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Determination of the catechol-O-methyltransferase inhibitor Ro 40-7592 in human plasma by high-performance liquid chromatography with coulometric detection

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ABSTRACT

A sensitive and specific high-performance liquid chromatographic method has been developed to measure the catechol-O-methyltransferase (COMT) inhibitor 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone (Ro 40-7592) in human plasma. The compound and the internal standard were extracted from plasma at pH 2 with *n*-butyl chloride–ethyl acetate (95:5, v/v). The extract was chromatographed on a reversed-phase column (Hypersil ODS, 5 μm) using a mixture of phosphate buffer (0.05 *M*, pH 2), methanol and tetrahydrofuran (45:55:5, v/v/v) as the mobile phase. Long-retained components were removed from the system by means of a simple column-switching system. Quantification of the catechol-O-methyltransferase inhibitor was performed by means of coulometric detection (0.15 V). The limit of quantification was about 1 ng/ml, using a 1-ml specimen of plasma. The recovery from human plasma was >88%. The mean inter-assay precision was 5.3% in the range 2.5–1000 ng/ml. Linearity of the standard curve was obtained in the concentration range 2.5–500 ng/ml. The catechol-O-methyltransferase inhibitor was stable in human plasma when stored for six months at −20°C and for 24 h at room temperature. The practicability of the new method was demonstrated by the analysis of more than 400 plasma samples from a tolerance study performed in human volunteers.

INTRODUCTION

Parkinson's disease is one of the most common chronic progressive neurological diseases, and is characterized by the gradual degeneration of a group of neurons located in the brain stem in the substantia nigra. Although Madopar and Sinemet [combinations of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) with the peripheral decarboxylase inhibitor benserazide or carbidopa, respectively] are still the best treatments available for this condition, many patients experience significant side-effects and require frequent dosing, more than three times a day. These problems are related to markedly fluctuating L-DOPA levels as a result of peripheral degradation of L-DOPA to 3-O-methyldopa by the enzyme catechol-O-methyltransferase (COMT) [1].

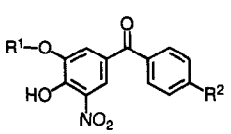

Fig. 1. Structures for the compounds referred to in the text: I, R^1 = H, R^2 = CH_3, parent compound; II, R^1 = CH_3, R^2 = CH_3, potential metabolite; III, R^1 = H, R^2 = Cl, internal standard.

In order to optimize L-DOPA therapy of Parkinson's desease, attempts have been made to discover compounds which would inhibit COMT. 3,4-Dihydroxy-4'-methyl-5-nitrobenzophenone (Ro 40-7592, I; see Fig. 1) is a novel nitrocatechol derivative which has been shown both *in vitro* and *in vivo* to be a potent, selective, competitive and reversible COMT inhibitor [2,3]. Adding I to L-DOPA-containing medications could optimize L-DOPA therapy of Parkinsonism.

A new reversed-phase high-performance liquid chromatography (HPLC) assay with coulometric

detection is described here for the determination of I in human plasma using the chlorinated analogue III as internal standard. Because of the high sensitivity of the assay (1 ng/ml, using 1-ml specimens) it was possible to follow plasma levels of I for a period of six elimination half-lives after a single 5-mg oral dose to human volunteers.

EXPERIMENTAL

Materials, reagents and solvents

Ethyl acetate (for pesticide residue analysis), methanol (gradient grade) and hydrochloric acid (titrisol, p.a.) were obtained from Merck (Darmstadt, Germany). *n*-Butyl chloride (HPLC grade) and tetrahydrofuran (unstabilized, HPLC grade) were purchased from Fisons (Loughborough, UK). Phosphate buffer (0.05 *M*, pH 2) was prepared by dissolving sodium dihydrogenphosphate monohydrate (p.a.; Merck) in water (HPLC grade; Baker, Deventer, Netherlands), titrating the solution with phosphoric acid (85%, suprapur; Merck) to pH 2 and filtering the buffer through a 0.22-μm membrane filter (Millipore, Bedford, MA, USA). Plasma standards were prepared using pre-tested fresh-frozen plasma, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland).

Preparation of standards

A stock solution was obtained by dissolving 10 mg of compound I in 10 ml of methanol. Aliquots of the stock solution were diluted in methanol, providing the working solutions. The plasma standards were obtained by spiking human blank plasma (25 ml) with 250 μl of the corresponding working solution, providing concentrations between 1 and 500 ng/ml. The plasma standards were divided into aliquots of 2.5 ml and stored deep frozen (−20°C) until required for analysis.

