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Note

Simple and rapid micromethod for the determination of levodopa and 3-O-methyldopa in human plasma by high-performance liquid chromatography with coulometric detection

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Levodopa alone or in combination with a peripheral decarboxylase inhibitor (both benserazide and carbidopa) remains the most effective agent in the treatment of Parkinson's disease. However, chronic levodopa therapy is associated with a number of complications, such as on—off effect, dyskinesias, myoclonus and psychiatric symptoms [1]

Levodopa has a short half-life (about 1 5 h), while its metabolite 3-O-methyldopa (OMD) has a plasma half-life ten-fold longer and so accumulates during chronic therapy [2] Elevated concentrations of this metabolite have been associated with levodopa-induced side-effects [3—5] and the monitoring of its plasma concentrations together with those of the parent drug may prove useful in the management of levodopa therapy.

Several papers dealing with methods for determining levodopa in plasma by high-performance liquid chromatography (HPLC) with electrochemical detection (ED) have been published almost all require time-consuming purification steps [6—10] and only in a few cases has simultaneous measurement of both levodopa and OMD been included [11, 12].

We developed a micromethod that is faster and simpler than previous ones and sensitive enough for the therapeutic monitoring of these compounds in plasma.

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EXPERIMENTAL

Reagents and standards

Levodopa and OMD were purchased from Sigma (St Louis, MO, U.S.A.) Acetonitrile and water were HPLC grade (Merck, Darmstadt, F.R G) All other chemicals were of reagent-grade quality

The phosphate—acetate buffer employed in the preparation of the mobile phase was composed of a solution of 50 m*M* dihydrogen phosphate, 50 m*M* sodium acetate, 0.7 m*M* sodium dodecyl sulphate and 2 m*M* ethylendiaminetetraacetic acid disodium salt. This solution was adjusted to pH 3.1 with 2 *M* phosphoric acid and filtered through a 0.22-μm membrane filter (GS type, Millipore, Molsheim, France) Stock solutions of levodopa and OMD were prepared at 5 and 4.4 mmol/l, respectively, in 0.1 *M* perchloric acid, and working concentrations achieved by serial dilutions in 0 1 *M* perchloric acid All working solutions were stored at 4°C and freshly prepared every week.

Plasma standards of 2.5, 6 25, 12 5 and 25.0 μmol/l levodopa and 4 4, 11 0, 22 0 and 44 0 μmol/l OMD (calibration samples) were prepared by adding 10 μl of working solutions of both compounds, at increasing concentrations, to 1-ml aliquots of blank pooled plasma A 100-μl volume of each sample was then treated exactly as a patient's specimen

Apparatus and chromatography

The HPLC system consisted of a Series 10 liquid chromatograph (Perkin-Elmer, Norwalk, CA, U.S A.), a Rheodyne Model 7125 S injection valve (Rheodyne, Cotati, CA, U.S A.) fitted with a 50-μl sample loop and a Nucleosil C_{18} (5 μm) reversed-phase column, 200 mm × 4 mm I D. (Macherey and Nagel, Düren, F R.G), protected by a C_{18} Guardpack precolumn insert (Waters Assoc., Milford, MA, U S A) The electrochemical detection system (Coulochem 5100 A, ESA, Bedford, MA, U S.A) comprised a Model 5021 conditioning cell and a standard analytical cell (Model 5010) containing dual coulometric electrodes The overall system operated in the redox mode. The conditioning cell was set at +0 35 V, the working potentials of the two electrodes of the analytical cell were +0 04 V for the first and —0 30 V for the second, respectively. Signals from the detector were recorded on a Perkin-Elmer Model 56 recorder (500 nA full-scale recorder sensitivity)

The mobile phase was prepared by mixing 87.5 parts of the phosphate—acetate buffer with 12 5 parts of acetonitrile. This solution was degassed before use in an ultrasonic bath (Branson, Soest, The Netherlands). The mobile phase flow-rate was 1 0 ml/min and the back-pressure was ca 13.8 MPa.

Procedure

Blood samples from patients receiving levodopa in combination with either benserazide or carbidopa were drawn by venipuncture, transferred to heparinized tubes (8—10 I.U./ml heparin in blood) and immediately centrifuged at 1500 *g* for 10 min at 4°C Plasma was immediately separated and 100-μl aliquots were deproteinized by addition of 100 μl of 1 2 *M* perchloric acid [13]. After dilution up to 1 ml with HPLC-grade water, in order to avoid excessive concentration of the acidic solution, the samples were vortexed for

30 s then centrifuged at 2500 *g* for 10 min at 4°C. A 30-μl volume of the clean upper layer was directly injected into the chromatographic system.

RESULTS AND DISCUSSION

Typical chromatograms obtained from a standard mixture of levodopa and OMD, a blank plasma and a patient's plasma are shown in Fig. 1. There is no interference from endogenous plasma substances and metabolites. Moreover, the co-administration of bromocriptine, amantadine and anticholinergic drugs as biperiden, procyclidine, orphenadrine and trihexyphenidyl were not found to interfere with the analysis.

