Journal of Chromatography, 568 (1991) 45–54
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5915

Method for measuring endogenous 3-O-methyldopa in urine and plasma

INES ARMANDO*, EHUD GROSSMAN, AARON HOFFMAN and DAVID S. GOLDSTEIN

Clinical Neuroscience Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892 (USA)

(First received September 4th, 1990; revised manuscript received March 21st, 1991)

ABSTRACT

The present report describes a method using column liquid chromatography with electrochemical detection for assaying concentrations of 3-O-methyldopa in urine and plasma. The technique combines a one-step sample preparation scheme with post-column flow-through electrodes in series, allowing adequate chromatographic separation of 3-O-methyldopa from other endogenous substances in urine. The validity of the method was confirmed by markedly decreased urinary 3-O-methyldopa levels after administration of an inhibitor of catechol-O-methyltransferase to rats, radioactivity in chromatographic fractions corresponding to 3-O-methyldopa in urine of rats undergoing infusion of [^{3}H]-L-DOPA, and correlations between excretion rates of 3-O-methyldopa and catechols in humans. In healthy humans, urinary excretion of 3-O-methyldopa averaged 974 ± 707 (S.D.) nmol per day, and plasma levels of 3-O-methyldopa averaged 89 ± 32 nmol/l. The method should be useful in studies about the metabolism of endogenous and exogenous DOPA.

INTRODUCTION

L-DOPA is the immediate product of the rate-limiting step in catecholamine biosynthesis and the precursor of all the endogenous catecholamines. Recently, interest in the role and metabolic fate of endogenous L-DOPA has increased, since it has been suggested that L-DOPA in plasma is derived at least partly from sympathic nerve endings and is a major source of renal production of dopamine (DA) [1–3]. L-DOPA is also a mainstay in the treatment of Parkinson's disease. About 10% of circulating L-DOPA enters the brain (unpublished observation) and the resulting increase in DA levels in the basal ganglia [4] is thought to be the basis for the amelioration of Parkinsonism by L-DOPA therapy.

The two major known routes of metabolism of L-DOPA are decarboxylation to form DA and O-methylation to form 3-methoxy-4-hydroxyphenylalanine (3-O-methyldopa, 3-methoxytyrosine, OMeDOPA). After oral administration of L-DOPA, the two most prominent metabolites found in plasma are homovanillic acid (HVA), which is an end-product of DA metabolism, and OMeDOPA [5].

In man and in several species of laboratory animals, OMeDOPA is a normal constituent of plasma [6,7]. Because of the long half-life of OMeDOPA in plasma, the metabolite accumulates during chronic treatment with L-DOPA [8], and plasma levels of endogenous OMeDOPA average about 10 times that of DOPA and about 100 times that of norepinephrine (NE), the sympathetic neurotransmitter.

Several assays have been developed to measure OMeDOPA in plasma and cerebrospinal fluid. These include fluorimetry [5], gas chromatography–mass spectrometry [9], and liquid chromatography with electrochemical detection (LC–ED) [7,10–12]. These approaches have not been succesfully employed to measure endogenous concentrations of OMeDOPA in urine. In particular, application of available LC–ED methodologies for this purpose was unsatisfactory in our laboratory, because many substances in urine co-chromatographed with and obscured the OMeDOPA peak.

By combining a new sample preparation scheme with flow-through electrochemical detectors in series, we developed and report here a method for measuring urinary OMeDOPA that has adequate sensitivity and specificity to assay endogenous levels of this metabolite in urine from humans and rats. The one-step sample preparation was based on the finding that after loading urine onto a standard cation-exchange column, washing the column with acid resulted in elution of potentially contamining substances, leaving on column relatively purified OMeDOPA that could be eluted by phosphate buffer. The method was tested by: (1) assessing effects of inhibition of catechol-O-methyltransferase (COMT) on urinary excretion of OMeDOPA; (2) contents of ^{3}H in chromatographic fractions corresponding to OMeDOPA in animals undergoing infusion of [^{3}H]-L-DOPA; (3) comparison of absolute levels of plasma OMeDOPA with levels reported in other studies; and (4) correlations between urinary excretion rates of OMeDOPA and catechols among healthy humans.

