Journal of Chromatography, 530 (1990) 253–262
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5365

Simple automated high-performance liquid chromatographic column-switching technique for the measurement of dopa and 3-O-methyldopa in plasma

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(First received January 19th, 1990; revised manuscript received March 23rd, 1990)

ABSTRACT

A reliable assay procedure for the determination of 3,4-dihydroxyphenyl-L-alanine (dopa) and its O-methylated metabolite 3-O-methyldopa (3-OMD) is described. Supernatants from deproteinized plasma samples were directly injected into a column-switching system, allowing on-line prepurification of the samples by cation-exchange chromatography followed by reversed-phase high-performance liquid chromatography with electrochemical detection. With this method, the detector response was linear from endogenous levels of dopa and 3-OMD up to microgram concentrations. This technique combines the simplicity of direct injection methods with advantages of procedures using a sample clean-up. It represents a valid tool for the rapid and accurate measurement of large numbers of plasma samples in animals treated with levodopa and in clinical trials with new levodopa formulations.

INTRODUCTION

3,4-Dihydroxyphenyl-L-alanine (dopa) combined with inhibitors of the peripheral aromatic L-amino acid decarboxylase (AADC, *e.g.* benserazide in Madopar® or carbidopa in Sinemet®) provides the most effective therapy for the treatment of Parkinson's disease [1–3].

In humans, dopa is eliminated from plasma with a half-life of *ca.* 1 h [4]. It is well known that a high percentage of dopa, given in combination with peripheral AADC inhibitors, is O-methylated by catechol-O-methyltransferase (COMT). In contrast to dopa, 3-O-methyldopa (3-OMD) has a half-life of *ca.* 15 h and therefore accumulates during chronic therapy [4]. Dopa and 3-OMD are transported into the brain by the large neutral amino acid (LNAA) carrier system. Under chronic therapy with Madopar or Sinemet, the high plasma concentrations of 3-OMD produced might impair the transport of dopa into the brain [4–6]. Accordingly, it was reported that parkinsonian patients with poor response to dopa therapy showed particularly high plasma 3-OMD levels [7,8]. To improve levodopa therapy, coadministration of COMT inhibitors was recently suggested as a means of suppressing the formation of 3-OMD and increasing the bioavailability of dopa, especially with slow-release formulations of dopa, *e.g.* Madopar HBS [4].

0378-4347/90/$03.50

To gain more insight into the pharmacokinetics and metabolism of dopa, and to perform more accurate and extended preclinical and clinical investigations, methods of increased simplicity, sensitivity and reliability are needed.

In the past ten years a number of analytical methods for the monitoring of plasma concentrations of dopa have been published. Radioenzymic methods [9–12] and techniques based on the isolation of dopa by adsorption on aluminium oxide [13–17], solid-phase extraction procedures [18–20] or solvent extraction [21] are sensitive and permit the measurement of endogenous levels of dopa. However, these methods are time-consuming and/or do not allow for the simultaneous measurement of 3-OMD, the main derivative formed under levodopa therapy with Madopar of Sinemet. Direct injection of deproteinized plasma supernatants into a high-performance liquid chromatographic (HPLC) system, as proposed by some authors [22–25], offers a simplified alternative for the rapid assay of dopa and 3-OMD in plasma. It should be stressed that these procedures hardly allow the endogenous concentrations of dopa and 3-OMD in plasma to be detected reliably. In any case, in setting up these procedures we were faced with major problems, *e.g.* unstable baseline, fast contaminations of the HPLC column and late-eluting peaks.

The method presented here combines simplicity, specificity, sensitivity and high stability by on-line prepurification of deproteinized plasma samples using cation-exchange chromatography, followed by reversed-phase chromatography and dual-electrode electrochemical detection.

EXPERIMENTAL

Reagents

3-OMD was purchased from Regis (Merlen Grove, IL, U.S.A.). L-Dopa, benserazide, α-methyldopa and Ro 40-7592 were synthesized at F. Hoffmann-La Roche (Basel, Switzerland). Carbidopa was a gift of Merck Sharp and Dohme (West Point, PA, U.S.A.). All other chemicals were obtained from Fluka (Buchs, Switzerland).

