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Determination of free DOPA and 3-O-methyl-DOPA in human plasma by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A procedure was devised for the determination of the unconjugated non-protein-bound fraction of 3,4-dihydroxyphenylalanine (DOPA) and 3-O-methyl-DOPA (3-OMD) in plasma using a reversed-phase liquid chromatographic system coupled with electrochemical detection. Sample preparation involves rapid isolation of the unbound drugs from the drug–protein complex by ultrafiltration through a membrane with a molecular weight cut-off of 10 000 dalton. One chromatographic run requires less than 10 min. The relative standard deviation is < 3% for the within-assay imprecision and < 4% for the between-assay imprecision. The detection limits for DOPA and 3-OMD are 0.2 and 1.3 ng/ml, respectively.

INTRODUCTION

Treatment of Parkinson's disease is still based on substitution therapy with 3,4-dihydroxyphenylalanine (DOPA), alone or in combination with a peripheral decarboxylase inhibitor, in spite of the side-effects that may arise (dyskinesia, on–off phenomenon and psychoses), particularly during long-term therapy [1,2]. Elevated concentrations of 3-O-methyl-DOPA (3-OMD) have been associated with DOPA-induced side-effects [3,4].

For a more complete understanding of the mechanism of action of this drug, we consider that its plasma concentration together with that of its metabolite should be monitored in order to establish the amount that could cross the blood–brain barrier. In addition, it is important to assess whether DOPA and 3-OMD bind significantly to plasma proteins. In fact, it is generally accepted that only the free (non-protein-bound) fraction of drugs is available to equilibrate with receptor sites in tissues [5], even though with DOPA the existence of an active uptake system in the blood–brain barrier [6] may represent a complicating factor. Papers dealing with methods for determining DOPA and 3-OMD in plasma by high-performance liquid chromatography (HPLC) with electrochemical detection have been published [7–10], but none has taken into account their unconjugated free (non-protein-bound) fraction.

This paper describes a micro-scale method for the determination of free DOPA

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and 3-OMD not protein bound in plasma; no sample pretreatment other than ultrafiltration is needed.

EXPERIMENTAL

Reagents and standards

DOPA and 3-OMD were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and water were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

The elution buffer was composed of a solution of 50 mmol/l sodium dodecyl sulphate and 100 mg/l ethylenediaminetetraacetic acid disodium salt adjusted to pH 2.5 with 2 *M* phosphoric acid.

Stock solutions of DOPA and 3-OMD were prepared at 1 mg/ml in 0.1 *M* perchloric acid. Working standard solutions of 20, 40 and 80 ng/ml DOPA and 0.5, 1.0 and 2.0 μg/ml 3-OMD were prepared by serial dilutions with filtered isotonic saline. The stock standard solutions were stored at −20°C and working solutions were prepared every day. A 500-μl volume of the working standard solutions was treated as if it was a specimen from a patient.

Apparatus and chromatography

The HPLC system consisted of a Model 1350 pump (Bio-Rad Labs., Richmond, CA, U.S.A.) equipped with a Rheodyne Model 7125S injection valve fitted with a 20-μl sample loop. The chromatographic column (150 mm × 4.6 mm I.D.) was packed with Rosil C_{18} HL, particle size 3 μm (Bio-Rad Labs.). A cartridge head with interchangeable cartridges (40 mm × 4.6 mm I.D.) packed with ODS-5S (particle size 5 μm) (Bio-Rad Labs.) was screwed onto the column head. The electrochemical detection system (Coulochem 5100 A; ESA, Bedford, MA, U.S.A.) consisted of a conditioning cell (Model 5021) and an analytical cell (Model 5010) containing dual coulometric electrodes. The overall system operated in the redox mode. The conditioning cell was set at +0.30 V. The working potentials of the two electrodes of the analytical cell were 0.00 for the first and −0.30 V for the second. Signals from the detector were converted to a chromatographic trace by a Bio-Rad Labs. Model 1322 recorder. The mobile phase was elution buffer–acetonitrile (88:12), filtered through a 0.22-μm membrane filter (GS type; Millipore, Molsheim, France). Isocratic elution was carried out at room temperature at a flow-rate of 1.0 ml/min.

