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Determination of the catechol-*O*-methyltransferase inhibitor tolcapone and three of its metabolites in animal and human plasma and urine by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

Reversed-phase HPLC procedures were developed for the determination of tolcapone (Ro 40-7592) and its metabolites Ro 40-7591, Ro 61-1448, and Ro 47-1669 in plasma and in urine samples. One of the procedures for plasma involved the determination of tolcapone and its metabolite Ro 40-7591 and the other, the determination of the two other metabolites. The urine assay enabled the simultaneous determination of tolcapone and all metabolites in one run. Sample preparation in plasma involved protein precipitation with acetonitrile. Urine was simply diluted. The compounds of interest were monitored in the UV at 270 nm. The limits of quantification were 0.05 μg/ml for each compound (plasma assay) and 0.2 μg/ml for the urine assay. The mean inter-assay precisions (C.V.) were ≤6% (plasma assay) and ≤8% (urine assay). The procedures were successfully applied to the sample analysis of animal pharmacokinetic (rat, dog, mouse, rabbit and cynomolgus monkey) and clinical pharmacology studies.

Keywords: Tolcapone

1. Introduction

Tolcapone (Ro 40-7592) is a novel orally active inhibitor of catechol-*O*-methyltransferase (COMT) and is active both in extracerebral tissues and in the central nervous system (CNS). The compound is used in the treatment of Parkinson's disease as add-on therapy to Madopar and Sinemet [1]. The drug is almost completely metabolised, and glucuronidation is the main metabolic pathway [2]. The 3-*O*-methyl metabolite (Ro 40-7591), the carboxylic acid metabolite (Ro 47-1669) and an alcohol metabolite have been identified as minor plasma metabolites [1,2]. The *O*-methyl metabolite and the glucuronide metabolite (Ro 61-1448) are pharmacologically inactive, whereas the two oxidated metabolites exert COMT-inhibitory effects similar to that of the parent drug [1].

An HPLC procedure for the determination of the parent drug in plasma had been developed [3] and applied to the sample analysis of early tolerance and dose finding studies, where low doses had been administered. The procedure involved liquid–liquid extraction and coulometric detection to enhance both sensitivity and selectivity, and column switching to remove late eluting peaks. The high assay sensitivity

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(1 ng/ml) was later on not needed in animal and human pharmacology studies. A more simple HPLC–UV procedure (based on protein precipitation) was developed, and the determination of the 3-*O*-methyl metabolite included. The new procedure was applied to the sample analysis of animal pharmacokinetic and most human pharmacology studies.

To evaluate the effect of hepatic disease on the pharmacokinetics and the elimination mechanism of tolcapone, a procedure for the determination of the metabolites Ro 61-1448 and Ro 47-1669 in human plasma and a procedure for the determination of the unchanged drug and its metabolites in urine were developed at a later stage of the drug development in addition to the existing procedures. The determination of the alcohol metabolite was not included.

2. Experimental

2.1. Materials, chemicals and solutions

Methanol (LiChrosolv), tetrahydrofuran (LiChrosolv), sodium dihydrogenphosphate (p.a.) and phosphoric acid (Suprapur, 85%) were purchased from E. Merck, (Darmstadt, Germany); acetonitrile (HPLC grade) was from Rathburn (Walkerburn, UK); and *N*-hexylmethylamine (purum) from Fluka (Buchs, Switzerland).

Ro 40-7592 (tolcapone, parent drug, III), Ro 40-7591 (3-*O*-methyl metabolite, IV), Ro 61-1448 (glucuronide metabolite, I), Ro 47-1669 (acid metabolite, II), and Ro 40-6031 (internal standard, V) were synthesised at Hoffmann-La Roche Ltd (for structures see Fig. 1). Stock solutions of the compounds were prepared in methanol (with the aid of ultrasonication). Appropriate working solutions for the preparation of the calibration standards were then prepared by further diluting aliquots of the stock solution with methanol. Working solutions of the internal standard were prepared by diluting the stock solution with acetonitrile. Stock solutions were stored at 4–6°C in a refrigerator.