EXPERIMENTAL

Sample collection

Urine specimens from humans or rats were collected into plastic containers containing 6 mol/l HCl. The urine pH was 2–3 in most cases. Urinary volume was measured, and aliquots from each sample were stored frozen at −70°C until assayed. Collections of urine (24 h) were obtained from 46 normal volunteers (21–74 years old) all of whom were normotensive and were not taking any medications chronically.

Forearm venous blood was obtained from seven subjects after an overnight fast, 20 min after placement of an indwelling intravenous needle.

Sample preparation

Two 1-ml aliquots of each urine sample were carried through the whole procedure. OMeDOPA (2.5 μg/ml) was added to one of these aliquots for recovery

calculation, since recovery of α-MeDOPA through the sample preparation steps was found to differ from that of OMeDOPA. When plasma samples were assayed, 500-μl aliquots were used.

The pH of the samples was adjusted to 2. Each sample was applied to a plastic column containing 0.5 ml of AG 50W-X8 (200–400 mesh) cation-exchange resin (Bio-Rad Labs. Richmond, CA, USA). Before use, the resin, in the hydrogen form, was washed with 10 ml of 2 mol/l HCl, 10 ml of deionized water, 10 ml of 1 mol/l sodium acetate buffer (pH 6.5) and 10 ml of deionized water [5]. After the samples had passed through the column, the resin was washed with 10 ml of deionized water, 2 ml of 2 mol/l HCl and 10 ml of water in succession, and OMeDOPA was eluted with 2.5 ml of 0.5 mol/l potassium phosphate buffer (pH 6.5).

Aliquots of the eluates were transferred to plastic inserts and subjected to reversed-phase LC–ED. A fixed amount of dihydroxybenzylamine (DHBA) or N-methyldopamine (NMDA) was added to each sample before the chromatographic step, in order to correct for differences in injection volume from sample to sample.

Chromatography

Aliquots (15 μl) of the eluate were injected onto a μBondapak C_{18} (Waters Assoc., Milford, MA, USA) reversed-phase stainless-steel column (300 mm × 3.9 mm I.D.; 10 μm average particle size) using a Model 710B automated sample processor (Waters Assoc.).

The mobile phase contained 0.1 mol/l NaH_2PO_4, 100 mg/l EDTA, 3 ml/l sodium octanesulfonate (PIC B8, Waters Assoc.) and 14 ml/l acetonitrile. The pH was adjusted to 3.85 using 85% (w/v) phosphoric acid and was filtered and degassed before use. The mobile phase was pumped at a flow-rate of 1.0 ml/min using a Waters Model 590 programmable solvent-delivery system.

A triple-electrode system (Environmental Sciences Assoc., Bedford, MA, USA) was used to quantify OMeDOPA eluting from the column. To maximize sensitivity and specificity, a series of three post-column detectors was used with oxidation at the first and reduction at the last, so that only reversibly oxidized species were detected at the last recording electrode. The first electrode in the post-column series was set at 0.30 V, the second at 0.15 V and the third at −0.35 V. The output of the third electrode was recorded using a Waters Model 730 data module.

Catechol assay

L-DOPA and DA concentrations in urine were measured using LC–ED after partial purification by absorption on alumina [13]. The limit of detection for each catechol was about 15 pg per volume assayed for each catechol. [^{3}H]-L-DOPA and [^{3}H]-DA in plasma and urine were determined by liquid scintillation spectrometry of fractions of the column effluent corresponding in retention times to those of standard DOPA and DA.

COMT inhibition study

Urine samples were collected from four individually caged, male Sprague–Dawley rats treated with OR-462 [3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione], a potent inhibitor of COMT [14]. The drug was dissolved in polyethylene glycol and 30 mg/kg injected intraperitoneally. On the day of drug administration, rats underwent injection of OR-462 at 09:00 h and 17:00 h, and the urine was collected throughout a 24-h period starting at 09:00 h. For comparison purposes the same rats received two injections of vehicle on the day before the experiment, and urine was collected according to the same schedule.