Sample preparation procedure

The samples were thawed at room temperature and homogenized by vortex-mixing. An aliquot of plasma (1 ml) was mixed with 50 μl of internal standard solution containing 250 ng of III in hydrochloric acid (5 *M*). The sample was extracted with 5 ml of *n*-butyl chloride–ethyl acetate (95:5, v/v) by shaking for 15 min at 15 rpm on a rotating shaker (Heidolph, Kelheim, Germany). After centrifugation for 5 min, 4 ml of the separated organic phase were transferred to a tapered tube and evaporated to dryness at 45°C by means of a gentle stream of pure (99.999%) nitrogen. For HPLC analysis, the extraction residue was dissolved in 200 μl of mobile phase by vortex-mixing for 15 s.

Instrumentation

A schematic representation of the column-switching system is given in Fig. 2. It consisted of a double-piston pump (P1; Model L6200; Merck), an automatic sample injector (A; Model Wisp 712; Waters, Milford, MA, USA), an analytical column (C1; 125 × 4 mm; Merck), slurry-packed with Hypersil ODS 5 μm (Shandon) in our laboratory, a stainless-steel precolumn (C2; 30 × 4 mm; Merck), slurry-packed with 5-μm Hypersil ODS (Shandon,

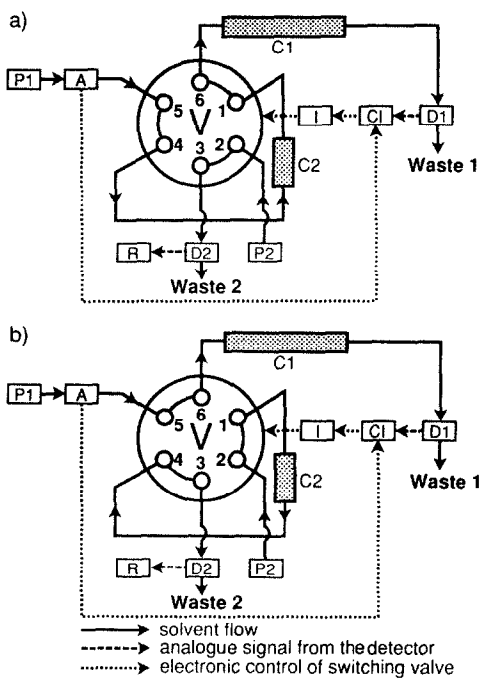

Fig. 2. Schematic representation of the column-switching system assembled for the analysis of I in plasma (see text for details). (a) Valve position 0–4 min and 16–18 min after injection. (b) Valve position 4–16 min after injection. P1 = double-piston pump; A = autosampler; C1 = analytical column; C2 = precolumn; D1 = electrochemical detector; P2 = single-piston pump; D2 = LC–UV detector; R = recorder; V = automatic switching valve; CI = computing integrator; I = interface.

Astmoor, UK), a column oven operating at 30°C (Model 7930; Jones, Hengoed, UK), a coulometric detector (D1; Model 5100, potential 0.15 V; ESA, Bedford, MA, USA), an analytical cell (Model 5011; ESA), a single-piston pump (P2; Model L6000; Merck), an air-actuated switching valve (V; Model 7000 assembled with a Model 7001 pneumatic actuator and a Model 7163 solenoid valve; Rheodyne, Cotati, CA, USA) and a laboratory-made interface (I) for actuating the switching valve by means of a computing integrator (CI; Model SP 4200; Spectra Physics, San Jose, CA, USA) [4]. Although not necessary, a LC–UV detector (D2; Model Spectroflow 773, 254 nm; Kratos, Ramsey, NJ, USA) and a recorder (R; Model W+W 600; Kontron, Zurich, Switzerland) were desirable for proper control of the column-switching process.

Data acquisition was performed by means of the computing integrator (CI), working with a special BASIC program originally developed for the integrator SP 4100 [5]. The acquired data were sent via Ethernet to the host computer for further data reduction and reporting by the KINLIMS system, recently developed in our company [6].

Chromatography and system operation

A mixture of phosphate buffer (0.05 *M*, pH 2), methanol and tetrahydrofuran (45:55:5, v/v/v) was used as the mobile phase for both pumps. The mobile phase was degassed before use with a stream of helium for 1 min. The flow-rates of the two pumps, P1 and P2, were adjusted to 1 ml/min.

The extraction residue was dissolved in 200 μl of the mobile phase. After mixing for 30 s on a vortex mixer, an aliquot (100 μl) of the clear solution was injected by the autoinjector A onto the precolumn (C2) (Fig. 2a). Immediately after the elution of compounds I and III from the precolumn (about 4 min), the HPLC valve (V) was switched to the second position by means of the computing integrator (CI) (Fig. 2b). The two compounds of interest were then chromatographed in the usual manner on the analytical column (C1), while longer-retained plasma constituents were removed from the HPLC system by back-flushing the precolumn (C2). When the chromatography of I and III was completed (about 16 min) the valve (V) switched back to the initial position (Fig. 2a). After an equilibrium time of about 1–2 min the system was ready for the next injection.

