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DETERMINATION OF L-3,4-DIHYDROXYPHENYLALANINE IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER SOLVENT EXTRACTION

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SUMMARY

The use of high-performance liquid chromatography combined with solvent extraction for sample purification is described for the determination of L-3,4-dihydroxyphenylalanine in blood plasma. It is extracted into *n*-hexanol via complexation of its catechol moiety with diphenyl borate and ion-pair formation of its carboxylic group with tetrapentylammonium ion in an alkaline buffer. Under optimal extraction conditions, L-3,4-dihydroxyphenylalanine and 3,4-dihydroxybenzylamine used as an internal standard are extracted from blood plasma by a simple procedure and in a short time and then separated by reversed-phase ion-pair chromatography. The analytical recovery (100.8%) and reproducibility (coefficient of variation = 2.3% for $n = 6$) from plasma samples are good enough for routine analysis. L-3,4-Dihydroxyphenylalanine levels in blood can be monitored by this method after oral intake of the substance.

INTRODUCTION

L-3,4-Dihydroxyphenylalanine (L-DOPA) is a direct precursor amino acid of dopamine. L-DOPA is formed by hydroxylation of tyrosine and it plays an important role as an intermediate in the subsequent biosynthesis of catecholamine. Metabolic potency to dopamine is depressed in dopaminergic neurons

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of patients with Parkinson's disease and dopamine antagonists induce symptoms characteristic of parkinsonism. The conversion of tyrosine into L-DOPA is thought to be the rate-limiting step in catecholamine biosynthesis [1]. Thus, L-DOPA has been widely used as a therapeutic agent for Parkinson's disease because dopamine does not cross the blood–brain barrier [2]. Therapy with L-DOPA requires that the daily dosage be gradually increased until the optimum response occurs [3]. The therapeutic effects of L-DOPA are influenced by the dosage, which is closely related to side-effects with possible toxicity from overdose [4,5]. The monitoring of L-DOPA levels in blood is necessary to evaluate the therapeutic effects and the pharmacokinetics in the planning of an effective therapy.

High-performance liquid chromatography (HPLC) is most suitable for such studies because of its superior resolution, detection sensitivity and useability. HPLC methods reported for L-DOPA analysis have involved electrochemical detection [6–9], pre-column fluorescence derivatization [10], UV detection [11], etc. Since blood samples contain various interfering compounds, purification of L-DOPA is essential prior to HPLC separation. Column chromatography [8,10] and alumina treatment [6,7,9] have been widely used to purify L-DOPA. However, such methods are not necessarily either simple or specific, as evidenced by the presence of several peaks other than L-DOPA on chromatograms [8,9]. Furthermore, sample preparation and chromatographic analysis time are too long to permit routine multiple sample analysis.

Compounds with 1,2- and 1,3-diol moieties generally react with borates to form relatively stable complexes [12]. This property has been successfully used for purification of catecholamines by column chromatography using boric acid gel [13] and liquid–liquid extraction using diphenyl borate [14–16]. We considered that the formation of an L-DOPA complex with a borate, which would be extractable into an organic solvent, should enable purification of L-DOPA from blood samples by simple extraction. This consideration prompted us to study an HPLC method coupled with such complex extraction for determining L-DOPA in blood. Changes of L-DOPA levels in blood plasma were followed by HPLC with native fluorescence detection after oral intake of L-DOPA.

EXPERIMENTAL

Chemicals

L-DOPA and 3,4-dihydroxybenzylamine hydrobromide (DHBA), used as an internal standard, were purchased from Wako Pure Chemicals (Osaka, Japan) and Aldrich (Milwaukee, WI, U.S.A.), respectively. Their standard solutions were prepared by dissolving them in 10 m*M* hydrochloric acid containing 5 m*M* sodium pyrosulphite (2.0 μmol/ml each) as a preservative and were stored at 4 °C. The stock solutions were appropriately diluted daily with water before use. Diphenyl borate–ethanolamine complex (DPB), *n*-butylboronic acid

(BB), phenylboronic acid (PB) and ferroceneboronic acid (FB) were obtained from Tokyo Kasei (Tokyo, Japan) and used without any purification. Sodium octanesulphonate of ion-pair chromatographic grade (Nakarai, Kyoto, Japan) was used. Other chemicals were of analytical grade. Water was redistilled in all-glass apparatus after purification with a Milli-Q water purification system (Nihon Millipore, Tokyo, Japan).