[^{3}H]-L-DOPA infusion

Three male, halothane-anesthetized Sprague–Dawley rats received an intravenous infusion of 0.55 MBq/h [^{3}H]-L-DOPA of high specific activity (1113.7 GBq/mmol, DuPont New England Nuclear, Boston, MA, USA) in physiological saline for at least 90 min. Urine from a cannulated ureter was collected for 10–30 min. The content of OMeDOPA and the amounts of ^{3}H in fractions of the column effluent corresponding in retention to that of standard OMeDOPA were determined in alumina supernates.

Data analysis

Results are expressed as means ± S.D. A p value less than 0.05 defined statistical significance. Dependent-means t-tests were used to assess differences between treated and untreated rats. Correlation coefficients were calculated by linear regression analysis.

RESULTS

The response of the electrochemical detector was linear (r = 0.997) from 100 pg to 100 ng of injected OMeDOPA standard. The detection limit (three times the baseline noise) was 50 pg. Recovery of OMeDOPA standard added to the samples averaged 34 ± 8% during the sample preparation step.

The intra-assay coefficient of variation (C.V.), determined from ten aliquots of the same urine sample, was 11.1% at a mean concentration of 237 nmol/l, and the inter-assay C.V. for the same urine sample was 13.2%.

Fig. 1A and B show chromatographic recordings from a urine and a plasma sample, respectively, without and with added OMeDOPA standard. No interference from other substances was observed. The retention time of OMeDOPA was less than 10 min; however, longer run times (about 45 min) were required to avoid contamination of subsequent chromatograms by late-eluting compounds.

Fig. 2 shows voltammetric curves relating current detected, expressed as peak height, and applied potential for OMeDOPA in a standard solution and in a urine sample. The curves obtained were similar, pointing to the identity of both substances.

