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Note

Simultaneous determination of L-dopa and 3-O-methyldopa in human serum by high-performance liquid chromatography

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Levodopa (3,4-dihydroxyphenylalanine), in combination with carbidopa (L-α-hydrazino-3,4-dihydroxy-L-α-methylcinnamic acid), a peripheral decarboxylase inhibitor, has been shown to improve motor function in the majority of patients with Parkinson's disease [1]. However, long-term treatment with levodopa/carbidopa is associated with a variety of untoward effects, including unpredictable patterns of motor fluctuation ("on-off phenomenon"), abnormal involuntary movements, and end-of-dose akinesia ("wearing off") [2]. Elevated blood levels of 3-O-methyl dopa (3-methoxy-tyrosine, OMD), a major metabolite of L-dopa, have been associated with the

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occurrence of L-dopa-induced dyskinesias in Parkinsonian patients; furthermore, several groups have suggested that plasma OMD levels and/or OMD/L-dopa ratios may be predictive indicators of the long term response to L-dopa therapy [3, 4]. This hypothesis has not been experimentally verified, mostly because of the lack of simple, rapid, and reliable quantitation of blood OMD/L-dopa levels in human plasma.

Heretofore, plasma levodopa and OMD have been determined separately by the time-consuming and tedious method of fluorescent analysis [5]. Although high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been used successfully for determination of catecholamines in most tissues [6], simultaneous determinations of L-dopa and metabolites by HPLC and ED so far have not included measurements of OMD [7, 8], or sample preparations and elution patterns have been too long (40 min to 20 h per sample) to realistically permit multiple sample analysis [9, 10].

We report here a rapid, sensitive, and specific method for the simultaneous determinaton of these compounds in deproteinized plasma through separation by reversed-phase HPLC and ED.

MATERIALS AND METHODS

Standards of L-dopa and its metabolites were purchased from Sigma (St. Louis, MO, U.S.A.). Carbidopa was the generous gift of Merck Sharp & Dohme (West Point, PA, U.S.A.). Ammonium phosphate was HPLC grade and was purchased from A.H. Thomas (Philadelphia, PA, U.S.A.). All other chemicals were of reagent-grade quality. All solvents used in the HPLC system were HPLC grade, were solubilized with doubly distilled water further treated with a Millipore Milli-Q system (Milford, MA, U.S.A.), and were degassed and filtered under vacuum with a 0.45-μm Millipore HAWP-type filter before use.

High-performance liquid chromatography

The HPLC system from Waters Assoc. included a Model 6000A solvent delivery system, a Model U6K loop injector, and a Model RCM100 radial compression module. The column, also from Waters, was a Radial-Pak reversed-phase Partisil column (10 cm $\times$ 8 mm μBondapak C_{18}, 10 μm particle size). The system was also fitted with a 30 $\times$ 2.9 mm guard column composed of Waters C_{18} Corasil, 37—50 μm particle size range.

The electrochemical detector consisted of a TL5 glassy carbon electrode and an LC4A potentiostat (BioAnalytical Systems, West Lafayette, IN, U.S.A.). The working electrode was set at +660 mV relative to an Ag/AgCl reference electrode. Potentiostat sensitivity ranged from 5 to 100 nA. Elution profiles were continuously recorded on a Pedersen 37 MR strip-chart recorder at 1 V full scale. A good quantitative correlation was observed between peak heights and concentration of various standards injected. The elution of compounds from the column was carried out at ambient temperature in an isocratic mode. The mobile phase consisted of 100 m*M* ammonium phosphate, pH 4.3, and was pumped across the column at a flow-rate of 3.0 ml/min.

Sample preparation

Blood samples (5—10 ml) were drawn by venipuncture, transferred to agar separation tubes, and centrifuged at 1000 *g* for 5 min at 4°C. Serum (1 ml) was withdrawn and deproteinized by addition of 50 μl of 72% perchloric acid (PCA) and mixing on ice for 10 min. PCA was then removed by precipitation with 500 μl of 1 *M* dipotassium hydrogen phosphate, pH 11, with 5 m*M* NaEDTA (ethylenediamine tetraacetic acid), and final sample pH was adjusted to 8 with 1 *M* potassium hydroxide (typically 700 μl), resulting in formation of insoluble K^+ perchlorate. Samples were spun at 11,000 *g* for 4 min at 4°C. The supernatant was removed, reacidified to pH < 5 with 5 *M* hydrochloric acid or 2.5 *M* sulfuric acid, and aliquots of 20 μl were injected for HPLC determination.

RESULTS AND DISCUSSION

A representative chromatogram illustrating resolution of standard mixtures of L-dopa, 3-O-methyl dopa, and carbidopa, as well as norepinephrine and epinephrine, is shown in Fig. 1A. The complete separation of these compounds occurs within 10 min. Other major metabolites of L-dopa metabolism: 3,4-dihydroxyphenylacetic acid, vanilmandelic acid, homovanillic acid, as well as α-methyl dopa (a commonly used antihypertensive medication) do not interfere with the resolution of L-dopa or OMD. Dopamine, the carboxylated