Plasma samples

Healthy male subjects, who were not taking any drugs, were given an oral dose of L-DOPA (1 mg/kg body weight), which was administered with 200 ml of orange juice at ca. 11:00 a.m. Heparinized blood was obtained from the forearm vein before and after dosing at appropriate time intervals. Plasma samples were analysed immediately after collection or, if not, plasma (1.0 ml) was mixed with Na_2EDTA (1 mg) and reduced-form glutathione (1 mg) and then stored at $-80°C$ till analysis.

Extraction procedure

To 0.5 ml of blood plasma, 0.1 ml of a DHBA solution (200.0 pmol/ml), 3.0 ml of *n*-hexanol (containing 0.8% tetrapentylammonium bromide and 0.4% tetrapropylammonium bromide) and 1.0 ml of 2.0 *M* ammonium (NH_4Cl–NH_4OH) buffer (containing 0.3% DPB and 0.5% Na_2EDTA, pH 8.6) were added. The mixture was vortex-mixed for 2 min and centrifuged (ca. 1000 *g* for 1.5 min). To the organic phase, 0.4 ml of 0.1 *M* hydrochloric acid was added and the mixture was vortex-mixed for 2 min. After centrifuging (ca. 1000 *g* for 1.5 min), an aliquot (0.25–0.30 ml) of the aqueous phase was analysed by HPLC.

HPLC separation

A Model LC-4A high-performance liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan), equipped with a Model RF-540 spectrofluorimeter (flow-cell volume of 120 μl) and a Chromatopac C-R 2AX data analyser, was used for separation of L-DOPA. A mobile phase consisting of 20 m*M* sodium octanesulphonate, 5% (v/v) acetonitrile and 95% (v/v) 0.25 *M* sodium phosphate buffer (containing 0.1 m*M* Na_2EDTA, pH 2.30) was pumped through a 250 mm$\times$4.6 mm I.D. stainless-steel column, packed in the laboratory with NS Gel C_{18} (5 μm particle size; Sakata, Tokyo, Japan) at a flow-rate of 1.5 ml/min and at a column temperature of 50°C. L-DOPA and DHBA eluted from the column were detected by their native fluorescence (280 nm for excitation and 325 nm for emission wavelength).

RESULTS AND DISCUSSION

Several methods have been reported for purification of L-DOPA from biological fluids [6–10]. However, they lack simplicity or specificity, or are not

adaptable to the analysis of multiple samples. In the present study, we attempted to simplify the analytical procedure.

Borates react with catechol compounds to form specific and stable complexes [12]. This property was used for the extraction of L-DOPA according to other reports [14–16]. After forming complexes with several borate compounds, L-DOPA and DHBA were extracted by DPB into organic solvents, as shown in Fig. 1. The extraction efficiency reached a maximum at 0.3% DPB in an alkaline buffer. Neither PB nor BB gave efficient extraction, which may be due to their low partition coefficients, with respect to *n*-hexanol. The effect of FB on extraction was also negligible (data not shown).

Complexation of catechols with borates has generally been carried out in weakly alkaline solutions [12]. When several buffers (2.0 *M*, pH 8.6), such as ammonium, potassium phosphate, ammonium phosphate and Tris–HCl buffers were used, all except the Tris–HCl buffer gave good extraction of both L-DOPA and DHBA. Aliphatic alcohols, aliphatic and aromatic hydrocarbons, diethyl ether, ethyl acetate and chloroform were also investigated. Both *n*-hexanol and *n*-heptane afforded high extractability, although unknown interfering peaks appeared on chromatograms following extraction with *n*-heptane. The other organic solvents were not effective for extraction of L-DOPA and DHBA. Among the alcohols, *n*-hexanol gave the most favourable result in terms of efficiency and specificity.

