimitsu Co., Tokyo). The effluent from the column was first monitored with a wavelength-tunable effluent monitor (Hitachi Ltd., Tokyo) for absorbance at 277 nm and then mixed with o-pthalidialdehyde reagent, which contained 0.08% (w/v) o-pthalidialdehyde, 0.2% (v/v) 2-mercaptoethanol and 0.1% (w/v) Brij 35 in 0.4 mol borate buffer (pH 10.0), delivered at a flow rate of 0.92 ml/min. The generated fluorescence was monitored with a fluorescence detector (model FLD-1, Shimadzu Seisakusho Ltd., Kyoto). The intensities were recorded with a pen recorder (Matsushita Electric Co., Tokyo) or Chromatopac C-R1A chromatography integrator (Shimadzu Seisakusho Ltd.).

Preparation of Urine Sample—A 0.2 ml portion of urine was mixed with 0.8 ml of 0.1 mol acetic acid containing 4 μg of DHBA hydrobromide and 100 μg of HFA as internal standards. Twenty μl of this solution was subjected to HPLC.

Clean-up Procedures for Confirmation of the Accuracy of the Method—For confirmation of the accuracy of the method, the amounts determined by the present method with some randomly chosen urine samples were compared with those obtained for the same samples treated by the following clean-up procedures.

For the clean-up of DOPA, DM, and DOPAC, alumina extraction was performed according to the previous papers. Briefly, 0.2 mg of DHBA hydrobromide, 1 g of acid-washed neutral alumina, 1 ml of 0.2 mol EDTA-2Na and 7 ml of 0.5 mol Tris-HCl buffer (pH 8.5) were added to a 2 ml urine sample, and the whole was stirred mechanically for 10 min. The supernatant was discarded and the alumina was transferred into a glass chromatographic tube, and eluted with 0.4 mol acetic acid. The first 1 ml portion was discarded and the next 3 ml portion was collected. Twenty μl of the eluate was subjected to HPLC.

Ethyl acetate extraction was performed for HVA according to the previous paper. Five hundred μl of urine was acified to pH 1 with 6 N hydrochloric acid, then 40 μg of HPA and 0.2 g of sodium chloride were added, and the whole was extracted twice with 1 ml each of ethyl acetate. The upper layer was evaporated to dryness, and the residue was dissolved in 1 ml of 0.1 mol acetic acid; 20 μl of this solution was injected into the HPLC column.

Standard compounds were added, together with certain amounts of internal standards, to a urine sample collected before the administration of L-DOPA, and the proportionality between the peak height ratios and the added amounts of CA-S were tested (C.V.: DOPA, 7.9%; DM, 6.8%; DOPAC, 8.6%; HVA, 4.6%), and used for calculation of the amounts of CA-S in the urine samples.

Results

Method for the Measurement of DOPA and Its Metabolites

A sulfonated polystyrene, TSK gel LS 212, was found to be suitable for the separation of DOPA, DOPAC and HVA under ordinary conditions. However, it strongly adsorbed DM as a cation. Elution of DM from the resin was achieved by chelate formation with boric acid. Thus, the following stepwise elution was selected: DOPA and the acidic metabolites, DOPAC and HVA, were eluted first with 67 mol citrate buffer (pH 4.15, 0.2 n Na+, 0.1% Brij

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Fig. 1. Chromatograms of Urine Samples
---: The urine excreted before drug administration, ---: The urine excreted at 2-4 hr after drug administration. DM, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DHBA, 3,4-dihydroxybenzylamine (an internal standard); HPA, p-hydroxyphenylacetic acid (an internal standard). Elution was performed with the citrate buffer for 25 min and then with the borate buffer for 25 min. Details are given in the text.

After Al₂O₃ Ex.*  After EtOAc Ex.**

Fig. 2. Correlation between the Amounts (nmol) in the Samples Determined with or without Clean-up Procedure

The urine samples were chosen at random. *Al₂O₃ Ex.: alumina extraction. **EtOAc Ex.: ethyl acetate extraction. Details are given in the text. The lines drawn in the figures represent the theoretical correlations.

Fig. 3. Urinary Excretion of DOPA after Oral Administration of 500 mg (2.5 mmol) of l-DOPA

The vertical bars in the plot for the control subjects show the standard deviations. Symbols in the plot for the patients with Parkinson's disease represent different individuals. On the abscissa, 0 means the urine excreted just before drug administration.
and then DM was eluted with 0.2 M borate buffer (pH 8.5, 0.2 n Na, 0.1% Brij 35) at a column temperature of 60° and a flow rate of 0.46 ml/min. Under these conditions, the compounds were well separated from the other constituents in urine samples together with internal standards (DHBA and HPA), as shown in Fig. 1.

