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# QUANTIFICATION OF L-3-(3-HYDROXY-4-PIVALOYLOXYPHENYL)ALANINE (NB-355) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING *o*-PHTHALALDEHYDE/N-ACETYL-L-CYSTEINE DERIVATIZATION

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## SUMMARY

A new and rapid high-performance liquid chromatographic assay has been developed for the determination of L-3-(3-hydroxy-4-pivaloyloxyphenyl)alanine (NB-355, I), a novel prodrug of L-DOPA The method involves precolumn derivatization of the drug in biological samples with *o*-phthalaldehyde (OPA) and N-acetyl-L-cysteine (NAC) in a triethanolamine buffer (pH 8 0), giving a fluorescent compound that is stable for 2 h at 4 °C Use of an internal standard improved the assay in accuracy and reliability A programmable injector allowed automatic derivatization of large numbers of samples Chromatographic separation was performed on a reversed-phase column (Capcell Pak $C_{18}$) in which the silica gel was coated with silicone polymer The peaks corresponding to compound I and the internal standard were eluted within 16 min with a mobile phase of acetonitrile–phosphate buffer (pH 7 1) The reliable limit of quantification was 0 5 pmol per injection (0 05 μg equivalents of L-DOPA per ml in plasma) The method was successfully applied for the measurements of dog plasma concentrations after oral dosing of compound I

## INTRODUCTION

L-3-(3-Hydroxy-4-pivaloyloxyphenyl)alanine hemihydrate (NB-355, I) is a novel prodrug of L-DOPA developed for the treatment of Parkinson's disease Compound I provides a flat and prolonged shape of a plasma L-DOPA profile after oral dosing in animals [1] A highly sensitive and convenient method for the quantification of compound I is indispensable for pharmacokinetic studies

A simple high-performance liquid chromatographic (HPLC) method seems to be promising, although the derivatization of compound I is necessary because spectrophotometric or electrochemical detection is insufficient The derivatization must be completed rapidly in moderate conditions Compound I isomerizes between 3- and 4-O-pivaloyl forms in aqueous solutions, and the pivaloyl ester is gradually hydrolysed above pH 7 to produce L-DOPA Several methods have been used for the fluorescent derivatization of amino acids or amines using *o*-phthalaldehyde (OPA) and thiols [2–13] The reaction is rapid and quantitative in moderate conditions, but the stabilities of the isoindoles produced are usually poor [14, 15] N-Acetyl-L-cysteine (NAC) was originally introduced to resolve optical isomers of amino acids in the OPA method [9–13], and was reported to offer stable fluorescent derivatives

In this study, we utilized the OPA/NAC reaction for a precolumn derivatization of compound I The method was successfully applied to the quantification of dog plasma concentrations after oral dosing of compound I Preliminary results of this study have been reported [1]

## EXPERIMENTAL

### *Materials*

Compound I and L-3-[4-(3,3-dimethylbutyryloxy)-3-hydroxyphenyl]-alanine (internal standard, AW-357, II) were synthesized in the organic chemistry section of our laboratory The method has been described elsewhere [1] L-DOPA was purchased from Nakarai (Kyoto, Japan) The chemical structures of these compounds are shown in Fig 1 OPA was purchased from Tokyo Kasei (Tokyo, Japan) NAC was purchased from Wako (Tokyo, Japan) and was recrystallized from water. Carbidopa used was a product of our company Tetra-*n*-butylammonium bromide (Tokyo Kasei) and acetonitrile (Wako) were of HPLC grade All other chemicals and solvents were of analytical-reagent grade Water was purified by Milli Q purification system (Millipore, Bedford, MA, U S A ).