Ion-pair formation between the carboxyl group of L-DOPA and tetraalkylammonium ion was used to increase the extractability of L-DOPA according

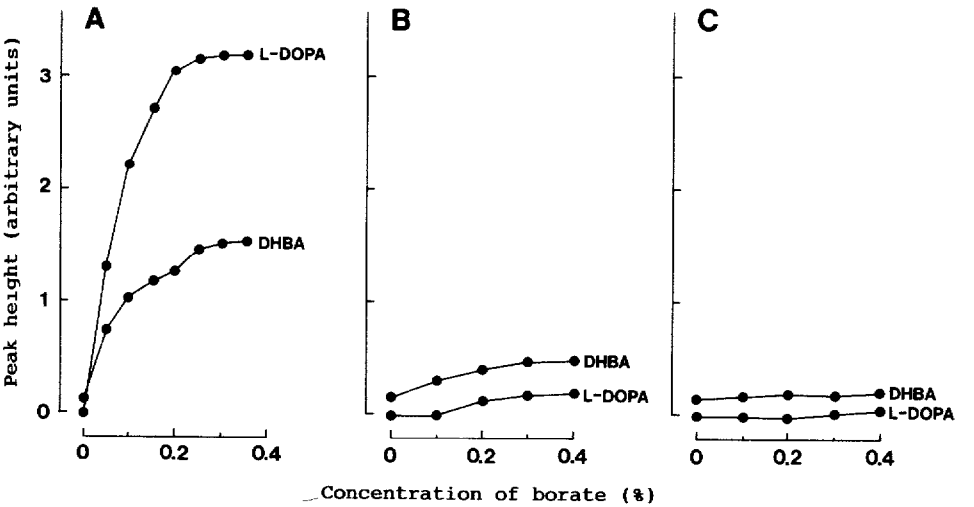

Fig. 1. Effect of borate on extraction of L-DOPA and DHBA. L-DOPA (400.0 pmol/ml, 0.5 ml) and DHBA (400.0 pmol/ml, 0.1 ml) were extracted from ammonium buffers containing DPB (A), PB (B) and BB (C), followed by HPLC separation.

to the method of Smedes et al. [14]. All tetraalkylammonium bromides increased the extractability of L-DOPA, depending on their concentrations, whereas that of DHBA remained unchanged as shown in Fig. 2A. Other tetraalkylammonium bromides of various alkyl chain lengths (methyl to dodecyl) were investigated and tetrapentylammonium gave the best results. In the course of that investigation, it was observed that the peak height of DHBA was increased (but the peak area was scarcely influenced) by adding tetrapropylammonium bromide, although the reason for this is not clear. The peak height increased with increasing tetrapropylammonium concentration, as shown in Fig. 2B. Such an increase was not observed for the peak of L-DOPA.

Extraction of L-DOPA–DPB complexes into *n*-hexanol proceeded rapidly, and the plateau was achieved by vortex-mixing for 2 min, as shown in Fig. 3A. Since complexes of catechols with borates readily dissociate under acidic conditions [12], L-DOPA and DHBA were reextracted into an aqueous phase acidified with hydrochloric acid. Reextraction was also completed in 2 min, as shown in Fig. 3B.

Although different detection systems have been reported for L-DOPA [6–11], native fluorescence detection was used in this study because of its simplicity. Representative chromatograms are shown in Fig. 4. The results for one subject dosed with L-DOPA (1 mg/kg body weight) show that L-DOPA and DHBA are separated in a short time without any major interfering peaks, indicating the relatively high selectivity of our extraction method. The calibra-

