Journal of Chromatography, 277 (1983) 115—123
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1776

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION OF PLASMA L-3,4-DIHYDROXYPHENYLALANINE IN PARKINSONIAN PATIENTS

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(First received January 26th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

An assay for the quantitative estimation of L-3,4-dihydroxyphenylalanine in human plasma has been developed, using α-methylnoradrenaline as internal standard and ion-pair reversed-phase (C_8), isocratic high-performance liquid chromatography with amperometric detection. A citrate—phosphate buffer, pH 3.1, containing 2 m*M* EDTA disodium salt, 6.5 m*M* 1-octanesulphonic acid and 14% v/v methanol, provided good separation of the analytes from each other and from the carbidopa present in the plasma of Sinemet-treated Parkinsonian patients. The sensitivity, selectivity and precision of the method were found to be sufficient to enable its routine use for pharmacodynamic studies.

INTRODUCTION

L-3,4-Dihydroxyphenylalanine (L-dopa) a catechol α-amino acid, is widely used in the treatment of Parkinson's disease [1—4], usually in combination with a peripheral dopa decarboxylase inhibitor such as L-α-hydrazino-3,4-dihydroxy-L-α-methylbenzenepropanoic acid (carbidopa) [5] (Fig. 1).

Following the introduction of L-dopa for Parkinson's disease an extensive literature of fluorimetric analyses appeared [6—12]. However, the patient compliance and the considerable individual variation in therapeutic dosage, have

0378-4347/83/$03.00

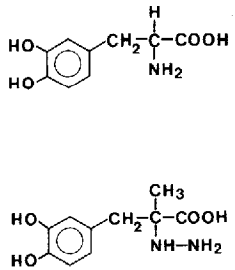

Fig. 1. Molecular structures of (a) L-dopa and (b) carbidopa.

prevented the widespread routine monitoring of L-dopa in plasma or urine; and as a consequence there has been comparatively little use of modern methods of analysis.

Radioenzymatic assays for L-dopa have been described [13, 14], but these tend to be expensive, time-consuming and unsuitable for small numbers of samples. Furthermore, there is the possibility of crossover interference from carbidopa in Sinemet-treated patients. High-performance liquid chromatography (HPLC) methods utilising ultraviolet detection [15, 16] or fluorescence detection [17] have the sensitivity to measure L-dopa in urine and pharmaceutical preparations; but the reliable measurement of plasma levels has awaited the development of a highly sensitive detector. The amperometric (electrochemical) detector introduced by Kissinger and co-workers [18, 19] has picomole sensitivity, is compatable with most reversed-phase HPLC systems; and now that problems with electroactive contaminants and electrical noise have been overcome, HPLC with amperometric detection is frequently the method of choice for catecholamines and related compounds [20]. Riggin et al. [21] described an HPLC method for measuring serum L-dopa and dopamine, using a bonded phase cation-exchange column and amperometric detection. More recently Freed and Asmus [22] reported a reversed-phase HPLC method suitable for plasma L-dopa, which gave improved resolution, but which was subject to undefined interfering peaks. Contrary to the experience of these workers we have found that reversed-phase ion-pairing HPLC systems based on citrate—phosphate (McIllvaine) buffers are ideally suited to separation and amperometric detection of catecholamines and related compounds [23, 24]. The advantages of citrate buffers have been appreciated by others [25, 26] but their acceptance has awaited the description of the mechanism of action, as recently given by Krstulović et al. [27].

This present paper deals with the development of an HPLC—amperometric method to measure L-dopa in the plasma of Parkinsonian patients treated with Sinemet, as part of a study of the effects of L-dopa on cerebral blood flow and metabolism, as assessed by positron emission (ECAT) scanning. The clinical results of this study will be published separately.

EXPERIMENTAL

Apparatus

Chromatography was performed using a Model 100A solvent delivery pump and a Model 210 sample injection valve (Altex Scientific, Berkeley, CA,

U.S.A.). The separations were achieved with a 15 cm × 4.6 mm I.D. Ultrasphere octyl (5 μm particle size) column (Altex), and detection was accomplished using a TL-4 glassy carbon electrode held at +0.72 V vs. Ag/AgCl reference electrode.