Chromatographic system

The apparatus is schematically shown in Fig. 1. The chromatographic system consisted of an autosampler (Model 360; Kontron Analytic, Zürich, Switzerland), which is able to initiate time-programmed switchings of an automatic six-port valve (Rheodyne 7010P with pneumatic actuator, controlled by a solenoid 7163-030 air valve), two HPLC pumps (pump 1 Model 420, and pump 2 Model 414; Kontron, Zürich, Switzerland), each equipped with a pulse-damping system (Portmann, Therwil, Switzerland) and a dual-electrode electrochemical detector (ESA, Model 5100; Bedford, U.S.A.) with a high sensitivity analytical cell (ESA, Model 5011) set at a potential of +0.30 V (first electrode) and −0.20 V (second electrode). Each electrode was connected either to an integrator (3396A; Hewlett-

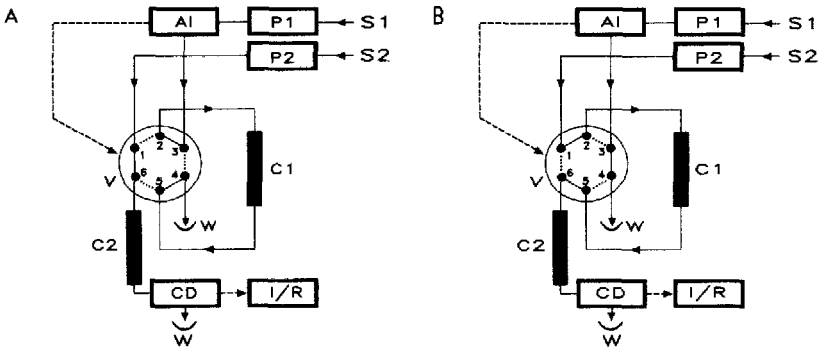

Fig. 1. Configuration of the column-switching system at the valve positions A and B. S = solvent; P = pump; AI = autoinjector; V = switching valve; C = column; CD = dual-electrode electrochemical detector; I/R = integrator or recorder; W = waste; continuous lines = liquid connections; dashed lines = electrical connections.

Packard, Avondale, PA, U.S.A.) or to a two-channel recorder (BD41; Kipp & Zonen, Delft, The Netherlands).

Column C1 (10 cm × 4 mm I.D.) used for the prepurification of plasma samples, was dry-filled with strong acidic cation-exchange pellicular particles (Zipax SCX; Du Pont, Wilmington, DE, U.S.A.). Column C2 (30 cm × 3.9 mm I.D.) used for the separation of the compounds eluted from the column C1, was slurry-packed with reversed-phase material (Nucleosil 100-7 C_{18}; Macherey-Nagel, Düren, F.R.G.).

Solvent 1 consisted of a 0.05 *M* solution of perchloric acid, and solvent 2 was prepared by dissolving citric acid (0.064 *M*), disodium hydrogenphosphate (0.175 *M*), EDTA (0.1 m*M*), 1-octanesulphonic acid sodium salt (0.50 m*M*) and methanol (100 ml) in glass-tridistilled water (final volume 1 l, pH 3.2). Both mobile phases were sterilized by filtration through 0.45-μm celolate filters (HVLP, Millipore, Bedford, MA, U.S.A.) and degassed by ultrasonification. The flow-rate through both pumps was 1.0 ml/min.

Sample preparation

Human blood was collected from the cubital vein using vacutainers containing sodium heparinate as anticoagulant. Male albino rats (stock Füllinsdorf, SPF, 200–250 g body weight) were anaesthetized with Vetanarcol (0.5 ml/kg i.p.; Veterinaria, Zürich, Switzerland) and blood was drawn from the abdominal aorta using EDTA (1% in saline, 1:10, v/v) as anticoagulant. Human and rat blood cells were sedimented by centrifugation (1000 *g* for 10 min), and the plasma collected into new polypropylene tubes was stored at −80°C until analysis.