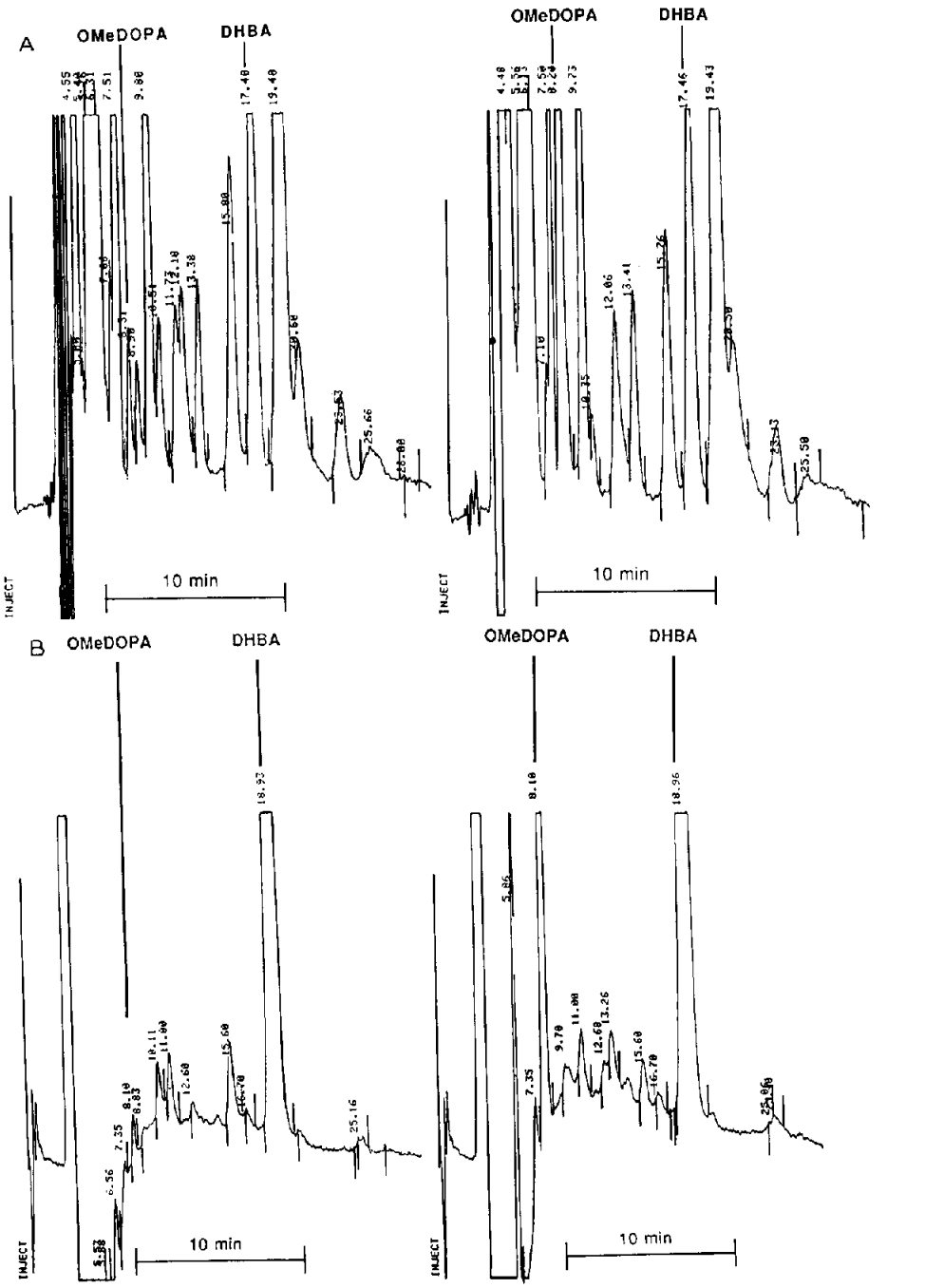

Fig. 1. (A) Left panel: chromatographic recording of a partially purified urine sample from a human subject. OMeDOPA = 3-O-methyldopa; DHBA = dihydroxybenzylamine. Right panel: as in left, except that OMeDOPA standard was added to the urine sample. Full scale = 10 nA. (B) Left panel: chromatographic recording of a partially purified plasma sample from a human subject. OMeDOPA = 3-O-methyldopa; DHBA = dihydroxybenzylamine. Right panel: as in left, except that OMeDOPA standard was added to the plasma sample. Full scale = 10 nA.

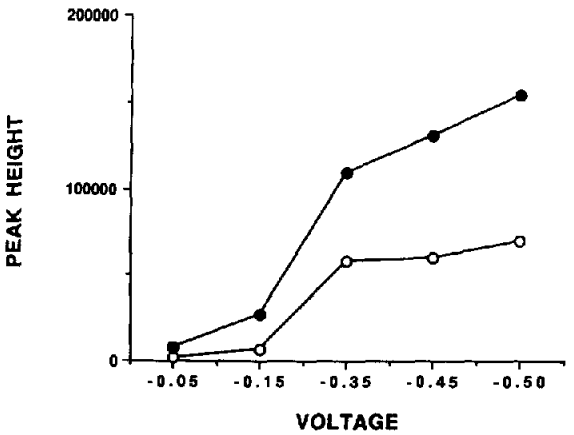

Fig. 2. Current (peak height) *versus* applied potential (voltage) curves from OMeDOPA standard (●) and a urine sample (○).

Treatment with OR 462 decreased the excretion rate of OMeDOPA by about two thirds, whereas excretion of DOPA tended to increase (Fig. 3). The urinary ratio of L-DOPA to OMeDOPA therefore was increased significantly by about three-fold in rats treated with OR 462 (0.90 ± 0.36 *versus* 0.31 ± 0.13; $p < 0.05$).