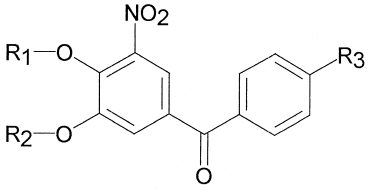

Fig. 1. Chemical structures of the compounds referred to in the text. Tolcapone (III): R_1=H, R_2=H, R_3=CH_3; Ro 61-1488 (I): R_1=H, R_2=Glycuronyl, R_3=CH_3; Ro 47-1669 (II): R_1=H, R_2=H, R_3=COOH; Ro 40-7591 (IV): R_1=CH_3, R_2=H, R_3=CH_3; I.S. (V): R_1=H, R_2=H, R_3=Cl; (alcohol metabolite: R_1=H, R_2=H, R_3=CH_2OH).

Drug free human plasma (anticoagulants: potassium oxalate, sodium citrate) was obtained from a blood bank; drug free animal plasma (anticoagulant potassium oxalate) was from Hoffmann-La Roche Ltd.

2.2. Calibration standards and quality control samples

Calibration standards were prepared by adding aliquots of the stock or working solutions to pretested blank plasma and urine. The usual calibration ranges for the determination of Ro 40-7592 and Ro 40-7591 were: 0.05 to 2.5 μg/ml in human plasma, 0.1 to 50 μg/ml in animal plasma, and 0.2 to 100 μg/ml in human urine. The usual calibration ranges for the determination of Ro 61-1448 and Ro 47-1699 were: 0.05 to 5 μg/ml in human plasma, and 0.2 to 100 μg/ml in human urine. Quality control samples (QCs) were prepared in a similar way.

Calibration standards and QCs were stored frozen in small portions at −20°C until needed.

2.3. Chromatographic system

The HPLC system consisted of the following modular components: Pump L-6200 (Merck-Hitachi, Darmstadt, Germany), autosampler Gilson 231 (Abimed, Langenfeld, Germany), UV-detector SPD-10A (Shimadzu, Duisburg, Germany) and a PE-Nelson 2600 data system (Perkin-Elmer, Überlingen, Germany). Detection was at 270 nm.

2.3.1. Determination of Ro 40-7592 and Ro 40-7591 in plasma

The analytical column (125×4 mm) and the guard column (10×4 mm) were packed with Hypersil ODS, 5 μm (Grom, Herrenberg, Germany). The mobile phase consisted of a (550:450, v/v) mixture of methanol (containing 1.15 g of *N*-hexyl-

methylamine per litre) and 0.05 *M* sodium dihydrogenphosphate. The apparent pH of this mixture was adjusted to 2.1 with phosphoric acid before finally adding 50 ml of tetrahydrofuran.

At a flow-rate of 1 ml/min, the compounds eluted at approximately 7 min (Ro 40-7592), 9 min (Ro 40-7591) and 10.5 min (internal standard).

2.3.2. Determination of the metabolites Ro 61-1488 and Ro 47-1669 in plasma

The analytical column (125×4 mm) and the guard column (10×4 mm) were packed with Hypersil SAS 3 μm (Grom, Herrenberg, Germany). The mobile phase consisted of 0.05 *M* sodium dihydrogenphosphate (650 ml) and a (4:1, v/v) mixture of methanol and tetrahydrofuran (350 ml). The final mixture was adjusted to an apparent pH of 2.2 with phosphoric acid.

At a flow-rate of 1 ml/min, the compounds eluted at approximately 8 min (Ro 61-1448), 11 min (Ro 47-1669), and 32 min (internal standard).

2.3.3. Determination of Ro 40-7592 and its metabolites Ro 40-7591, Ro 61-1488 and Ro 47-1669 in urine

The analytical column (125×4 mm) and the guard column (10×4 mm) were packed with Spherisorb ODS-1, 3 μm (Grom, Herrenberg, Germany). The mobile phase consisted of 0.05 *M* sodium dihydrogenphosphate (700 ml), acetonitrile (170 ml) and tetrahydrofuran (130 ml). The final mixture was adjusted to pH 2.1 with phosphoric acid. The column was run at 25°C.

At a flow-rate of 1 ml/min, the compounds eluted at approximately 5 min (Ro 61-1448), 8 min (Ro 47-1669), 18 min (Ro 40-7592), 22 min (Ro 40-7591) and 27 min (internal standard).