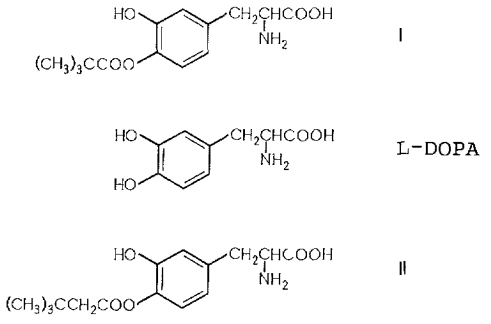


Fig 1 Structures of compound I, L-DOPA and internal standard (II)

*Precolumn derivatization*

The derivatization reagent consisted of 5 ml of ethanol containing OPA (4 mg) and NAC (4 mg), and 5 ml of 1 *M* triethanolamine buffer (pH 8 0) The triethanolamine buffer was prepared from aqueous triethanolamine and diluted HCl The derivatization reagent (40 $\mu$l) was added to a deproteinized biological sample (10 $\mu$l in 0 5 *M* perchloric acid, PCA) and mixed The mixture was allowed to stand for 5 min at room temperature, and then a 10-$\mu$l portion of the mixture was injected into the column The steps of addition, mixing and injection were performed by a programmable sample injector (231-401, Gilson Medical Electronics, Middleton, WI, U S A ), unless otherwise specified

*Chromatography*

The chromatographic system consisted of a DG-3510 degasser, an 880-PU pump, an 860-CO column oven, an 801-SC system controller, an 805-GI graphic integrator (Japan Spectroscopic, Tokyo, Japan), an HP-1046A fluorescence detector (Hewlett-Packard, Waldbronn, F R G ), and the programmable sample injector Samples were maintained at 4 °C in a temperature-controlled rack on the injector A guard column of LiChrospher RP-18e, 5 $\mu$m particle size, 4 mm×4 mm I D (E. Merck, Darmstadt, F R G ) was used throughout the study Peak heights were calculated by the integrator

For the separation of compound I, an analytical column of Capcell Pak $C_{18}$ (SG120), 5 $\mu$m particle size, 250 mm×4 6 mm I D (Shiseido, Tokyo, Japan) was used The mobile phase composition was acetonitrile and 25 m*M* phosphate buffer (pH 7 1) (38 62, v/v) The phosphate buffer contained 0 25% tetra-*n*-butylammonium bromide as an ion-pairing reagent The mobile phase was filtered through an FP-450 membrane filter (0 45 $\mu$m, Gelman Science, Ann Arbor, MI, U S A ) before use Chromatography was performed at 40 °C with a flow-rate of 1 0 ml/min The column pressure was 97–130 bar These standard samples were injected into the system for conditioning, and after use, the columns were washed with acetonitrile–water (50 50) The excitation and emission wavelengths of the fluorescence detector were set at 230 and 440 nm, respectively

For the separation of L-DOPA, an analytical column packed with Nucleosil 100-5$C_{18}$ (250 mm×4 6 mm I D , Macherey-Nagel, Duren, F R G ) was used An aliquot (10 $\mu$l) of a deproteinized sample was directly injected into the HPLC system equipped with an electrochemical detector (VMD-501, Yanaco, Japan) The mobile phase composition was 0 1 *M* sodium citrate buffer (pH 4 1) containing 0 1 m*M* EDTA (sodium salt) and 1 $\mu$g/ml $NaN_3$ The temperature of the column was 40 °C and the flow-rate was 0 8 ml/min The applied voltage of the electrochemical detector was 600 mV vs Ag/AgCl

*Animals, dosing and sample collection*

Male beagle dogs, 16–18 months of age, were fasted for 18 h (water ad libitum) prior to dosing and received regular meals 4 h after dosing Animals were

pretreated with haloperidol 15 min before each dosing to prevent L-DOPA-dependent emesis (i.v 0.05 mg/kg, 0.1 ml/kg in saline) Prodrug I (100 mg) and carbidopa (25 mg) were suspended in 50 ml of 0 5% sodium carboxymethyl cellulose solution and administered orally Whole blood was collected at pre-selected time points and centrifuged (3000 *g*, 10 min, 4°C) An aliquot (200 $\mu$l) of the plasma sample was immediately treated with 0 5 *M* PCA (800 $\mu$l) containing 1 mg/ml EDTA and centrifuged again. The supernatant was stored at −80°C until assay.