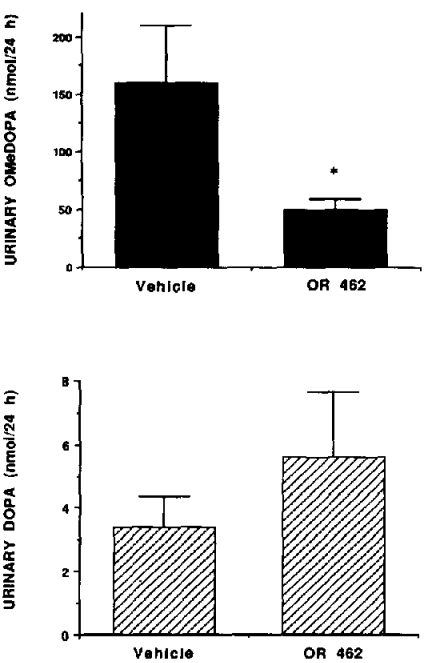

Fig. 3. Effect of OR 462 (30 mg/kg; two injections) on urinary excretion rates of OMeDOPA and DOPA in rats (n = 4). Values shown are means ± S.E.M. (*) $p < 0.05$ *versus* vehicle (paired t-test).

Intravenous infusion of [^{3}H]-L-DOPA resulted in the appearance in urine of ^{3}H in the fraction of the column effluent corresponding in retention to that of standard OMeDOPA. The concentration of OMeDOPA averaged 68 ± 5 pmol/ml, and ^{3}H in this fraction averaged 40 ± 20 dpm/pmol. The ratio of specific activity of [^{3}H]-L-DOPA in plasma to that of [^{3}H]-OMeDOPA in urine averaged 42 ± 25. [^{3}H]-L-DOPA and [^{3}H]-DA accounted for 5.7 ± 2.5 and 38 ± 14% of the total radioactivity in the urine, whereas 1.2 ± 0.5% corresponded to [^{3}H]-OMeDOPA.

In the 46 human subjects, urinary excretion of OMeDOPA averaged 974 ± 707 nmol per 24 h, with individual values ranging widely from 97 to 3620 nmol per 24 h. The excretion rate of the L-DOPA metabolite averaged about five times that of L-DOPA and about half that of DA (Table I). Although urinary excretion of OMeDOPA varied substantially across subjects, about 65% of the subjects had rates between 300 and 1000 nmol per 24 h.

TABLE I

EXCRETION RATES AND PLASMA LEVELS OF 3-O-METHYLDOPA, DOPA AND DA IN HUMANS AND RATS

Catecholamine	Urinary excretion rate (mean ± S.D.) (nmol per 24 h)	Plasma level (mean ± S.D.) (nmol/l)
Humans		
OMeDOPA	974 ± 707	89.0 ± 32.0
DOPA	190 ± 119	9.6 ± 1.7
DA	2042 ± 1160	N.D.[a]
Rats		
OMeDOPA	16.0 ± 9.0	35.0 ± 12.5
DOPA	0.9 ± 0.7	2.9 ± 0.8
DA	9.4 ± 0.2	N.D.[a]

[a] N.D. = not detected.

Mean plasma levels of OMeDOPA in seven healthy, normotensive subjects averaged 89 ± 32 nmol/l. The concentration of OMeDOPA in plasma was ten times that of L-DOPA (Table I).

In rat plasma, the concentration of OMeDOPA also averaged about ten times of that of L-DOPA. In rat urine the OMeDOPA concentration was twenty times that of L-DOPA. In humans the urine OMeDOPA concentration was five times that of L-DOPA (Table I).

The urinary output of OMeDOPA was determined daily over a four-day period in four healthy subjects on a normal diet. Excretion of the metabolite was fairly reproducible (day 1: 688 ± 284; day 2: 507 ± 320; day 3: 566 ± 316; day 4: 593 ± 270 nmol per 24 h).

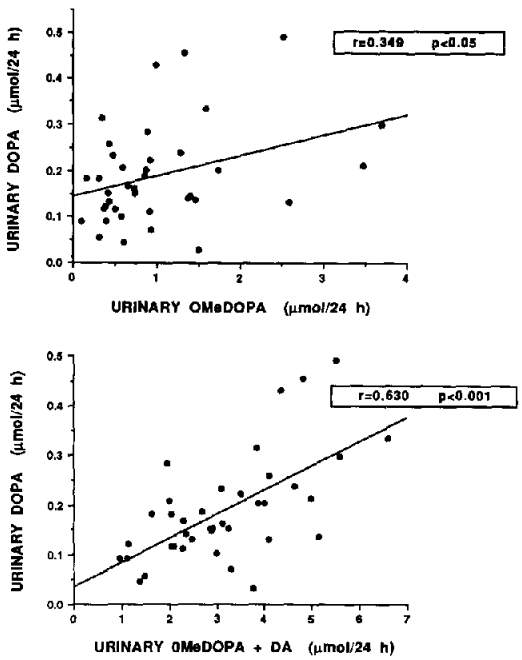

Fig. 4. Relationship between urinary excretion rates of L-DOPA and OMeDOPA and between L-DOPA and the sum of urinary excretion rates of OMeDOPA and DA in healthy human subjects (n = 46).