2.4. Sample preparation procedures

2.4.1. Procedure for plasma samples

Plasma (0.2 ml) was mixed with a solution of the internal standard in acetonitrile (25 μl). Proteins were then precipitated by the addition of acetonitrile (0.3 ml). The samples were kept at 4°C (15 min) and centrifuged (5 min). An aliquot of the supernatant (100 μl) was mixed with 100 μl of a solution of 0.05 *M* sodium dihydrogenphosphate (adjusted to pH 2 with phosphoric acid) and injected for analysis (80 μl).

For the determination of the metabolites Ro 61-1488 and Ro 47-1669, an aliquot of the supernatant (100 μl) was mixed with a solution of diluted phosphoric acid (10%, 150 μl) and injected for analysis (100 μl).

2.4.2. Procedure for urine samples

To 25 μl of urine (taken from a thoroughly mixed and representative aliquot of the whole urine fraction) were added a solution of the internal standard (25 μl) and 0.2 ml of diluting solvent (175:25, v/v, mixture of 10% phosphoric acid and acetonitrile). Following vortex mixing and centrifuging, 50 μl of the mixture were injected for analysis.

2.5. Calibration

For each analytical series, five to six calibration standards covering the expected concentration range were worked up as described and run together with the unknowns. Internal standardisation was used. Calibration was performed by computing a weighted ($1/Y^2$) least-squares linear regression line of the measured peak height ratios (Y) (analyte to the internal standard), versus the spiked concentrations (X). The concentration of the unknown samples was calculated from this regression line. A special software package (RECAL) for the PE-Nelson 2600 data system was used for regression calculation.

3. Results

3.1. Linearity and limits of quantification

Correlation between peak height ratio and concentration was linear within the described concentration ranges. The limits of quantification of the plasma assays were 0.05 μg/ml for Ro 40-7592, Ro 40-7591, Ro 61-1448 and Ro 47-1669, respectively. The limits of quantification of the urine procedures were 0.2 μg/ml for each compound.

3.2. Precision

Inter-assay precision (C.V. (%)) of the analytical procedures was evaluated by analysing quality control samples at different concentrations on different days. For human plasma it was ≤6.0% for Ro 40-7592 and its metabolites (Table 1). For animal plasma, precision for Ro 40-7592 and Ro 40-7591 was in the same range (not presented). For the urine assay, precision normally was ≤8.0% (Table 2).

3.3. Recovery

Experiments were carried out to assess recoveries from human plasma. Peak heights, obtained from worked up spiked plasma samples, were compared to peak heights, obtained from worked up drug free plasma, to which the analytes had been added at the end of the sample preparation procedure. Mean recoveries from human plasma were in the range of 80–90% for Ro 40-7592 and its metabolites, and 100% for the internal standard (Table 3).

In addition, recoveries of Ro 40-7592 and Ro 40-7591 from animal plasma were determined relative to those from human plasma by spiking animal drug free plasma at defined drug concentrations and analysing them with calibration samples in human plasma. Similar recoveries were found for human,

Table 3
Recoveries from human plasma

Analyte	Conc. added (μg/ml)	Recovery (%)	Replicates (*N*)
Ro 40-7592	2.50	79	4
	0.25	77	4
Ro 40-7591	2.50	79	4
	0.25	84	4
Ro 61-1448	4.0	81	3
	0.2	87	3
Ro 47-1669	5.0	86	3
	0.25	93	3
Internal standard	2.5	100	10

Table 1
Inter-assay precision and accuracy of the procedure for human plasma (from replicate analysis of quality control samples)

Analyte	Conc. added (μg/ml)	Conc. found (μg/ml)	Precision C.V. (%)	Inaccuracy (%)	*N*
Ro 40-7592	1.00	1.11	4.0	+11.0	25
	0.20	0.21	5.9	+5.0	25
Ro 40-7591	1.00	1.11	3.2	+11.0	25
	0.20	0.20	5.9	±0.0	25
Ro 61-1448	2.40	2.28	3.2	−5.0	22
	0.20	0.18	4.9	−7.5	22
Ro 47-1669	3.00	3.08	3.2	+2.7	22
	0.25	0.25	4.8	±0.0	22

