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Note

Simultaneous determination of levodopa, its metabolites and carbidopa in clinical samples

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Investigations into the pharmacokinetics of levodopa (*l*-3,4-dihydroxyphenylalanine, *l*-DOPA) and levodopa/carbidopa (*S*,α-hydrazino-3,4-dihydroxy-α-methylbenzene propanoic acid) combinations have greatly aided our understanding of pharmacotherapy in Parkinsonian's disease [1–3]. A variety of methods have been described for the estimation of these compounds and their metabolites in body fluids, most recently relying on high-performance liquid chromatography with electrochemical detection (HPLC–ED) for separation and identification of the compounds of interest [4–6]. However, most of these methods rely upon separate isolation steps, usually alumina adsorption and cation exchange for the isolation of catechol compounds and their O-methylated metabolites (e.g. 3-O-methyldopa, OMD), respectively, or are unable to resolve OMD and carbidopa in clinical samples [7; Fig. 1 of ref. 8].

We now describe a simple assay procedure which allows simultaneous estimation of catechol and O-methylated compounds, as well as quantitation of the compounds of interest over an extended range of concentrations, using a single sample preparation step and a single injection.

EXPERIMENTAL

Instrumentation

The system employed consisted of a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injection valve or a Waters (Milford, MA, U.S.A.) Model 710B refrigerated

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automatic injector coupled to a Bioanalytical Systems (West Lafayette, IN, U.S.A.) LC-150 solvent delivery system. The electrochemical detector employed was a Bioanalytical Systems LC4B dual-electrode detector configured in the dual-parallel mode. The voltage on one channel was set at 700 mV and on the second at 560 mV, versus an Ag/AgCl reference electrode. The detector range for channel 1 (700 mV) was 200 nA f.s. for quantification of levodopa and OMD, which were present in patient samples in amounts up to 15 μg/ml. The range for channel 2 (560 mV) was set at 10 nA f.s. for quantitation of dopamine, carbidopa and dihydroxyphenylacetic acid (DOPAC), all of which were present in amounts less than 1 μg/ml. The recorder was a Waters Model 730 dual-pen data module.

Materials

3,4-Dihydroxybenzylamine (DHBA; internal standard), levodopa, dopamine, 3-O-methyldopa and DOPAC were obtained from Sigma (St. Louis, MO, U.S.A.). Carbidopa was a gift of Merck Sharp & Dohme (West Point, PA, U.S.A.) HPLC-grade water and methanol were obtained from Baker (Phillipsburg, NJ, U.S.A.).

Chromatographic conditions

The analytical column was either a Biophase ODS, 5-μm packing, or Phase II ODS, 5-μm packing, both 250×4.6 mm (Bioanalytical Systems), protected by two guard columns: Biophase ODS 5-μm, 30×4.6 mm (Bioanalytical Systems) and Pelliguard LC-18, 50×4.6 mm (Supelco, Bellefonte, PA, U.S.A.). The life of the analytical column was approximately 300 injections. Guard columns were changed weekly. The mobile phase consisted of 5% methanol in pH 3.2 citrate–phosphate buffer (20 m*M* sodium citrate and 100 m*M* NaH_2PO_4), containing 0.15 m*M* EDTA and 1.25 m*M* heptanesulfonic acid (Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase was filtered through a 0.45-μm cellulose membrane filter (Millipore, Milford, MA, U.S.A.), and vacuum-degassed. Flow-rate was approximately 1–1.5 ml/min. The temperature of the analytical column was kept at 28°C.

Sample preparation

Collection. Patient samples were obtained through an indwelling venous catheter ("heparin lock") at intervals following the administration of carbidopa/levodopa (Sinemet) to patients enrolled in a study of the safety and efficacy of two controlled-release formulations. The clinical results of these studies are being reported elsewhere. Blood was collected in EDTA-containing Vacutainer tubes, chilled immediately on ice and centrifuged as quickly as possible. Plasma (2 ml) was removed and placed in a polyethylene tube containing 100 μl of 10% sodium bisulfite as antioxidant, and the samples were frozen at −20 or −75°C until analyzed.

Deproteinization. Samples were allowed to thaw at room temperature. A 0.5-ml aliquot of plasma was placed in a round-bottomed polystyrene centrifuge tube, and 50 μl of 4 *M* perchloric acid and 50 μl 0.1 *M* perchloric acid containing 1 mg/l dihydroxybenzylamine as internal standard were added to each sample. The samples were centrifuged 10 min at 1500 *g*. A volume of 300 μl of the supernatant was

TABLE I

RECOVERY AND REPRODUCIBILITY FOR THE DETERMINATION OF LEVODOPA, ITS METABOLITES AND CARBIDOPA

Compound	Typical retention times (min)	Range of values* (ng/ml)	Recovery (%)	Coefficient of variation** (%)
Group A				
DOPA	5.1	10 000	98.7 ± 3.0	3.4
OMD	13.5	25 000	100.5 ± 2.8	2.5
Group B				
Dopamine	11.4	< 5	97.3 ± 4.25	2.7
Carbidopa	15.1	500	86.4 ± 3.8	3.9
DOPAC	17.3	250	82.1 ± 0.4	3.4