In normal subjects, the urinary rate of excretion of OMeDOPA was weakly but significantly positively correlated with the excretion rate of L-DOPA ($r = 0.349$, $p < 0.05$). No significant correlation was found between the urinary excretion rates of OMeDOPA and DA. When the excretion rates of DA and OMeDOPA were added, the sum was positively correlated with the excretion rate of DOPA ($r = 0.630$, $p < 0.001$, Fig. 4).

DISCUSSION

The present method was found to be adequate to measure urinary annd plasma concentrations of endogenous OMeDOPA in humans and rats. The sensitivity of the method is less than that reported using gas chromatography–mass spectrometry [9] but about ten times that reported using other LC–ED methods [7,10–12]. The increased sensitivity was achieved by the series electrode system, which also improved the specificity of the assay, because interfering electroactive species were irreversibly oxidized at electrodes at the beginning of the series and therefore were not detected by the recording electrode at the end of the series.

Recovery of the added internal standard averaged about 35%. Losses occurred mainly in the ion-exchange step, due to elution of OMeDOPA during washing of the resin; however, this scheme resulted in markedly improved chro-

matographic results. The within-assay percentage recovery of OMeDOPA was highly reproducible from sample to sample.

In order to validate the method, several experimental approaches were employed. First, rats were treated with OR 462, a COMT inhibitor that has been shown to reduce effectively the activity of the enzyme in peripheral tissues [14,15]. Treatment with OR 462 decreased the urinary excretion rate of OMeDOPA by about two thirds and increased the urinary ratio of DOPA to OMeDOPA by about three-fold. This supported the validity of the assay in measuring the O-methylated metabolite of L-DOPA.

A second approach was to infuse trace amounts of [^{3}H]-L-DOPA into animals. One of the major metabolites of exogenous DOPA in plasma is OMeDOPA, and the infusion resulted in excretion of [^{3}H]-OMeDOPA, since part of the infused ^{3}H appeared in the fraction eluting at the same retention time as that of standard OMeDOPA. The amount of [^{3}H]-OMeDOPA was only about 1/30 that of [^{3}H]-DA, indicating that in rats, circulating L-DOPA is extensively metabolized by decarboxylation to form DA.

The obtained absolute levels of OMeDOPA in human plasma agreed with those reported previously [6,7,9], further supporting the validity of the methodology, since if there were important contamination of the OMeDOPA peak by other compounds, the obtained values would have been higher than those reported by other methods.

Absolute levels of OMeDOPA have not been reported in the urine of drug-free humans, although excretion of this metabolite has been described to be small compared to that of HVA and dihydroxyphenylacetic acid (DOPAC) [9]. In the present study, excretion rates of OMeDOPA in humans were well below those reported by others for HVA excretion but in the range of values for DOPAC [16] and DA [16].

Per kg body weight, rats and humans had similar excretion rates of L-DOPA and DA, whereas the excretion rate of OMeDOPA in rats was about five times that in humans. These differences are consistent with more active O-methylation of L-DOPA in rats than in humans.

Goldstein *et al.* [17] reported that during manipulations of dietary salt intake, proportionate changes in excretion of L-DOPA and DA were similar. In the present study, a positive, although weak, correlation was obtained between concentrations of OMeDOPA and L-DOPA in human urine, and there was no relationship between concentrations of OMeDOPA and DA. In contrast, when levels of DA and OMeDOPA in urine were added, the sum was strongly positively correlated with urinary L-DOPA levels, consistent with both DA and OMeDOPA being important urinary metabolites of L-DOPA.