Table 2
Inter-assay precision and accuracy of the urine assay (from replicate analysis of quality control samples in human urine)

Analyte	Conc. added (μg/ml)	Conc. found (μg/ml)	Precision C.V. (%)	Inaccuracy (%)	*N*
Ro 40-7592	3.00	2.96	7.1	−1.3	18
	30.0	32.3	3.1	+7.7	18
Ro 40-7591	2.00	2.03	6.1	+1.6	18
	20.0	21.2	3.2	+6.0	18
Ro 61-1448	3.00	2.96	5.5	−1.2	18
	30.0	31.2	4.4	4.1	18
Ro 47-1669	4.00	3.99	6.4	−0.2	18
	40.0	42.0	4.8	+5.0	18

rat, dog, cynomolgus monkey and mouse plasma. Ro 61-1448 and Ro 47-1669 were not investigated.

Recoveries from urine were not investigated, as sample preparation was simply dilution.

3.4. Selectivity

The analytes were sufficiently separated from endogenous peaks arising in plasma and urine samples. Figs. 2–4 show representative chromatograms of blank plasma and urine samples as well as those after drug administration. Chromatograms from animal study samples were similar.

3.5. Stability

The stability of the compounds in plasma and urine was investigated. For this, plasma and urine were spiked at different concentrations. The spiked samples were stored at room temperature (24 h) and at −20°C (3–10.5 months) and were then analysed. With each set of stored samples a set of freshly prepared samples was analysed to provide the 100% values. The statistical interpretation of the data followed the procedure of Timm [4]. A concentration decrease of ≥15% after storage (zero not included in the calculated 90% confidence interval) was regarded as relevant. Ro 40-7592, Ro 40-7591, Ro 61-1448 and Ro 47-1669 were stable in human and animal plasma at room temperature for 24 h (Table 4) and at −20°C over a 3 month (Ro 61-1448 and Ro 47-1669) and a 10 month (Ro 40-7592 and Ro 40-7591) storage period (Table 5). The data are consistent with those previously reported for the parent drug [3].

In urine, the compounds were stable at room temperature for 24 h (Table 6). When stored at −20°C, Ro 40-7592 and Ro 40-7591 were stable over a 3 month storage period. Indications for instability (−15 to −20% decrease) were found after a 6 month storage period. Ro 61-1448 and Ro 47-

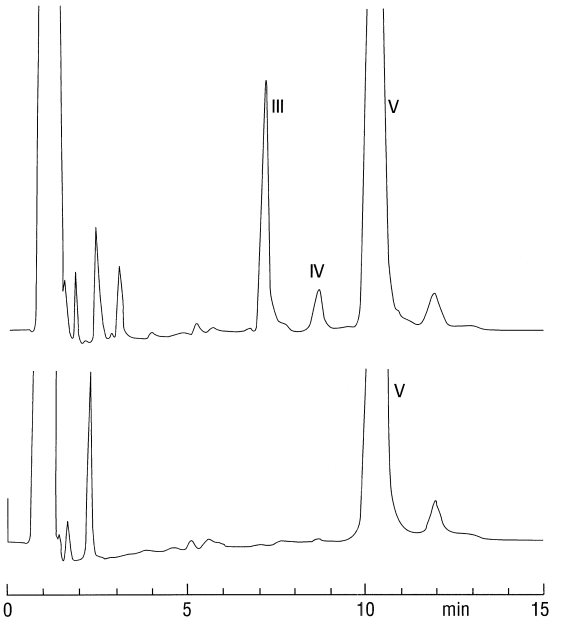

Fig. 2. Chromatograms of in vivo human plasma samples. (Assay for Ro 40-7592 and Ro 40-7591 in plasma); (above): post-dose; Ro 40-7592 (III): 0.97 μg/ml; Ro 40-7591 (IV): 0.18 μg/ml; (below): pre-dose (drug free, I.S. added (V)).