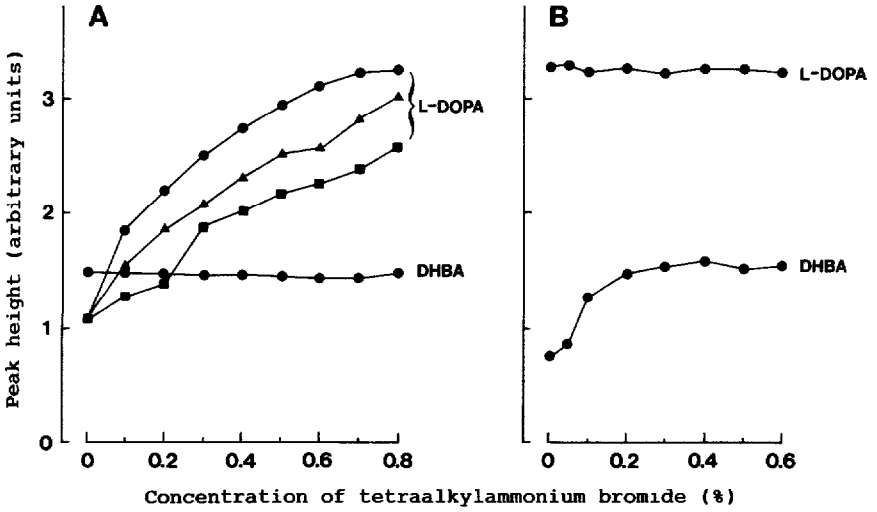

Fig. 2. Effect of counter-ion on extraction of L-DOPA and DHBA. L-DOPA (400.0 pmol/ml, 0.5 ml) and DHBA (400.0 pmol/ml, 0.1 ml) were extracted from *n*-hexanol containing tetraalkylammonium bromide, followed by HPLC separation. (A) 0.4% Tetrapropylammonium bromide plus (●) tetrapentyl-, (▲) tetraoctyl- or (■) tetradecylammonium bromide. (B) 0.8% Tetrapentylammonium bromide plus tetrapropylammonium bromide.

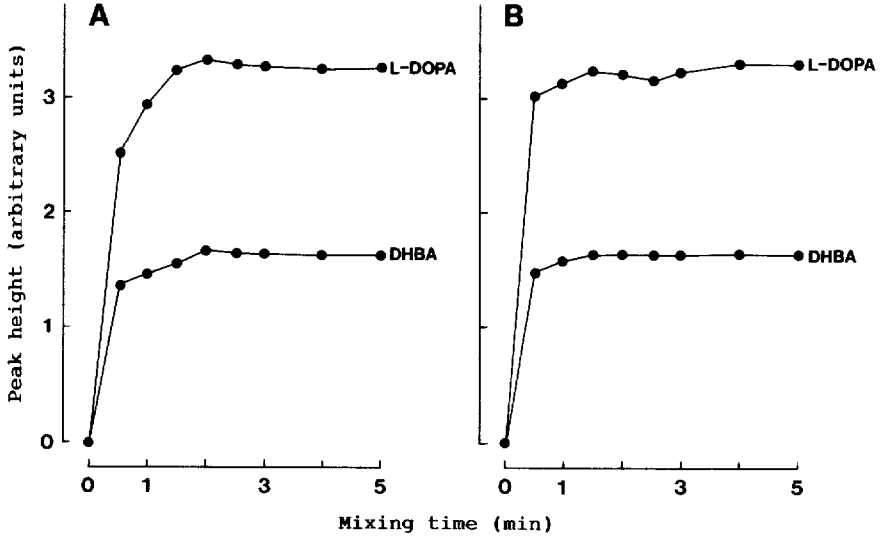

Fig. 3. Effect of length of mixing time on extraction of L-DOPA and DHBA. L-DOPA (400.0 pmol/ml, 0.5 ml) and DHBA (400.0 pmol/ml, 0.1 ml) were vortex-mixed to extract them into *n*-hexanol from an aqueous phase (A) and to reextract them into 0.1 *M* hydrochloric acid from an organic phase (B).