To 600 μl of plasma, 100 μl of 0.01 *M* hydrochloric acid and 300 μl of 1 *M* perchloric acid, containing 100–500 ng of α-methyldopa as internal standard were added. The mixture was well mixed and allowed to stand for 10 min in an ice-bath

to complete protein denaturation. Standard samples were prepared in the same way, using plasma from an untreated subject. A 100-μl volume of a standard solution containing 300 ng of dopa and 600 ng of 3-OMD replaced the hydrochloric acid. The same plasma used for the preparation of standards was analysed without addition of standards in order to subtract endogenous levels of dopa and 3-OMD from the standard samples. A 100-μl volume of the clear supernatant was injected into the HPLC system. For the analysis of plasma samples containing very high concentrations of dopa and 3-OMD (> 5 μg/ml), only 100 μl of plasma, diluted with 500 μl of water was deproteinized, and/or the injection volume was reduced to 20 μl.

Operation of the column-switching system

Deproteinized plasma samples were placed in an autosampler such that every six samples were followed by a standard. After injection the switching valve remained in position A (Fig. 1A) for exactly 1 min. Dopa, 3-OMD and α-methyldopa as well as some other compounds containing amino groups were retained by the cation-exchange column C1, whereas neutral and acidic compounds were washed out very quickly. Just before the first compound of interest (dopa) was eluted from C1, this column was switched in series with the column C2 (Fig. 1, configuration B) for a further 1.5 min. Within this time, dopa, 3-OMD and the internal standard were transferred almost completely to the column C2, and the switching valve was returned to its initial position (Fig. 1A). During the separation of dopa, 3-OMD and α-methyldopa on the analytical column C2, the ion-exchange column C1 was regenerated for the next switching cycle.

RESULTS AND DISCUSSION

Certain modern autosamplers (*e.g.* Kontron Model 360 or 460) are equipped with auxiliary devices that allow direct time-programmed switchings of a valve without involvement of an external programmer. In our laboratory, the sampler Model 360 was connected with a two-pump HPLC system with a pneumatic controlled switching-valve to obtain a very simple and easy to handle column-switching system.

Pressure jumps caused by switching of two HPLC columns in series often lead to unacceptable baseline disturbances. In the present system this problem was solved by filling column C1 with large-diameter pellicular particles. The resulting back-pressure of the isolated column C1 (Fig. 1A) was at least twenty-fold lower than that of column C2, so that only a minimal increase in pressure resulted when both columns were switched in series. Peak broadening caused by the use of large particles in column C1 was reduced to a minimum by the careful choice of the mobile phase for column C2 (high capacity to elute dopa and 3-OMD from column C1 to C2 in a concentrated fraction).

Memory effects, another problem occurring frequently using column-switch-

ing techniques, were never observed with our method. As expected, no traces of dopa or 3-OMD were retained on column C1 after re-equilibration with perchloric acid.

The switching times (1 and 2.5 min after injection) recommended in the experimental part are valid for a newly packed column (C1) with the dimensions given above (see Experimental). Usually these switching conditions provided virtually constant recoveries of dopa and 3-OMD from column C1 for at least 2 weeks, independently of the number of samples measured. The alterations of the column C1 could be corrected by a slight modification of the "time window" (*e.g.* 0.9 and 2.3 min). However, we preferred to refill the inexpensive column C1 every 2–3 weeks instead to modify the switching times. When the analysis is restricted to 3-OMD alone, very clean chromatograms with switching times set at 1.9 and 3.2 min can be obtained. Under our experimental conditions, using an injection volume of 100 μl and switching times of 1 and 2.5 min, recoveries of dopa, α-methyldopa and 3-OMD were (means ± S.D., $n=3$) 97.1 ± 0.2% 100.3 ± 0.7% and 98.6 ± 0.2%, respectively. The above recoveries were calculated by taking the peak areas generated by samples injected directly into the analytical column C2 as the 100% values.