*Highest concentrations observed in clinical material.
**Mean of six samples from single plasma pool assayed in triplicate.

aspirated and centrifuged once again at 1600 *g* through a 0.2-μm regenerated cellulose filter (Bioanalytical Systems). A volume of 20 μl of the filtrate was then injected directly into the HPLC system. Standards for quantitation were prepared daily by the addition of 50-μl aliquots of stock solutions containing known amounts of all compounds of interest plus internal standard in 0.1 *M* perchloric acid to blank plasma. The stock solutions were prepared by serial dilutions of a master stock solution containing 10 μg/ml each of levodopa and OMD and 2 μg/ml carbidopa, dopamine and DOPAC. The working dilutions were divided into aliquots which were frozen immediately at −75 °C and used only once.

Quantification

A four-point standard curve of peak-height ratios versus DHBA for all substances of interest and covering the range of expected patient values (usually up to 10 μg/ml for DOPA and OMD and up to 500 ng/ml for carbidopa, dopamine and DOPAC) was constructed daily, using the method of least-squares regression. Peak-height ratios were found to yield more reproducible results than area ratio determinations or external standardization using peak heights alone.

Recovery. Recovery was estimated by comparing peak heights of standard amounts of compounds of interest carried through the assay procedure to those resulting from addition of the same amount of standard stock solution to 0.1 *M* perchloric acid, followed immediately by injection into the HPLC system.

RESULTS

The recovery of the five compounds of interest from spiked plasma is shown in Table I. Coefficients of variation for repeated determinations from the same plasma sample were less than 4%. Repeated freezing and thawing of samples led to approximately a 10% loss of carbidopa; the other compounds were stable for periods up to one year.

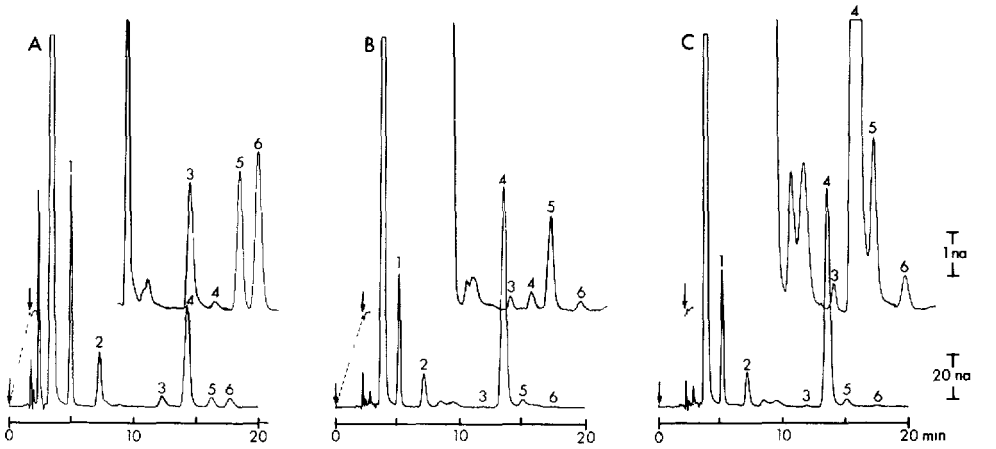

Fig. 1. Representative chromatograms of spiked plasma (A) and a patient sample (B and C). Peaks: 1 = levodopa; 2 = DHBA (internal standard); 3 = dopamine; 4 = OMD; 5 = carbidopa; 6 = DOPAC. Bottom tracing in each panel was recorded at an applied potential of 700 mV and 200 nA f.s.; top tracing (with first 7 min blanked out) at 560 mV and 10 nA f.s. (A and B) and 700 mV and 10 nA f.s. (C). Drug and metabolite concentrations (all in ng/ml) are as follows: levodopa 5000 (A) or 3459.7 (B and C); dopamine 500 (A) or 50.32 (B and C); OMD 10 000 (A) or 3372.1 (B and C); carbidopa 500 (A) or 519.5 (B and C); DOPAC 500 (A) or 38.4 (B and C).

The assay was highly linear ($r = 0.999$) for all compounds tested over the entire range examined, from the lower limits of detection (1 ng/ml for levodopa and 6 ng/ml for carbidopa) up to 15 μg/ml for levodopa and 3-O-methyldopa (group A) and 0.5 μg/ml for carbidopa, DOPAC and dopamine (group B). The ranges of observed plasma concentrations of all compounds measured is given in Table I. The use of the dual-electrode detector made it possible to accurately quantify compounds in both groups A and B in a single injection without altering the sensitivity of the detector. Since the retention times of OMD and carbidopa were similar in a variety of mobile phases tested and their separation varied with the age of the mobile phase, we took advantage of the greater half-wave potential of O-methylated compounds [8] to eliminate the OMD peak from the second channel in order to eliminate its interference with the determination of carbidopa. Representative chromatograms of spiked plasma and the same patient sample, recorded at 700 mV on both channels and 700 mV on channel 1 asnd 560 mV on channel 2, are shown in Fig. 1.