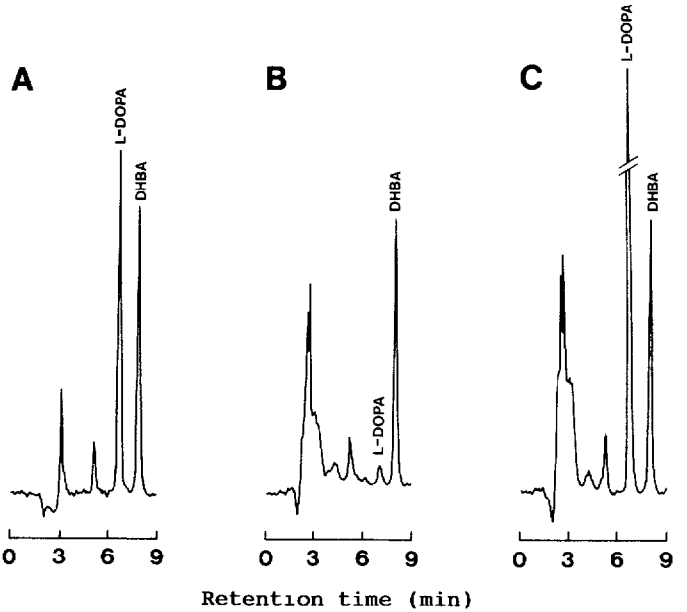

Fig. 4. High-performance liquid chromatograms obtained from standards (A) and plasma samples before (B) and 30 min after (C) intake of L-DOPA (1 mg/kg body weight). For chromatographic conditions see Experimental.

tion graph for L-DOPA obtained through all the procedures showed good linearity over the range 10.0–1000.0 pmol/ml of samples subjected to extraction. When plasma samples spiked with standard L-DOPA (250.0 pmol/ml) were analysed repeatedly, the relative mean recovery and the coefficient of variation (C.V.) for L-DOPA were 100.8 and 2.3% ($n=6$). The absolute values were 77.2 and 5.1% ($n=8$). When the same plasma samples were analysed by our HPLC method and gas chromatography–mass spectrometry (GC–MS) [17], quantitative results obtained by the two different methods were in good agreement, with a correlation coefficient of 0.994 ($n=20$).

Catechol compounds generally decompose at room temperature [18], and this may constitute a problem in routine multiple sample analysis. Acidic extract solutions of L-DOPA and DHBA (50.0–200.0 pmol/ml) were allowed to stand at room temperature and were analysed at appropriate time intervals. Peak heights of both compounds had decreased scarcely at all after standing for 24 h, hence L-DOPA and DHBA are stable in extracts at room temperature for practical analytical times. Our method permits automatic analysis of L-DOPA if an autosampler is used.

After healthy subjects were dosed with L-DOPA (1 mg/kg body weight), time-dependent changes of L-DOPA levels in blood were followed by sampling blood plasma at appropriate intervals. A typical result is shown in Fig. 5, which was obtained from a male subject aged 44 years. The L-DOPA levels increased immediately after intake and the maximum concentration was reached in 20–30 min. Since L-DOPA decarboxylase activity is high in the peripheral systems, concomitant use of decarboxylase inhibitors (carbidopa and benserazide) is frequently employed to reduce the dosage and to minimize side-effects [4,19,20].

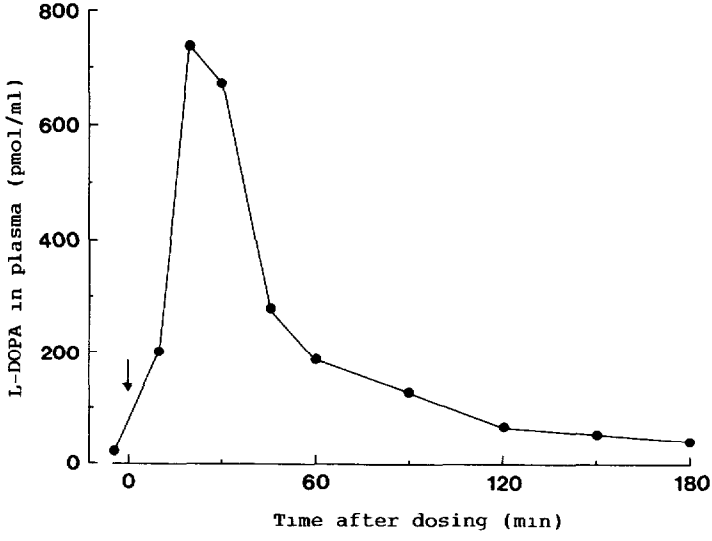

Fig. 5. L-DOPA levels in plasma after dosing with L-DOPA (1 mg/kg body weight). The arrow indicates the intake time.