The electrode was controlled by an LC-4 amperometric detector (Bio-Analytical Systems, West Lafayette, IN, U.S.A.). Typical amperometric detector sensitivity settings for plasma L-dopa were 200 nA full scale on filter C. An aluminium Faraday cage enclosed the column, electrodes and amperometric detector and this was earthed to the Model RE. 541.20 Servoscribe chart recorder (Smiths Industries, London, U.K.).

Reagents

The ion-pairing agent 1-octane sulphonic acid was obtained from Fisons (Loughborough, U.K.). AnalaR grade methanol from May and Baker (Dagenham, U.K.) was found adequate. The standard L-dopa was obtained from Sigma (Poole, U.K.) and α-methylnoradrenaline was a gift from Hoechst Pharmaceuticals (Hounslow, U.K.). All other chemicals were of analytical grade and obtained from BDH Chemicals (Enfield, U.K.). The alumina (Brockmann grade 1, active neutral) was washed and activated as described previously [23].

Chromatography

Mobile phases consisted of citrate—phosphate buffer (pH 3.1) containing 6.5 m*M* 1-octanesulphonic acid and 14% methanol as organic modifier. The disodium salt of EDTA was added to a final concentration of 2 m*M*. Prior to use the mobile phase was filtered through Gf/f glass microfibre paper (Whatman Lab. Sales, Maidstone, U.K.) and helium degassed (BOC Special Gases, London, U.K.). All separations were carried out at ambient temperature.

Once set up, the system was run continuously to ensure stability and sensitivity. During sample injections the flow-rate was usually 1.2 ml/min and this was reduced to 0.1 ml/min when the system was not in use.

Dosing

Sinemet (Merck Sharp & Dohme, Hoddesdon, U.K.) was obtained from the hospital pharmacy as Sinemet 110 (10 mg carbidopa with 100 mg L-dopa) or Sinemet 275 (25 mg carbidopa with 250 mg L-dopa). Combinations of these two formulations were used in patient dosing.

Plasma samples

A cannula was inserted into a radial artery prior to the scanning procedure and blood was taken into lithium heparin tubes. These were immediately centrifuged (2050 *g*, 5 min, room temperature) and the separated plasma frozen and stored at −70° C prior to the analysis. The protocol outlined below (Fig. 2), generated four samples per patient, from scans during acute and chronic L-dopa treatment. The protocol was approved by the Research Ethics Committee of the Royal Postgraduate Medical School and Hammersmith Hospital and all patients gave their informed consent.

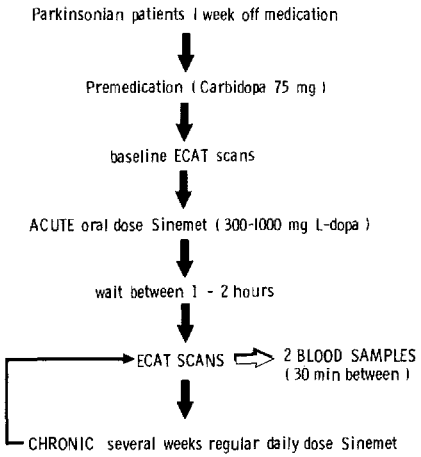

Fig. 2. Protocol for Parkinsonian patient study.

Quantitation

A stock standard of 10 μg/ml of L-dopa was prepared in 0.1 *M* hydrochloric acid and stored at 4°C. Spiked standards were prepared in drug-free plasma on the day of the assay, typically a range from 0—2000 ng/ml was employed. An internal standard of α-methylnoradrenaline was chosen to allow for variable recovery of L-dopa. Quantitation was thus achieved by comparison of the peak height ratio of L-dopa to α-methylnoradrenaline. A calibration curve of at least six spiked plasmas was run with every batch of samples.