Fig. 2 shows plots of applied voltage versus peak height and peak-height ratio versus DHBA for the compounds of interest. Of note is that OMD is undetectable at applied voltages less than 580 mV, whereas there is only a minor reduction in peak height and no change in peak-height ratio for the other compounds of interest.

The major drawback of the present system is the short column life, even with the utilization of two guard columns totalling 80 mm in length. The guard columns in turn resulted in significant peak broadening. Saturation of the guard columns with particulate and proteinaceous substances caused the appearance of extraneous peaks and an unstable baseline, necessitating weekly changes. As the mobile phases aged, the separation between dopamine, OMD and carbidopa less-

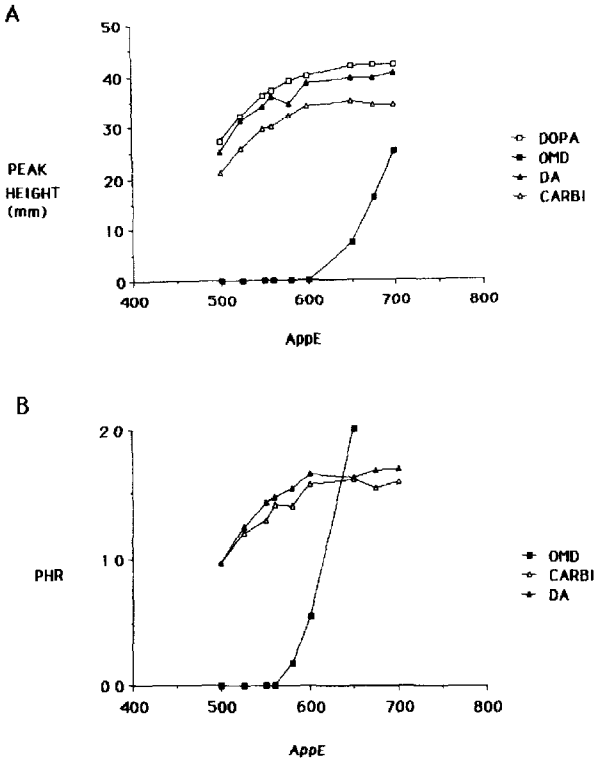

Fig. 2. Partial voltammograms of absolute peak height (in mm) (A) and peak-height ratio (versus DHBA) (B) versus applied potential (AppE). Both are constant for DOPA, dopamine (DA) and carbidopa (CARBI) above an AppE of 560 mV. Each point represents the mean of two determinations.

ened, requiring preparation of a new mobile phase approximately every two weeks. However, the elimination of the OMD peak in the 560-mV chromatograms allowed prolongation of the life of the mobile phase by eliminating potential obscuration of the much smaller dopamine and carbidopa peaks.

DISCUSSION

In performing pharmacokinetic studies involving large numbers of plasma samples, rapidity and ease of sample preparation are important considerations. Using this method, preparation time for a set of eleven patient samples and appropriate standards is approximately 45 min. Chromatographic run time averaged less than 20 min, allowing processing of up to 72 samples in a 24-h period using the automatic injection system. The necessity for separate extractions and chromatographic conditions for catechol and O-methylated compounds is eliminated, and the recovery of all compounds of interest, especially of carbidopa, is greatly improved.

The recovery of carbidopa, using the procedure described above, was $86.4 \pm 3.8\%$, versus approximately 50% using conventional alumina-adsorption methods [4,6].

This improved recovery presumably results from the more rapid sample preparation, since the compound is easily oxidized, even in acid pH at room temperature [9]. The recovery of DHBA, which is not as easily oxidized, using alumina-adsorption methods is considerably greater than that of carbidopa. This discrepancy may lead to erroneous results when DHBA is used as an internal standard for carbidopa determinations under the conditions of alumina extraction. Thus our method provides increased sensitivity and reliability for quantification of carbidopa.

The understanding of the relationship between plasma levels of OMD and levodopa and the therapeutic response to levodopa in patients has come under increasing scrutiny, since it has been reported that co-administration of OMD reduces the clinical effectiveness of levodopa [10], presumably by interfering with the entry of the latter compound into the brain [11]. Previous methods have relied upon alumina extraction for separation of catechol compounds [4–8,12], with a separate ion-exchange isolation step for OMD and other O-methylated catechol metabolites or, because of the similar retention times of OMD and carbidopa, have been unable to resolve the two in clinical samples [7,8].

CONCLUSIONS

We have described a rapid, single-step procedure for the determination of levodopa, carbidopa and related compounds in clinical samples from patients being treated with levodopa/carbidopa combination medications. The assay is reproducible and offers improved recovery of all compounds, as well as the ability to quantify compounds present over ranges of concentration from a few to 15 μg/ml. This is facilitated by cleaning up the region of the chromatogram containing OMD and carbidopa by eliminating the OMD peak, achieved through the use of a second electrode with an applied voltage too low to oxidize OMD. This assay has been used to advantage in our laboratory in the investigation of the clinical efficacy of controlled-release levodopa/carbidopa preparations [13].

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