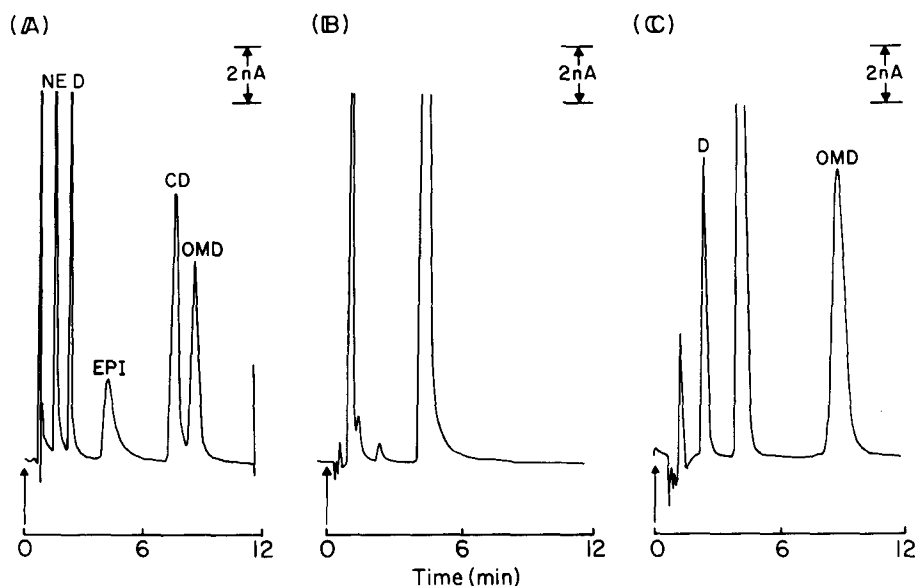

Fig. 1. HPLC elution profiles of: (A) standard mixture of L-dopa metabolites after single injection. The 10-μl injection consisted of aqueous mixture containing 100 pmol each of L-dopa (D), 3-O-methyl dopa (OMD), epinephrine (EPI), norepinephrine (NE), and carbidopa (CD); (B) 20 μl control serum obtained from healthy male volunteers prepared as described in the text; (C) 20 μl identically prepared serum obtained from a Parkinsonian patient 2 h after administration of 200 mg of Sinemet. See text for details on the mobile and stationary phases, flow-rates, and detector settings.

product of L-dopa conversion by dopa decarboxylase, has a retention time closest to that of OMD, but does not interfere with quantitation of OMD peak heights. In addition, dopamine is usually present at concentrations 10^4 times less than that of typical OMD blood levels found in Sinemet (levodopa/carbidopa) treated Parkinsonian patients and was not detectable in any of our plasma chromatograms. Carbidopa, the dopa decarboxylase inhibitor, has a retention time similar to dopamine, but likewise was found not to inhibit quantitation of OMD peak heights.

A chromatogram of control serum (Fig. 1B) obtained from healthy male volunteers demonstrates that no contaminating peaks are introduced by our serum preparation process. Additionally, plasma obtained by separation of blood containing the anti-coagulant EDTA can also be utilized for analysis without alteration of resolution of the L-dopa/OMD peaks (not shown). However, EDTA (which elutes immediately after the dead volume) does overlap with the peak of norepinephrine, and therefore, under these particular chromatographic conditions, quantitation of norepinephrine would be difficult.

Fig. 1C is a chromatogram of a serum sample obtained from a patient with advanced Parkinson's disease 2 h after administration of 200 mg of Sinemet. This patient, who has severe involuntary movements and random fluctuations in motor performance, can be seen to have clearly detectable levels of L-dopa and OMD in this chromatographic profile.

The absolute recovery of L-dopa and OMD from human serum was estimated by comparing peak heights obtained from the injection of known quantities of the compounds with peak heights obtained from injection of extracts prepared from control serum spiked with the compounds. This gave values of 98% for L-dopa and 102% for OMD (n = 4). Since alumina extraction of samples is not utilized and analytical recovery is close to 100% for both compounds, the use of extracted, spiked serum standards in quantitations was found to be unnecessary.

Repeated determinations (n = 5) of standard preparations at a concentration of 1 μg/ml gave coefficients of variation of 2.6% for L-dopa and 2.4% for OMD. Although all of the measurements were carried out in frozen samples within three days of deproteination, standards frozen under identical conditions were found to be relatively stable over much longer periods of storage. A decay in activity of 8.8% and 8.0% was observed for a month's storage at -18°C for L-dopa and OMD, respectively.

Standard curves for L-dopa and OMD were prepared from injecton of pure stock solutions over a wide range of concentrations. Linearity of our chromatographic technique and detector was routinely observed for both compounds over a concentration range of nearly three orders of magnitude (2—1000 pmol injected compounds). Measurement of L-dopa and OMD was achieved at a sensitivity of 10—100 nA/V full scale at an applied electrode potential of +660 mV. Under these conditions, the limits of detection were 10 ng/ml (1 pmol injected compound) with a signal-to-noise ratio greater than 10 for both L-dopa and OMD. Additional improvements on these lower limits of detection could be achieved simply by making the applied voltage across the detector more positive (e.g. +720 mV), or by increasing the amount of sample injected. Since serum levels of L-dopa and OMD typically range from 0.10 to 10 μg/ml, our

described settings were clearly adequate for detection of L-dopa/OMD in all samples assayed from our patient population.

CONCLUSIONS

We have presented here a rapid, reliable, and sensitive assay for simultaneous detection of L-dopa and its metabolite 3-O-methyl dopa in human sera using reversed-phase HPLC and amperometric detection without interference from other known L-dopa metabolites. Sample preparation has been simplified, requiring only precipitation of serum proteins, and does not require more time-consuming steps such as pH-dependent alumina absorption/elution. The described technique therefore seems ideally suited for the study of L-dopa metabolism requiring multiple sample analyses in Parkinsonian patients currently on levodopa/carbidopa combination therapy.

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