Procedure

Blood samples from a patients receiving DOPA were drawn by venipuncture, transferred into heparinized tubes and centrifuged at 3000 *g* for 10 min at 4°C. Plasma was immediately separated and 500 μl were centrifuged at 3000 *g* for 30 min through a membrane Minicent 10 (Bio-Rad Labs.), which restricts the passage of species larger than 10 000 dalton. Then 20 μl of the ultrafiltrate were injected directly into the chromatographic system. Samples were eluted isocratically and quantified by comparing the height of the peaks with those of standard solutions that had also been ultrafiltered. When operating for more than 24 h, 100 μl methanol should be injected into the column instead of the sample. This eliminates the lipids that have coated the electrodes and would be responsible for shifting the potential of the reference and auxiliary electrodes.

RESULTS AND DISCUSSION

The most delicate aspect of this procedure was to obtain a plasma free from protein without using either aggressive reagents, such as acids, or organic solvents, as both break the drug–protein bonds. To collect plasma free from proteins we suggest using an ultrafiltration membrane that has a molecular weight cut-off of 10 000 dalton. With the present procedure we obtained 200 μl of ultrafiltrate by centrifuging 500 μl of plasma for 30 min at 3000 *g*. The potentials of the three electrodes were selected after injection of fixed amounts of the standards. The choice of +0.30, 0.00 and −0.30 V, similar to those used by Baruzzi *et al.* [10], was a good compromise between high sensitivity and low noise. In fact, the use of a higher potential to oxidize 3-OMD completely gives rise to a significant increase in the baseline noise [9].