Calibration curves obtained with internal standards were linear from 50 pmol to 1 nmo for DOPA and DM using 180 pmol of DHBA, and from 2.5 nmol to 20 nmol for DOPAC and HVA using 50 nmol of HPA. Detection limits (signal-to-noise ratio=2) were 2 pmol of DOPA, 3 pmol of DM, 100 pmol of DOPAC and 200 pmol of HVA.

Fig. 4. Urinary Excretion of DM after Oral Administration of 500 mg (2.5 mmol) of L-DOPA
Symbols are the same as in Fig. 3.

Fig. 5. Urinary Excretion of DOPAC after Oral Administration of 500 mg (2.5 mmol) of L-DOPA
Symbols are the same as in Fig. 3.
When a urine sample excreted in 2—4 hr after L-DOPA administration was measured 6 times, the C.V. values of the determinations were 2% (DOPA), 4% (DM), 2% (DOPAC) and 1% (HVA).

The amounts determined with or without clean-up procedures were in good agreement (Fig. 2), which supports the accuracy of the method.

**Excretion of DOPA and Its Metabolites**

The excretion patterns of DOPA, DM, DOPAC and HVA are shown in Fig. 3—6. The excretion patterns in control patients and normal subjects were quite similar to each other and the maximum excretions of these compounds were observed mostly in 0—2 hr urine, whereas in patients with Parkinson’s disease the patterns varied and the excretions of all the compounds were delayed in all the patients except two (□▲), whose excretion patterns were similar to those of control subjects. Furthermore, in some patients for whom L-DOPA was not very effective (■×■▲□ in Fig. 3—6), the values of all the measured compounds were rather low.

![Graph showing excretion of HVA](image)

**Fig. 6. Urinary Excretion of HVA after Oral Administration of 500 mg (2.5 mmol) of L-DOPA**

Symbols are the same as in Fig. 3.

**Discussion**

Among the simultaneous assay methods for L-DOPA and its metabolites, the use of an amino acid analyzer appears to be the best. de Belleruche et al. used an analyzer to determine DM and its metabolites in the medium after incubation of synaptosomes with 14C-DL-DOPA. However, more than 4 hr was required for the elution, and the chromatographic separation in their method seems to be inadequate for the measurement of DOPA and its metabolites in physiological fluids, which contain many interfering substances.

On the other hand, in this work, a rapid, accurate and reliable method was developed for the assay of L-DOPA and its metabolites excreted in human urine after L-DOPA administration. Because of its simplicity, the method should be suitable for laboratory work, where the measurement of many specimens is required. With the use of an automatic sampling system more than 15 samples could be analyzed in a day. The method should also be applicable to blood samples obtained within a few hours after administration with the aid of acid deproteinization and alumina extraction. In a preliminary experiment, when 3 ml of plasma was used for the analysis, only L-DOPA was detectable, while DM and DOPAC were detected after acid hydrolysis.

Delayed and/or suppressed urinary excretions of administered L-DOPA and its metabolites in patients with Parkinson’s disease were often observed in this experiment. Since such delayed excretions were not observed in the case of i.v. administration of L-DOPA, our
results suggest the occurrence of delayed absorption of l-DOPA in cases of Parkinson's disease.

The successive administration of l-DOPA supposedly affects its absorption, but in the present work, no significant difference in excretion was detected between patients receiving the drug for the first time (O▲□□□Xıldığı Fig. 3—6) and patients who had received successive administrations. The pH of the stomach and movement of the digestive tract are thought to affect the absorption, which may be regulated by the central nervous system (hypothalamus). In a preliminary experiment involving partial destruction of the hypothalamus in the cat, we observed a delay of absorption of the drug. Since many patients with Parkinson's disease have lesions of the hypothalamus as well as substantia nigra and corpus striatum, the delay of absorption in patients with Parkinson's disease might be ascribed to the lesions at this site.

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References and Notes

1) A part of this work was presented at the Annual Meeting of the Pharmaceutical Society of Japan (Arawake et al., Okayama, 1978; Seki et al., Sapporo, 1979). Abbreviations: l-DOPA, l-3,4-dihydroxyphenylalanine; DM, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DHBA, 3,4-dihydroxybenzylamine; HPA, p-hydroxyphenylacetic acid; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography.

2) Location: a) 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan; b) 2-2-1, Hitotsubashi, Chiyoda-ku, Tokyo 101, Japan; e) 3311, Yashiki, Minamikawachi, Tochigi 229-04, Japan; d) 2-6-1, Musashidai, Fuchu-shi, Tokyo 183, Japan; e) 3-7-2-1, Fukushima-cho, Akiyama-cho, Tokyo 196, Japan; f) 3-1-3, Hongo, Bunkyo-ku, Tokyo 113, Japan.