The detection of dopa by electrochemical oxidation followed by reductive detection provided the most successful procedure to suppress non-reversible electroactive components still present in the plasma. Unfortunately, 3-OMD produced too weak a signal to be detected at the same electrode as dopa. For this reason, the signals from two electrodes were needed; the first (+0.30 V) for detection of 3-OMD and the second (−0.20 V) for dopa and α-methyldopa.

Plasma concentrations of dopa and 3-OMD were calculated by comparing the internal standard ratios of the samples with those of the standards (plasma, spiked with known amounts of dopa and 3-OMD), corrected for the endogenous levels of dopa and 3-OMD.

Representative chromatograms are shown in Fig. 2. Fig. 2A shows a control human plasma from a healthy subject. The plasma profile of a healthy subject administered one capsule of Madopar 125 (100 mg of dopa plus 25 mg of benserazide) 6 h before blood collection is shown in Fig. 2B. The endogenous levels of dopa and 3-OMD measured in the plasma of untreated subjects, using this method, were (means ± S.D., $n=4$) 1.8 ± 0.7 and 19.2 ± 4.3 ng/ml, respectively. Using freshly prepared columns and switching times of 1 and 2.5 min, dopa, α-methyldopa and 3-OMD had retention times of 7.5, 12.9 and 14.2 min, respectively. As shown in Table I, several endogenous and exogenous compounds did not interfere with dopa and/or 3-OMD peaks. As expected, neutral and acidic metabolites of dopa, *e.g.* homovanillic acid and 3-methoxyhydroxyphenylethyleneglycol were eliminated on column C1 during the prepurification. Several unknown endogenous electroactive compounds, present in plasma, were eliminated by the prepurification step, or suppressed by the detection conditions, or exhibited retention times different from those of dopa and 3-OMD in the reversed-phase separation.

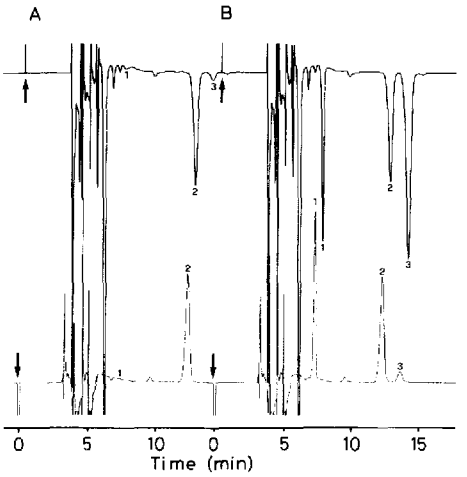

Fig. 2. Chromatogram of control human plasma (A) and of plasma obtained from a subject administered one capsule of Madopar 125, 6 h before blood collection (B). The concentrations of dopa (peak 1) and 3-OMD (peak 3) were 83 and 215 ng/ml plasma, respectively. In both A and B 125 ng/ml α-methyldopa was added as internal standard (peak 2). In the chromatogram of the untreated subject (A) endogenous 3-OMD but not dopa is seen at the detector sensitivity used. The electrical potentials of the electrochemical detector were set at +0.30 V (top) and −0.20 V (bottom), at a sensitivity gain of 60 (first electrode) and 170 (second electrode).

TABLE I

SPECIFICITY OF THE DOPA AND 3-OMD DETERMINATIONS

Retention times and relative detector responses of amino compounds.

Compound	Retention time (min)	Relative response at the oxidative electrode (3-OMD = 100)	Relative response at the reductive electrode (dopa = 100)
Norepinephrine	6.8	174	89
Dopa	7.4	283	100
Epinephrine	8.8	8	11
Tyrosine	8.9	0	0
Normetanephrine	11.5	43	31
Benserazide	11.6	0	0
α-Methyldopa	12.5	110	71
Dopamine	13.5	0	0
3-OMD	14.1	100	8
Carbidopa	14.5	133	0
4-OMD	16.1	43	17
5-Hydroxytryptophan	16.7	152	2
Serotonin	34.8	0	0
Tryptophan	43.4	0	0