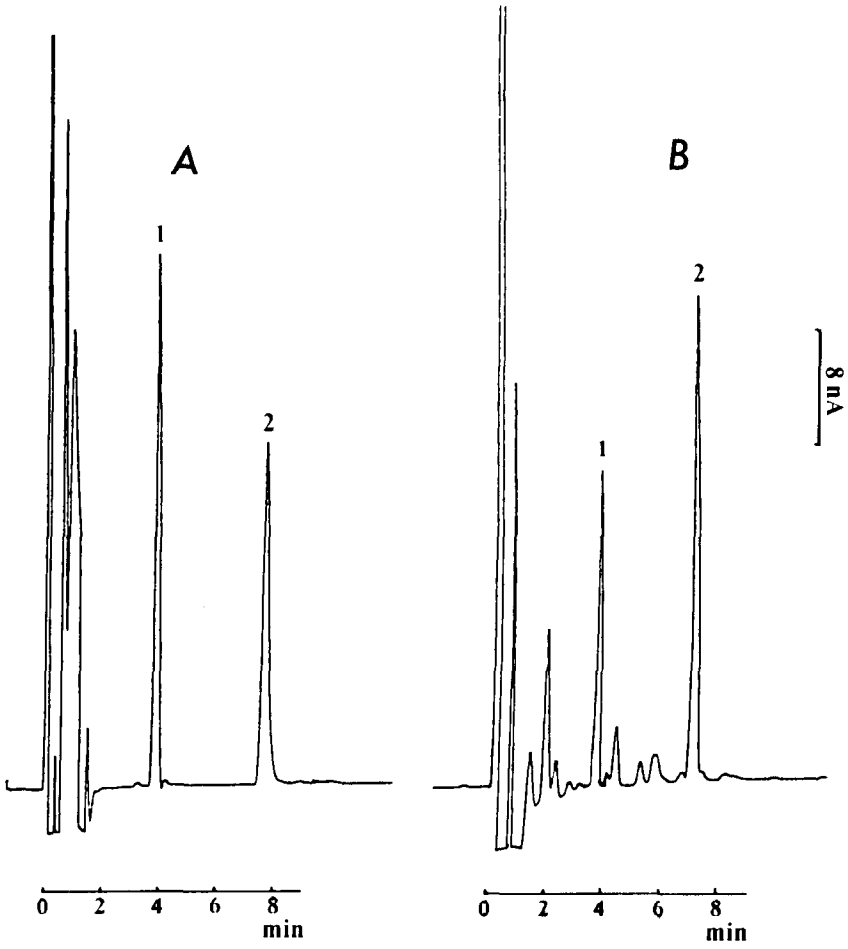

Fig. 1. Separation of (1) DOPA and (2) 3-OMD in (A) a standard solution of the compound and (B) an ultrafiltered plasma sample. The standard solution contained 20 ng/ml of DOPA and 1.0 μg/ml of 3-OMD. The plasma sample contained 11.7 ng/ml of DOPA and 1.42 μg/ml of 3-OMD.

A representative chromatogram showing the separation of a standard mixture of DOPA and 3-OMD is shown in Fig. 1A. Fig. 1B is the chromatogram of a plasma sample, prepared as described above, from a patient receiving DOPA. The separation and elution of the two compounds of interest were completed in 10 min.

From the analysis of ultrafiltered plasma, the identity of the chromatographic peaks is demonstrated in several ways. First, there is complete correspondence between the retention times of the samples and those of the standards. Second, on increasing the concentration of acetonitrile in the mobile phase, the retention times for standards and samples are markedly altered in the same manner. Third, the ratio of the peak areas of the standards and the samples measured at differed potentials is the same (signature) [11].

Other principal metabolites of DOPA, such as 3,4-dihydroxyphenylacetic acid (DOPAC), hydroxymethylmandelic acid (HMMA) and homovanillic acid (HVA), did not interfere in the separation of DOPA and 3-OMD. Dopamine, which arises after decarboxylation of DOPA, did not be interfere in the assay under the conditions described.

In order to investigate whether DOPA and 3-OMD bind to the filter membrane, we compared both the working standard solutions and the plasma samples deproteinized with 1.2 *M* perchloric acid [10] both before and after ultrafiltration. In the ultrafiltrate we found values ranging from 97% to 102% for both substances, showing that the membrane of the ultrafilter does not seem to bind at any level considered for DOPA and 3-OMD.

Linearity was assessed from injection of DOPA, 3-OMD standard mixtures in the concentration range 0.2–1000 ng/ml for DOPA and 1.0–5000 ng/ml for 3-OMD. For both substances a linear relationship between the amount injected and the detector signal was demonstrated. Under the assay conditions, the detection limits were 0.2 and 1.3 ng/ml for DOPA and 3-OMD, respectively, at a signal-to-noise ratio of 2.

To determine the reproducibility of the assay, a plasma from a patient receiving DOPA (125 mg, three times a day) was analysed ten times in the same run. The relative standard deviation (R.S.D.) was 2.5% for DOPA at a concentration of 106 ng/ml and 2.1% for 3-OMD at a concentration of 1.5 μg/ml. Analyses of the same sample over a 10-day span (the sample was stored in aliquots at −20°C between assays) yielded an R.S.D. of 3.4% for DOPA and 3.6% for 3-OMD.

In the plasma samples collected from four Parkinsonian patients (assuming doses of 125 mg of DOPA and 12.5 of carbidopa, three times a day) containing total DOPA ranging from 260 to 390 mg/ml, the free fraction was between 8% and 13% of the total concentration. In the same samples the total 3-OMD ranged from 0.90 to 1.55 μg/ml and the free fraction was between 18% and 21% of the total concentration.

In conclusion, the specific and sensitive method described may offer a means of determining the free unconjugated fractions of DOPA and its metabolite 3-OMD. The method is characterized by high recovery and good reproducibility; it is well suited for routine operation with extensive test runs.

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