Extraction

Conical polystyrene tubes were placed in an ice bath and the following reagents added: 60 mg activated alumina, 1 ml of 1 m*M* hydrochloric acid and 0.1 m*M* EDTA disodium salt, 100 μl of 10 μg/ml α-methylnoradrenaline and 1 ml of distilled water. Plasma samples were thawed, briefly centrifuged at 4°C to remove any fibrin aggregates and 1-ml portions were added to the tubes, followed by 1 ml of 3 *M* Tris—HCl buffer, pH 8.6. The tubes were mixed on a Spiramix rotary mixer (Denley, Bilinghurst, U.K.) for 15—20 min, then placed in ice and the fines of alumina allowed to settle. The supernatant was aspirated at the sink and the alumina washed three times with distilled water (previously adjusted to pH 7.0). In the final aspiration care was taken to remove all of the wash water, prior to elution with 200 μl of 0.1 *M* orthophosphoric acid — achieved by 2 min on the Spiramix mixer.

All tubes were centrifuged briefly at 2020 *g* at 4°C, finally removing the supernatant into small conical tubes (Eppendorf, 1.5 ml). Portions of the supernatant (usually 50 μl) were injected into the HPLC system, which was typically set at a sensitivity of 200 nA full scale in order to measure plasma L-dopa in the concentration range 100—4000 ng/ml. Storage of the separated eluent at —20°C had no deleterious effect on L-dopa levels for periods of up to five days.

RESULTS AND DISCUSSION

Optimization of pH

The pH was an important parameter to evaluate in the development of this

assay, since the pH of the mobile phase determines the concentration of the ionic forms of L-dopa and hence the degree of ion-pairing with citrate and 1-octanesulphonic acid [27]. Changing the pH from 6.0 to 3.1 increased the retention time of L-dopa from 1.0 to 2.5 min and increased the optimal applied potential from +0.50 V to +0.72 V vs. Ag/AgCl reference electrode.

Resolution

Using the HPLC–amperometric assay at a sensitiviity of 200 nA full scale, L-dopa and the internal standard, α-methylnoradrenaline, were resolved from other plasma constituents. This is illustrated with the chromatograms of extracted plasma samples from Sinemet-treated patients (Fig. 3).

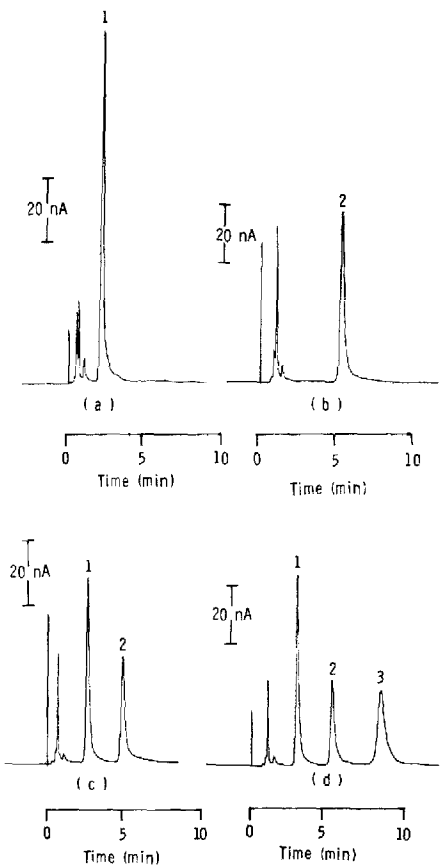

Fig. 3. Representative HPLC chromatograms of human plasma samples run through the assay. (a) Plasma from Sinemet-treated Parkinsonian patient without internal standard added, (b) drug-free plasma with internal standard added, (c) plasma from Sinemet-treated Parkinsonian patient with internal standard added (L-dopa = 1.60 μg/ml), (d) plasma from Sinemet-treated Parkinsonian patient with internal standard added and spiked with carbidopa (20 μg/ml). Chromatographic conditions: column, Ultrasphere octyl (5 μm particle size); eluent, 32 m*M* citric acid, 14 m*M* Na_2HPO_4, 2 m*M* Na_2EDTA, 6.5 m*M* 1-octanesulphonic acid, 14% methanol, pH 3.1; flow-rate, 1.2 ml/min; temperature, ambient; amperometric detector, +0.72 V vs. Ag/AgCl, 200 nA full scale, filter C. Peaks: 1 = L-dopa, 2 = α-methylnoradrenaline, 3 = carbidopa.