## RESULTS AND DISCUSSION

### *Derivatization*

The change in peak height of compound I after derivatization at various pH values is shown in Fig 2. The reaction was complete within 5 min at room temperature under these conditions. The fluorescent peak was not observed below pH 7 0 but, as the pH increased, the peak height increased steeply and then reached a plateau above pH 8.0. The fluorescent peak was stable for at least 2 h at 4°C, but the stability slightly decreased at elevated pH This may be due to hydrolysis of the pivaloyl moiety, as indicated by the compensatory appearance of a peak corresponding to L-DOPA We adopted a pH of 8 0 for the derivatization because of both high fluorescence intensity and adequate stability.

The peak-height ratio of compounds I to II (I.S.) is also shown in Fig 2 Since the peaks corresponding to compounds I and II decreased at a similar

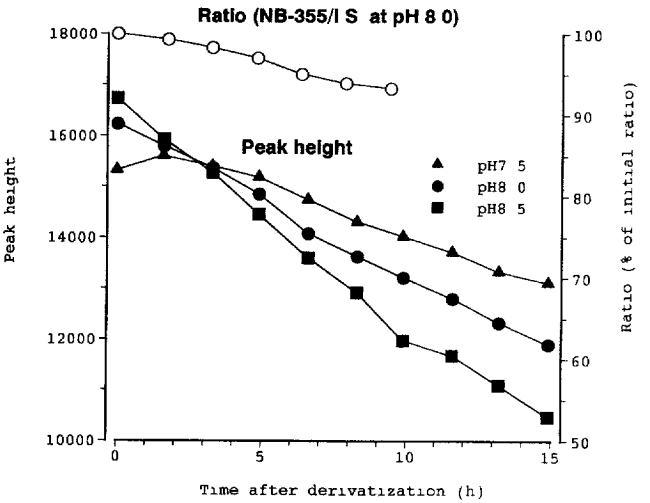


Fig 2 Time courses of peak height and peak-height ratio of compound I after derivatization The sample was derivatized manually at various pH values and successively injected into the HPLC system Each point represents the mean of two determinations

rate, the ratio retained the same value for 6 h, indicating the convenience and reliability of the method

The reproducibility of the automatic derivatization was examined, the coefficient of variation for the peak height of compound I was 0 64% after twelve injections. The automatic derivatization was quite efficient for large numbers of samples However, for small numbers (less than twenty samples per day), sufficient accuracy could be obtained by simultaneous derivatization in manual operation, followed by the usual HPLC autosampling technique

*Chromatography*

Several reversed-phase columns, such as Zorbax $C_8$ and Nucleosil $5C_{18}$, were examined, but resulted in a broad peak of compound I However, sharp and well defined chromatographic peaks of compounds I and II were obtained on Capcell Pak $C_{18}$, in which the silica gel was coated with silicone polymer and then modified with octadecyl group This silicone coating reduced the interaction between silanol groups on the column and polar groups of compound I, such as phenolic hydroxy and amino functions In addition, the coating improved the resistance of the column in neutral or weakly basic (less than pH 10) mobile phases Typical chromatograms are shown in Fig 3 Under these conditions, the peak corresponding to L-DOPA eluted with the front peaks Gradient elution was necessary for the simultaneous assay of compound I and L-DOPA The method was possible but not convenient, so that we measured concentrations of compound I and L-DOPA separately