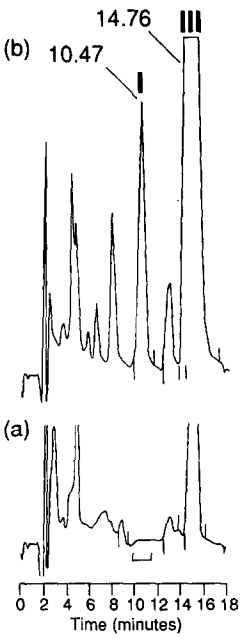

Fig. 3. Chromatograms of human plasma samples: (a) before application; (b) collected 6 h after a single oral dose of 5 mg I; measured concentration, 8.1 ng/ml. Detector, D1; potential, 0.15 V; gain, 5 × 10; response time, 10 s.

The retention times of I and III were about 10.5 and 14.8 min, respectively (Fig. 3b). A complete cycle lasted about 18 min.

Calibration and calculations

At least six different plasma standards covering the expected concentration range were processed as described above, together with the biological samples. The standard curve was established by weighted linear least-squares regression (weighting factor $= 1/y^2$) of the measured peak-height ratios I/III (y) *versus* the concentrations of I (x) added to the plasma. This regression equation was then used to calculate concentrations of I in unknown plasma samples from the measured peak-height ratios I/III.

RESULTS AND DISCUSSION

Sample preparation procedure

Because of the acidic nature of the nitrocatechol group ($pK_a = 4.3$), maximum recovery of I was obtained around pH 2; this was achieved by mixing the plasma samples with 50 μl of hydrochloric acid (5 *M*; also containing the internal standard) before

extraction. Several non-polar solvents (*e.g.* hexane, *n*-butyl chloride, chloroform) were tested as extractants; however, owing to the polar character of I, a more polar solvent was required to isolate the compound from plasma. A mixture of *n*-butyl chloride and ethyl acetate (95:5, v/v) provided the best compromise between high recovery of I and an acceptable degree of coextraction of interfering plasma components.

Analytical system and chromatography

From various suitable 5-μm stationary phases (*e.g.* Spherisorb ODS-1, Spherisorb ODS-2), Hypersil ODS 5 μm was selected as packing material for the analytical separation. However, long-term experience with this material showed that broad and tailing peaks for I and III were obtained for certain batches of Hypersil. This problem was overcome by purging new columns for 48 h with methanol–water, containing *n*-hexylmethyl amine (10 m*M*) as modifier. After removing the excess of amine with methanol–water, the columns were ready for use.

Good separation between I and III was achieved with the mobile phase system described in the Experimental section. Phosphate buffer (0.05 *M*, pH 2) was used as aqueous constituent, in order to suppress ionization of the nitrocatechol group in I and III during chromatography, and to provide a certain conductivity in the mobile phase, which was necessary for the electrochemical detection.

For some samples, late-eluting plasma constituents were observed which interfered in subsequent chromatograms. In order to keep the analysis time for these samples acceptable, the chromatographic system was extended by a simple column-switching unit, allowing a back-flush of the precolumn.

The relatively polar extraction conditions led to considerable coextraction of plasma constituents, which could not be separated completely from I and III during chromatography. The problem was overcome by replacing the UV detector by a highly specific coulometric detector. The COMT inhibitor is a suitable candidate for electrochemical detection, because of the nitrocatechol group, which easily undergoes electrochemical oxidation. However, the voltage of the detector had to be kept at low values (around 0.15 V), in order to suppress the electrochemical response of plasma interferences. Unfortunately, the potential 3-O-methylated metabolite II could not be detected under these conditions, because the nitrophenol group needed a higher potential (*ca.* 0.6 V) for electrochemical oxidation than the nitrocatechol system. Further work on a simultaneous assay for I and II is planned.

Recovery

Human blank plasma was spiked with compounds I and III at concentrations of 10, 50 and 250 ng/ml. The plasma was divided into 1-ml aliquots, extracted as described but without adding the internal standard. The extraction residues were reconstituted in mobile phase and chromatographed as described.

A second series of control samples, providing the 100% values, was prepared by extracting 1-ml aliquots of human blank plasma and then adding equimolar amounts of I and III in mobile phase to the dry residues.

The analytical recoveries for compounds I and III were calculated by comparing the peak heights of the extracted samples to the peak heights obtained from the control samples to which I and III had been added after extraction. The overall recoveries were calculated by correcting the analytical recoveries with the aliquot factors.