Linearity was demonstrated by processing human plasma samples with added dopa and 3-OMD ranging from 2.5 to 5000 ng/ml. Within this range there was an excellent linearity ($r^2 > 0.999$) between detector response and dopa/3-OMD concentrations (Fig. 3). The intersection of the 3-OMD standard curve at a positive point of the vertical axis was caused by endogenous 3-OMD (13 ng/ml) present in the plasma sample. Using a time constant of 2 s and a signal-to-noise ratio of 3 for the electrochemical detector, the detection limits for dopa and 3-OMD were 0.1 and 0.7 ng/ml plasma, respectively.

Reproducibility of the determination was established by 20 parallel analyses of a pool of human plasma, spiked with dopa and 3-OMD (1000 ng/ml each). After subtraction of the endogenous levels, 983 (dopa) and 987 (3-OMD) ng/ml were found with a coefficient of variation (C.V.) of 2.6% and 1.8% for dopa and 3-OMD, respectively. The day-to-day variability was ascertained by repeated analyses for 2 months of a pool of human plasma containing 250 ng of dopa and 3-OMD per ml. The C.V. were 2.7% and 3.5% for dopa and 3-OMD, respectively. In this experiment, the plasma samples were stored at −80°C. As shown in Fig. 4, the storage temperature plays an important role in view of the stability of dopa in human plasma. 3-OMD was stable at all temperatures tested (results not shown). Although certain antioxidants slightly reduced the instability of dopa at −25°C (results not shown) we did not use these preservatives because they significantly lowered the 3-OMD concentrations.

A representative example of the measurement of dopa and 3-OMD in human plasma is given in Fig. 5. It shows the time-course of dopa and 3-OMD concentrations in a healthy subject after ingestion of one capsule of Madopar 125 (100

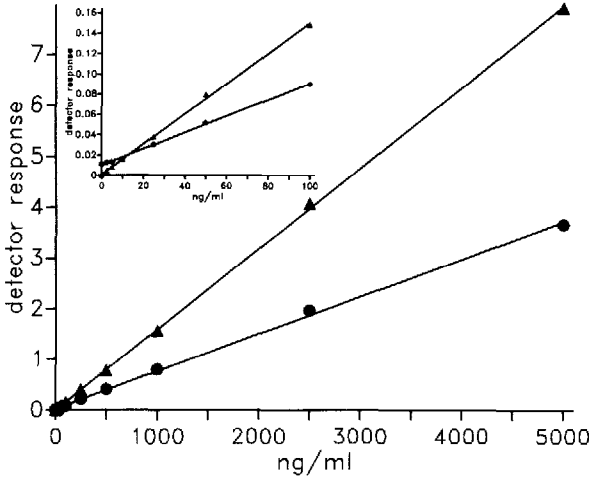

Fig. 3. Linearity of the dopa and 3-OMD assay. Dopa (▲) and 3-OMD (●) were added at various concentrations to the plasma and analysed in duplicate. The correlation coefficients were 0.9998 and 0.9989 for dopa and 3-OMD, respectively. Detector response is expressed as the peak-height ratio of dopa or 3-OMD to the internal standard.

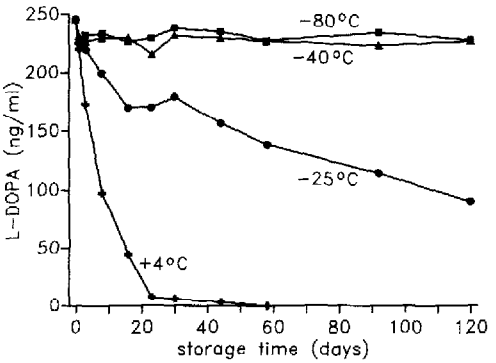

Fig. 4. Stability of dopa in human plasma. Human plasma was spiked with dopa (250 ng/ml) and analysed immediately and after different times at +4, −25, −40 and −80°C.