TABLE I

RETENTION TIMES FOR CATECHOL COMPOUNDS

For conditions, see text.

Compound	Retention time (min)
DOPAC	1.8
L-Dopa	2.5
α-Methylnoradrenaline	5.1
3-O-Methyl-dopa	5.5
α-Methyl-dopa	6.0
Carbidopa	6.8
Dopamine	8.4

Blank plasma chromatograms showed no interference which corresponded to the retention time of L-dopa or α-methylnoradrenaline. The reversed-phase ion-pairing HPLC system provided a good separation of L-dopa from carbidopa and other catechol compounds, with the compounds eluting in order of decreasing polarity (Table I). Since 3-O-methyl-dopa is a major metabolite of L-dopa [28] the possibility of its interference with plasma L-dopa measurements was investigated. In plasma samples from Sinemet-treated patients no 3-3-O-methyl-dopa could be detected after the alumina extraction, thus ruling out its interference in our samples.

Calibration

Fig. 4 shows a typical mean standard curve ($n = 6$) in plasma over a concentration range of 50--2000 ng/ml L-dopa, where peak height ratio was plotted as a function of L-dopa concentration. Linearity in standard curves of L-dopa was established over an extended range of up to 5 μg/ml in drug-free human plasma.

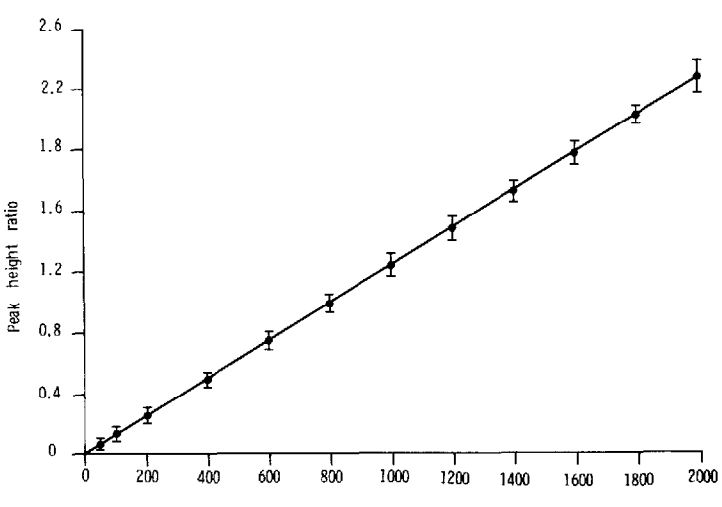

Fig. 4. Mean standard curve for L-dopa in plasma. Each point is the mean ± S.D. of six determinations.

Recovery

The absolute analytical recovery of L-dopa and α-methylnoradrenaline from human plasma was estimated by comparing the peak heights obtained from the injection of known quantities of the analytes with peak heights obtained from the injection of extracts of plasma samples spiked with the analytes. This gave values of 72% for L-dopa and 70% for α-methylnoradrenaline (n = 6).

Limit of detection

Measurement of L-dopa in the therapeutic range (and above) in 1-ml plasma samples was possible at a sensitivity of 200 nA full scale at +0.72 V vs. Ag/AgCl reference electrode on the amperometric detector. At this setting the absolute limit of detection was 25 ng/ml at a signal-to-noise ratio of 2. Since the therapeutic range is in the region of 0.10—3.0 μg/ml this is clearly satisfactory. A more sensitive assay could be achieved by increasing the current amplification on the detector, but this might lead to interferences e.g. from 3-O-methyl-dopa and so would need to be investigated.