TABLE I

RECOVERY OF THE COMT INHIBITOR FROM HUMAN PLASMA

Concentration added (ng/ml)	Concentration found (ng/ml)	Number of replicates (*n*)	Relative standard deviation (%)	Recovery (%)
10.0	9.11	5	8.71	91.1
50.0	44.0	5	2.93	88.0
250	244	5	4.60	97.6

TABLE II

RECOVERY OF THE INTERNAL STANDARD FROM HUMAN PLASMA

Concentration added (ng/ml)	Concentration found (ng/ml)	Number of replicates (*n*)	Relative standard deviation (%)	Recovery (%)
10.0	8.58	5	8.91	85.8
50.0	42.2	5	2.14	84.3
250	233	5	4.49	93.1

The data in Tables I and II indicate a satisfactory overall recovery of >88% and >84% for compound I and the internal standard III, respectively.

Selectivity

The electrochemical method was very specific with respect to endogenous components coextracted from plasma. In more than 100 clinical blank plasma samples (pre-dose, placebo) from 18 different volunteers analysed so far, in only one case was an interfering compound observed coeluting with the COMT inhibitor. Fig. 3a shows a representative chromatogram of a human plasma sample collected before administration.

Precision, accuracy and linearity

The inter-assay precision was measured at different concentration levels around therapeutic concentrations in human plasma. For each level (10, 50 and 250 ng/ml) a spiked plasma sample was prepared and analysed on different days (using a separate calibration line on each day). The data in Table III demonstrate an acceptable precision and accuracy over the concentration range investigated.

The correlation between the peak-height ratio I/III *versus* the concentration of I was linear in the range 2.5–500 ng/ml of plasma. Some clinical samples contained more than 500 ng of COMT inhibitor per ml. In this case the samples were diluted before work-up and the calculated concentration was corrected by means of a scale factor.

According to Table III, the standard deviations and, therefore, also the variances were not constant over this wide concentration range. For this reason, the calibration curve had to be calculated by means of a weighted linear least-squares regression procedure, using $1/y^2$ as weighting factor [7]. The standard software of the computing integrator provided

TABLE III

INTER-ASSAY PRECISION

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Number of replicates (*n*)	Relative standard deviation (%)	Inaccuracy[a] (%)
2.50	2.76 ± 0.4195	5	15.2	10.4
5.00	4.96 ± 0.3258	5	6.57	− 0.8
10.0	9.98 ± 0.4082	5	4.09	− 0.1
25.0	24.4 ± 1.210	5	4.96	− 2.5
50.0	50.9 ± 1.639	5	3.22	1.9
100	101 ± 2.151	5	2.13	0.6
250	247 ± 3.359	5	1.36	− 1.2
500	489 ± 25.53	5	5.22	− 2.4
10.3	10.3 ± 0.2482	34	2.41	0.4
51.1	51.0 ± 1.117	32	2.19	− 0.1
231	237 ± 10.50	35	4.43	2.4

[a] (Concentration found − concentration added) / concentration added × 100.

TABLE IV

STABILITY OF PARENT COMPOUND IN HUMAN PLASMA

Storage conditions	Concentration added (ng/ml)	Concentration found (ng/ml)	Change of concentration after storage (%)	90% Confidence interval (%)	Number of replicates (*n*)
Six months at −20°C	10.0	10.2	2.1	−8.5 to +13.8	5
	50.0	51.1	2.3	−1.7 to + 6.4	5
	250	260	3.9	−3.9 to +12.3	5
24 h at +25°C	10.0	10.0	0.0	−5.0 to + 5.3	5
	50.0	50.2	0.3	−2.7 to + 3.5	5
	250	253	1.1	−0.9 to + 3.2	5

only conventional linear regression and had, therefore, to be modified by means of additional programs [5].

Limit of quantification

By careful adjustment of the conditions of the coulometric detector, less than 1 ng/ml COMT inhibitor could be detected with a signal-to-noise ratio of 3:1. However, the limit of quantification, defined here as the minimum concentration that can be measured routinely with acceptable precision (<20%) and accuracy (>80%), was 1–2.5 ng/ml (see Table III).

Stability

Control plasma was prepared at concentrations of 10, 50 and 250 ng/ml. One portion of these samples was stored at room temperature for 24 h and then analysed. The other portion was frozen, stored at −20°C for six months and then analysed. With each set of stored samples, an equal number of freshly prepared samples was analysed to provide the 100% values. The statistical interpretation of the data followed the procedure recently developed [8]. The data presented in Table IV indicate that compound I was stable in human plasma under the storage conditions investigated.

Application to biological samples

The method has been applied successfully to the analysis of more than 400 plasma samples from a dose proportionality study performed in man. Fig. 3b shows a representative chromatogram from this study. All samples of the study could be analysed by means of the same precolumn and analytical column. Additionally, the electrochemical cell needed no special treatment or readjustment of potential, demonstrating the robustness of the new method. The method was sensitive enough to measure precisely the low concentrations of I in plasma for up to 10 h (corresponding to a period of approximately six elimination half-lives) after a single oral dose of 5 mg to human volunteers.

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