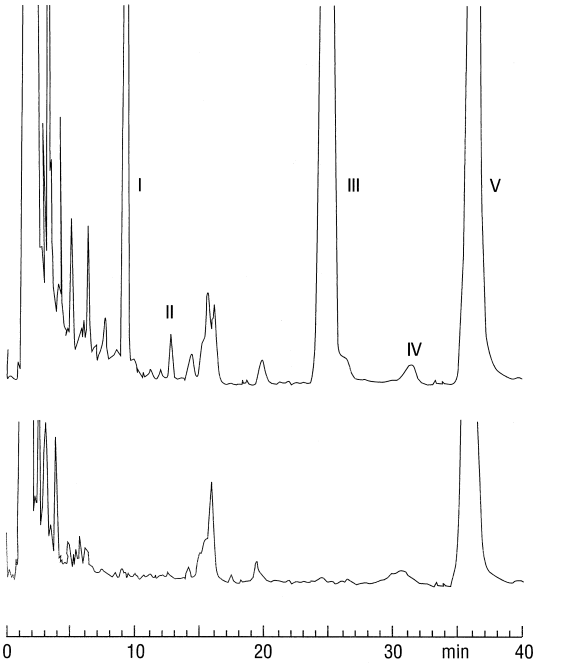

Fig. 3. Chromatograms of in vivo human plasma samples. (Procedure for Ro 61-1448 and Ro 47-1669 in plasma); (above): post-dose sample; Ro 61-1448 (I): 3.66 μg/ml; Ro 47-1669 (II): 0.12 μg/ml; III and IV denote the retention of (not quantified) Ro 40-7592 (III) and Ro 40- 7591 (IV); (below): pre-dose sample (drug free, I.S. added (V)).

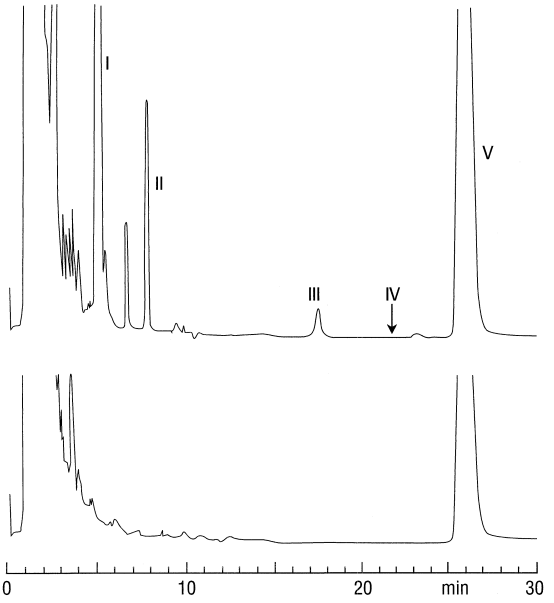

Fig. 4. Chromatograms of in vivo human urine samples; (above): post-dose sample; Ro 61-1448 (I): 118 μg/ml; Ro 47-1669 (II): 5.1 μg/ml; Ro 40-7592 (III): 0.8 μg/ml; Ro 40-7591 (IV) not found; (below): pre-dose sample (drug free, I.S. added (V)).

1669 were stable during 5 months storage (Table 7). It was concluded from these results, that urine samples should not be stored for more than 3 months at −20°C.

In addition, stability was investigated under the conditions of autosampler storage at room temperature. The compounds (internal standard included) were stable in the final injection solutions for at least 16 h.

When stored at 4–6°C in a refrigerator, the methanolic stock solutions of Ro 40-7592, Ro 40-7591 and Ro 47-1669 were stable for 12 months. Degradation (23%) was observed for Ro 61-1448.

4. Discussion

4.1. Procedures for plasma

The sensitivity of the protein precipitation method for Ro 40-7592 and Ro 40-7591 was sufficient for animal and human pharmacokinetic studies. Because of its greater simplicity, this method was favoured over the previously described extraction procedure [3]. Although it was generally possible, the determination of the parent drug was not included in the procedure for Ro 61-1448 and Ro 47-1669. The large run times would make its general use for parent drug determination (metabolites included) unfavourable. Attempts to reduce the run time failed within a reasonable time. A higher content of organic component in the mobile phase reduced retention times; however, the separation of Ro 61-1448 and Ro 47-1669 from endogenous matrix compounds then became critical. Analysis without internal standard reduced the run time to some extent, but led to worse precision. The procedure was therefore exclusively used for metabolite determinations in a limited number of studies.