During the course of our work on plasma L-dopa a more sensitive approach was described [29] utilising an HPLC system similar to that previously reported by us for catecholamines [24]. However, the elution order reported in this paper differs significantly from our own, in that the acidic metabolite dihydroxyphenylacetic acid (DOPAC) has the shortest retention in our system but an intermediate one in theirs. Furthermore in treated Parkinsonian patients, L-dopa is present in arterial plasma at μg/ml concentrations while DOPAC is in the ng/ml range. Thus for our application a sensitivity of 200 nA full scale on the amperometric detector enabled us to measure L-dopa without any interferences from DOPAC or 3-O-methyl-dopa.

Precision

Intra-assay. Replicate analysis of a pooled plasma sample containing L-dopa in the therapeutic range gave a coefficient of variation of 1.8% (n = 15, $\bar{X}$ = 720.3 ng/ml, S.D. = 13.1565).

Inter-assay. Analysis of a pooled plasma sample containing L-dopa in the therapeutic range over a 1-month period (the pool was stored in aliquots at —70°C between assays, once thawed the aliquots were not reused) yielded a coefficient of variation of 4.3% (n = 10, $\bar{X}$ = 693.5 ng/ml, S.D. = 29.7256).

Practical application

Using the method suggested, the plasma concentrations of L-dopa were monitored in Parkinsonian patients who had received Sinemet orally either following one week of drug abstinence, as an acute dose (range 300—1000 mg L-dopa, n = 8, $\bar{X}$ = 588 mg, S.D. = 219); or, following several weeks of daily optimal dosing, as a chronic dose (range 400—800 mg L-dopa, n = 8, $\bar{X}$ = 631 mg, S.D. = 150). The method described in this paper took advantage of an ECAT scanning study, which had been designed for other purposes; e.g. a positive clinical response had to be ensured after administering L-dopa.

This accounts for the differences in the doses given to the patients. The aim was to provide a single oral dose of L-dopa in the acute experiment equal to the total daily dose to which the patients were accustomed prior to the study.

TABLE II

PLASMA L-DOPA LEVELS IN SINEMET-TREATED PARKINSONIAN PATIENTS, DURING ECAT SCANNING (μg/ml)

Mean time interval A to B = 36 min, all values are the mean of two determinations.

Patient No.	Dyskinesis score	Actute treatment		Chronic treatment	
		A	B	A	B
1	+	3.19	2.83	0.75	0.59
2	—	2.20	2.05	0.50	0.51
3	—	2.70	2.20	0.18	0.26
4	—	2.80	2.28	1.16	0.94
5	++	3.55	3.25	0.48	0.48
6	+++	4.00	3.58	0.86	0.56
7	+	3.08	2.25	1.70	0.96

Samples were taken during ECAT scanning as indicated in Fig. 2, some preliminary results are shown in Table II. The determination of L-dopa in these patients was thought to be of value since previous workers have shown that clinical response can be related to plasma L-dopa concentration [30, 31]. Some evidence for this was obtained, as patients experiencing extra-pyramidal side effects following their acute dose tended to have higher levels of plasma L-dopa, viz. patients 5 and 6. The chromatograms from Sinemet-treated patients showed small carbidopa peaks, but no peaks corresponding to the retention time of DOPAC, 3-O-methyl-dopa or dopamine.

CONCLUSIONS

We have shown that citrate—phosphate buffers containing methanol and sulphonic acid ion-pairing agents give a good resolution of L-dopa and catecholamines on a reversed-phase (C_8) column. In contrast to Freed and Asmus [22] we found little column deterioration or aging with our system, and no interfering peaks in plasma samples. Any slight deterioration in column performance was usually due to proteinaceous material deposited on the head of the column — this was readily removed by use of a small spatula, followed by topping up with fresh packing material. With continuous pumping of mobile phase, resurfacing of the glassy carbon electrode was only occasionally necessary and the start-up time was a matter of minutes. The proposed method for the HPLC—amperometric assay of L-dopa in human plasma, would therefore seem to be suitable for the study of the effects of L-dopa (given as Sinemet) on the cerebral blood flow and metabolism of patients with Parkinson's disease and for therapeutic monitoring.

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