Thus, the maximum value in changes of L-DOPA levels would be higher and its half-life time in blood would be prolonged in such a therapy.

L-DOPA levels in blood can be monitored by our method after a single dose. In chronic treatment with L-DOPA, however, discrimination between endogenous L-DOPA and exogenous L-DOPA is necessary for pharmacokinetic studies. This requirement is met by the GC–MS method, using stable isotope-labelled L-DOPA, that we recently reported [17].

Compared with other methods [6–10], our purification procedure is simple and brief (less than 10 min per sample) and needs no deproteinization. It also permits relatively selective analysis of L-DOPA. The HPLC method coupled with extraction is suited for routine multiple sample analysis. Our method would be applicable to analysis of other catechol-type agents used in the therapy of parkinsonism, such as L-*threo*-3,4-dihydroxyphenylserine, carbidopa, benserazide, etc.

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REFERENCES

1 T. Nagatsu, M. Levitt and S. Udenfriend, J. Biol. Chem., 239 (1964) 2910.
2 J. Marka, The Treatment of Parkinsonism with L-DOPA, Elsevier, New York, 1974.
3 T.A. Hare, B.L. Beaseley, R.A. Chambers, D.H. Boehme and W.H. Vogel, Clin. Chem., 45 (1973) 273.
4 M. Uono, J. Jpn. Soc. Intern. Med., 71 (1982) 1217.
5 R.P. Lesser, S. Fahn, S.R. Snider, L.J. Cote, W.P. Isgreen and R.E. Barrett, Neurology, 29 (1979) 1253.
6 R.C. Causon, M.J. Brown, K.L. Leenders and L. Wolfson, J. Chromatogr., 277 (1983) 115.
7 S. Ito, T. Kato, K. Maruta, K. Fujita and T. Kurahashi, J. Chromatogr., 311 (1984) 154.
8 C.R. Benedict and M. Risk, J. Chromatogr., 317 (1984) 27.
9 D.S. Goldstein, R. Stull, R. Zimlichman, P.D. Levinson, H. Smith and H.R. Keiser, Clin. Chem., 30 (1984) 815.
10 M. Lee, H. Nohta, K. Ohtsubo, B. Yoo and Y. Ohkura, Chem. Pharm. Bull., 35 (1987) 235.
11 J. Mitchell and C.J. Coscia, J. Chromatogr., 145 (1978) 295.
12 A. Bergold and W.H. Scouten, in W.H. Scouten (Editor), Solid Phase Biochemistry, Analytical and Synthetic Aspects, Wiley Interscience, New York, 1983, Ch. 4, p. 149.
13 S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, Anal. Biochem., 77 (1977) 18.
14 F. Smedes, J.C. Kraak and H. Poppe, J. Chromatogr., 231 (1982) 25.
15 G. Kamperman and J.C. Kraak, J. Chromatogr., 337 (1985) 384.
16 P. Herregodts, Y. Michotte and G. Ebinger, J. Chromatogr., 345 (1985) 33.
17 T. Hayashi and H. Tsuchiya, Proc. Jpn Soc. Med. Mass Spectrom., 13 (1988) 229.
18 H. Tsuchiya, T. Koike and T. Hayashi, Anal. Chim. Acta, 218 (1989) 119.
19 S. Vickers, E.K. Stuart, H.B. Hucker and W.J.A. Vandenheuvel, J. Med. Chem., 18 (1974) 134.
20 U.K. Rinne and P. Molsa, Neurology, 29 (1979) 1584.