Situations associated with increased release of endogenous L-DOPA into the bloodstream would be expected to result in increased extraneuronal uptake and metabolism of L-DOPA, resulting in increased urinary excretion of OMeDOPA. In patients with decreased COMT activity or decreased uptake of L-DOPA into

cells containing COMT, the ratio of L-DOPA to OMeDOPA in urine would be expected to be increased. In patients with neuroblastoma, a condition where plasma L-DOPA levels are almost invariably increased [18], high urinary excretion rates of OMeDOPA could suggest the diagnosis. Treatment of Parkinsonian patients with a COMT inhibitor that does not penetrate the blood brain–barrier could result in improved delivery of exogenous L-DOPA to the brain, not only by increasing the amount of L-DOPA in plasma but also by decreasing OMeDOPA concentrations, since OMeDOPA may block the transport of L-DOPA into the brain [19]. The extent of inhibition of O-methylation of L-DOPA may be assessed by the urinary ratio of DOPA to OMeDOPA, aiding the development of drugs that inhibit COMT. The present method therefore should prove useful in studies about the metabolic fate of endogenous and exogenous L-DOPA in a variety of clinical settings.

REFERENCES

1 D. S. Goldstein, R. Udelsman, G. Eisenhofer, R. Stull, H. R. Keiser and I. J. Kopin, *J. Clin. Endocrinol. Metab.*, 64 (1987) 856.
2 R. Zimlichman, P. D. Levinson, G. Kelly, R. Stull, H. R. Keiser and D. S. Goldstein, *Clin. Sci.*, 75 (1988) 515.
3 N. E. Anden, M. Grabowska-Anden and J. Schwieler, *Acta Physiol. Scand.*, 136 (1989) 75.
4 D. B. Caine, J. W. Langston, W. E. W. Martin, A. J. Stoessl, T. J. Ruth, M. J. Adam, B. D. Pate and M. Schulzer, *Nature*, 317 (1985) 246.
5 N. S. Sharpless, M. D. Muenter, G. M. Tyce and C. A. Owen, *Clin. Chim. Acta*, 37 (1972) 359.
6 T. Ishimitsu and S. Hirose, *J. Chromatogr.*, 337 (1985) 239.
7 M. Dousa and G. Tyce, *Proc. Soc. Exp. Biol. Med.*, 188 (1988) 427.
8 J. G. Nutt and J. H. Fellmann, *Clin. Neuropharmacol.*, 7 (1984) 35.
9 A. P. J. M. de Jong, R. M. Kok, C. A. Cramers, S. K. Wadman and E. Haan, *Clin. Chim. Acta*, 171 (1988) 49.
10 M. F. Beers, M. Stern, H. Hurtig, G. Melvin and A. Scarpa, *J. Chromatogr.*, 336 (1984) 380.
11 A. Baruzzi, M. Contin, F. Albani and R. Riva, *J. Chromatogr.*, 375 (1986) 165.
12 S. Rose, P. Jenner and C. D. Marsden, *Movements Disorders*, 3 (1988) 117.
13 G. Eisenhofer, D. S. Goldstein, R. Stull, H. R. Keiser, T. Sunderland, D. L. Murphy and I. J. Kopin, *Clin. Chem.*, 32 (1986) 2030.
14 E. Nissinen, I.-B. Linden, E. Schultz, S. Kaakkola, P. Mannisto and P. Pohto, *Eur. J. Pharmacol.*, 153 (1988) 263.
15 P. T. Mannisto, S. Kaakkola, E. Nissinen, I.-B. Linden and P. Pohto, *Life Sci.*, 43 (1988) 1465.
16 I. J. Kopin, *Pharmacol. Rev.*, 37 (1985) 333.
17 D. S. Goldstein, R. Stull, G. Eisenhofer and J. Gill, Jr., *Clin. Sci.*, 76 (1989) 517.
18 D. S. Goldstein, R. Stull, G. Eisenhofer, J. C. Sisson, A. Weder, S. D. Averbush and H. R. Keiser, *Ann. Int. Med.*, 105 (1986) 887.
19 A. Reches and S. Fahn, *Ann. Neurol.*, 12 (1982) 267.