Isomers of compound I, 3- and 4-O-pivaloyl compounds, coeluted and could

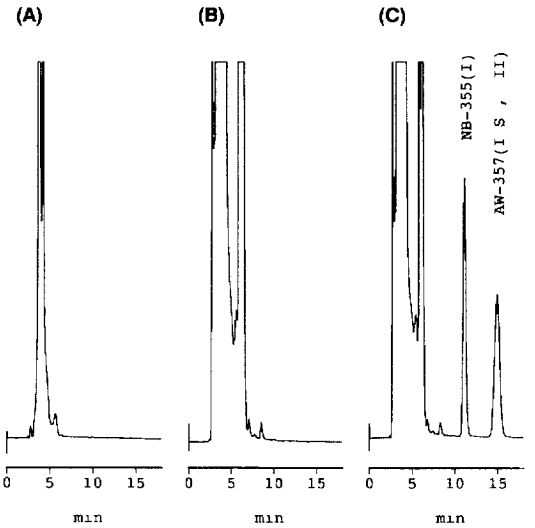


Fig 3 Chromatograms from (A) blank PCA solution, (B) blank dog plasma and (C) spiked plasma Concentrations compounds I and II each 10 $\mu M$

not be distinguished under the conditions described The peak broadened at lower pH of the mobile phase and/or at lower column temperature, suggesting a tendency toward isomeric resolution However, under physiological conditions, the interconversion of the isomers is so fast that resolution is not practical For this reason, we chose a relatively high pH for the mobile phase

*Linearity and precision*

The method provided good linearity over the range 0 05–20 $\mu M$ in plasma with sufficient accuracy, the correlation coefficient of the regression line was 0 9999 and the maximum deviation was 8 5% with fourteen standard samples Amino acids or amines contained in the plasma did not affect the peak height of compound I Standard curves from spiked samples and those obtained from deproteinized plasma were identical However, the reproducibility of the calibration curve depended on the column conditions So we prepared standard curves daily

*Stability in stock solution*

Stabilities of compounds I and II, after addition to the PCA-treated plasma, were checked. After 2 months of storage, recoveries of compounds I and II were 90 6% and 91.8% at −20°C, and 104 2% and 98 4% at −80°C, respectively. No degradation of L-DOPA was observed under similar conditions The biological samples containing compound I should be stored at −80°C after collection

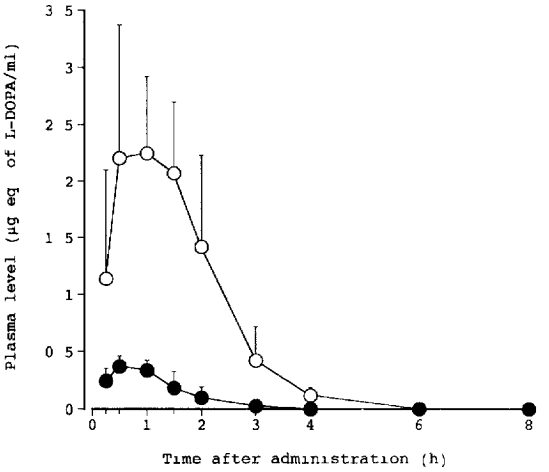


Fig 4 Plasma levels of compound I (●) and L-DOPA (○) after oral administration of compound I to dogs Compound I (100 mg equivalents of L-DOPA) was administered together with carbidopa (25 mg) Each point represents the mean ± S D of three animals

*Plasma concentration in dogs*

Plasma concentration of compound I and L-DOPA were measured after oral dosing of compound I with carbidopa in dogs Carbidopa, being a peripheral inhibitor of aromatic L-amino acid decarboxylase, prevents metabolic decomposition of L-DOPA and enhances the bioavailability after dosing of L-DOPA [16, 17] Carbidopa shows similar effects in the case of compound I [1] As shown in Fig 4, plasma concentrations of compound I were remarkably low compared with concentrations of L-DOPA The peak concentration of L-DOPA was $2\,93 \pm 0\,40$ $\mu$g/ml at $0\,9 \pm 0\,5$ h, and the peak value for compound I was $0\,42 \pm 0\,05$ $\mu$g equivalents of L-DOPA per ml at $0\,8 \pm 0\,5$ h. These data suggest that the conversion of compound I into L-DOPA in the systemic circulation contributed little to the prolonged plasma profile It is more likely that sustained intestinal absorption is responsible for the prolonged plasma concentrations in dogs, as was suggested for rats [1]

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