mg of dopa plus 25 mg of benserazide). An example with rat plasma is presented in Fig. 6, in which are illustrated the time-courses of dopa (Fig. 6A) and 3-OMD (Fig. 6B) in plasma of rats administered dopa (10 mg/kg p.o.) and benserazide (15 mg/kg p.o.) with and without coadministration of the COMT inhibitor Ro 40-7592 (3,4-dihydroxy-4'-methyl-5-nitrobenzoylphenone; 30 mg/kg p.o.) [26–28]. These results clearly show that Ro 40-7592 markedly increased the bioavailability of dopa and, at the same time, almost completely suppressed the conversion of dopa into 3-OMD.

The present method was developed during the past few years for a rapid and reliable analysis of plasma samples collected in several clinical trials. In an earlier version (not published), the clean-up was carried out by manual processing of deproteinized plasma through small cation-exchange columns followed by injection of the eluates into a reversed-phase HPLC column. Automation of this

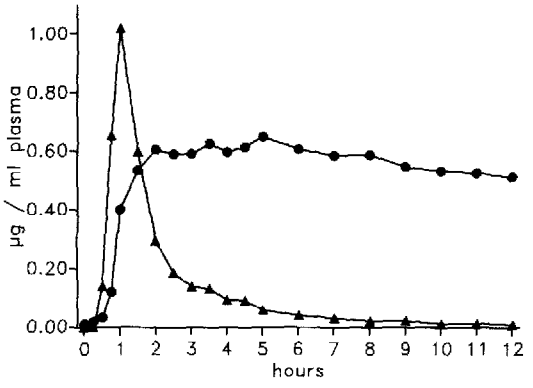

Fig. 5. Typical time-course of dopa (▲) and 3-OMD levels (●) in plasma of a healthy subject after ingestion of a Madopar 125 capsule (100 mg of dopa and 25 mg of benserazide).

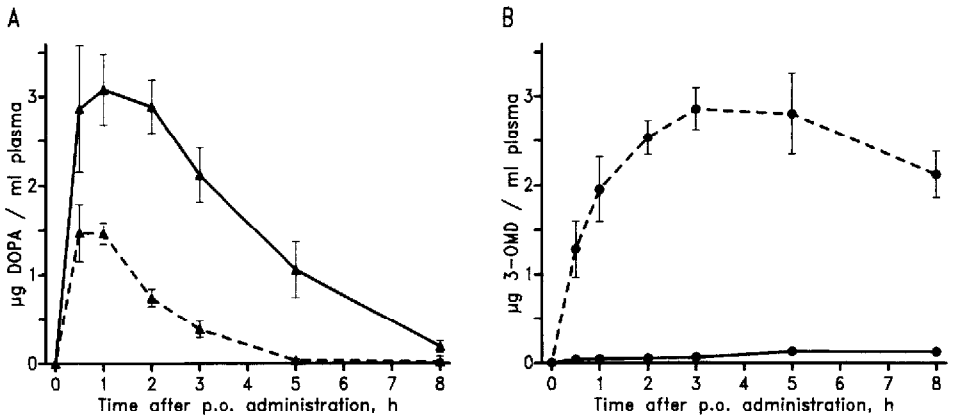

Fig. 6. Time-course of dopa (A) and 3-OMD (B) levels in plasma of rats after oral administration of dopa and benserazide, 10 and 15 mg/kg, respectively (dashed lines), or dopa, benserazide and the COMT inhibitor Ro 40-7592, 10, 15 and 30 mg/kg, respectively (continuous lines). Each point represents the mean ± S.D. of eight rats.

laborious step by column-switching led to this simplified procedure which, at the same time, reduced interferences and gave higher recoveries with lower variations. Using this method several thousands of human and animal plasma samples have been successfully analysed without major analytical problems.

ACKNOWLEDGEMENTS

The authors thank Drs. W. Haefely, W. Burkard and D. Dell for their helpful criticism of the manuscript. The excellent technical assistance of Miss N. Ceccaroni and Mr. M. Gengenbacher is gratefully acknowledged.

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