Carbidopa and benserazide were also checked, but under these chromatographic conditions they were not detectable in any of our patients' specimens Calibration curves showed a linear correlation between concentration and peak height, equations of the regression lines were· $y = 5\ 82 + 0\ 11x$ ($r = 0.99$) for levodopa and $y = 0\ 49 + 0.012x$ ($r = 0\ 99$) for OMD.

The absolute recovery of levodopa and OMD was calculated by comparing peak heights obtained from the injection of standard solutions with peak heights measured by injecting deproteinized blank pooled plasma, spiked with known quantities of the two compounds We found values ranging from 94 to 99% for both substances, over the concentration range of the calibration

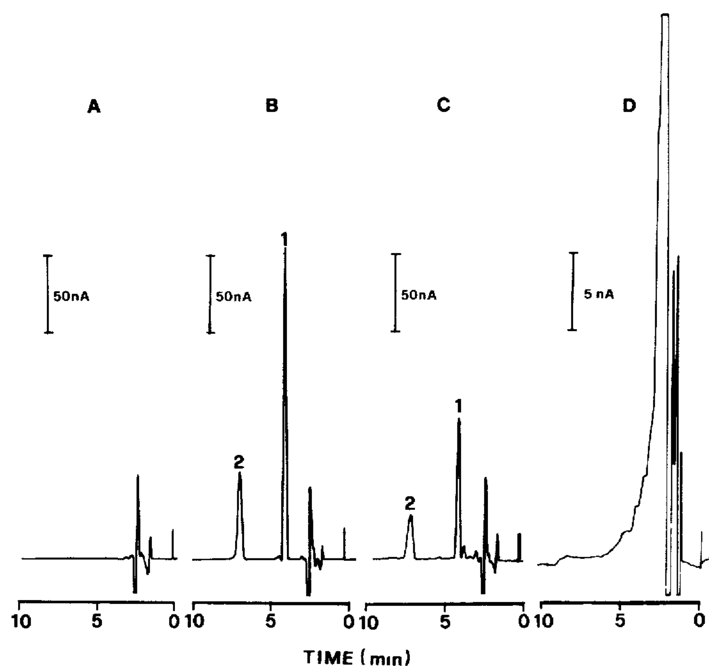

Fig 1 Liquid chromatographic responses obtained by injecting 30 μl of (A) deproteinized blank plasma, (B) standard mixture of 6 3 μmol/l levodopa and 22 0 μmol/l OMD, (C) deproteinized patient's plasma (2 8 μmol/l levodopa, 11 0 μmol/l OMD), at a detector sensitivity of 500 nA full scale Chromatogram D is obtained by injecting 30 μl of a deproteinized blank plasma at a ten-fold higher detector sensitivity (50 nA full scale) Peaks 1 = levodopa, 2 = OMD

curves. Since only deproteinization and direct injection of samples are involved in this assay, the addition of an internal standard was found to be unnecessary. The mean coefficient of variation over a two-month period was 2 8% for levodopa at a concentration of 6.3 μmol/l and 5.2% for OMD at a concentration of 11 0 μmol/l (n = 8).

In a series of fourteen patients, with a levodopa—carbidopa or benserazide combination dosage of 187 5—1125 mg per day (mean ± S.D = 542.8 ± 302 7), divided into three or four daily doses, we found plasma concentrations of 1.5—22.8 μmol/l for levodopa (mean ± S.D = 5 5 ± 6.0) and 3 2—47.5 μmol/l for OMD (mean ± S.D. = 17.9 ± 13.5), 1—2 h after the morning dose

The minimum detectable concentrations were 0.15 μmol/l for levodopa and 1 3 μmol/l for OMD. Since endogenous levodopa concentration in plasma is ca. 5 nmol/l [8, 9], it was below the detection limit under these chromatographic conditions. The ten-fold difference in sensitivity between these two analytes is largely due to the optimal oxidation of levodopa and to incomplete oxidation of OMD at our working potentials [12] Higher potentials are needed to completely oxidize OMD, but this results in a significant and intolerable increase in baseline noise. Our settings provided sufficient sensitivity for the determination of both levodopa and OMD in all patients' samples undergoing normal levodopa therapeutic treatment When lower limits of detection were required, as for acute kinetic studies, OMD could be measured at a ten-fold higher detector sensitivity (50 nA full scale, Fig 1D)

The present procedure, by omitting the neutralization step, further simplifies recently published methods for levodopa and OMD, based on protein precipitation by perchloric acid [10, 11]. Moreover, the low volume of plasma required for this assay makes it particularly suitable for experimental studies, when investigation of diurnal intra-individual variation of the ratio between levodopa and OMD needs more blood samples.

The only drawback we noted, arising from the direct injection of deproteinized plasma samples, was the gradual accumulation of contaminants on the precolumn, which caused changes in peak shapes We found it necessary to replace the precolumn after about sixty injections, without observing any loss of resolution of the analytical column over a period of several months of monitoring plasma specimens.

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