4.2. Procedure for urine

As for the plasma procedure for Ro 61-1448 and Ro 47-1669, the internal standard eluted late under

Table 4
Stability in human plasma at room temperature (storage time: 24 h)

Analyte	Conc. added (μg/ml)	Conc. change after storage (%)	90% Confidence interval of the change (%)	Replicates (*N*)
Ro 40-7592	2.5	+7.6	+4.0 to +11.6	7
	0.25	+8.7	+2.0 to +17.4	7
Ro 40-7591	2.5	+5.8	+1.9 to +10.2	7
	0.25	+2.3	+2.3 to +11.1	7
Ro 61-1448	2.0	−5.3	−6.7 to −3.9	4
	0.20	+7.9	+2.2 to +14.0	4
Ro 47-1669	2.5	−4.4	−5.2 to +3.7	4
	0.25	+8.1	+4.7 to +11.6	4

Table 5
Stability in human plasma at −20°C

Analyte	Storage period (months)	Conc. added (μg/ml)	Conc. change after storage (%)	90% Confidence interval of the change (%)	Replicates (*N*)
Ro 40-7592	3	0.25	+12.7	+8.2 to +19.5	7
	10	1.0	+1.2	−5.6 to +8.6	6
	10	10.0	+0.8	−3.9 to +5.7	5
Ro 40-7591	3	0.25	+2.3	−2.3 to +11.l	7
	10	1.0	+9.0	+2.4 to +16.2	6
	10	5.0	+0.7	−4.2 to +5.8	5
Ro 61-1448	3	2.0	−4.0	−7.3 to −0.6	5
	3	0.20	−10.1	−14.8 to −5.1	5
Ro47-1669	3	2.5	−7.9	−11.3 to −4.3	5
	3	0.25	−5.3	−9.0 to −1.5	5

Table 6
Stability in urine at room temperature (storage time: 24 h)

Analyte	Conc. added (μg/ml) (%)	Conc. change after storage (%)	90% Confidence interval of the change	Replicates (*N*)
Ro 40-7592	60.0	−5.1	−5.4 to −4.7	3
	0.60	+1.0	−2.4 to +4.6	3
Ro 40-7591	40.0	−4.8	−5.2 to −4.5	3
	0.40	+1.9	−0.9 to +4.8	3
Ro 61-1448	100	−4.7	−6.9 to −2.6	3
	1.00	+2.1	−2.0 to +6.4	3
Ro 47-1669	80.0	−4.1	−6.0 to −2.2	3
	0.80	−1.0	−3.6 to +1.6	3

Table 7
Stability in human urine at −20°C

Analyte	Storage period (months)	Conc. added (μg/ml)	Conc. change after storage (%)	90% Confidence interval of the change (%)	Replicates (*N*)
Ro 40-7592	3	7.5	−8.5	−9.9 to −7.0	6
	3	2.5	+3.3	0.9 to 5.9	6
	6	7.5	−16.6	−18.8 to −14.5	6
	6	2.5	−6.2	−9.7 to −2.6	6
Ro 40-7591	3	7.5	−6.8	−8.4 to −5.2	6
	3	2.5	+3.3	15 to 10.1	6
	6	7.5	−15.6	−17.7 to 13.4	6
	6	2.5	−18.5	−23.0 to −13.7	6
Ro 61-1448	5	30.0	−8.5	−14.2 to −2.5	5
		3.0	−9.9	−13.4 to −6.3	5
Ro 47-1669	5	30.0	+7.0	1.1 to 13.1	5
		3.0	+3.0	−0.9 to +7.1	5

the conditions of the urine procedure. Again, without internal standardisation, precision was worse. The exclusive determination of the parent drug and its metabolite Ro 40-7591 in urine could be achieved with shorter run times under the chromatographic conditions of the corresponding plasma procedure.

Thorough homogenisation of the urine sample prior to taking the sample aliquot was necessary to obtain a satisfactory assay precision.

5. Application

The procedure for Ro 40-7592 and Ro 40-5791 in plasma was applied successfully to the analysis of samples from animal pharmacokinetic (rat, dog, mouse, cynomolgus monkey) and most clinical pharmacology studies. The procedure for Ro 61-1448 and Ro 47-1669 in plasma and the urine assay were applied to sample analysis from a few therapeutic studies, only.

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