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DETERMINATION OF RIMANTADINE AND ITS HYDROXYLATED METABOLITES IN HUMAN PLASMA AND URINE

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SUMMARY

A gas chromatographic–mass spectrometric procedure has been developed for the quantitation of the antiviral agent rimantadine and its *meta-* and *para*-hydroxylated metabolites in human plasma and urine. The assay utilizes an extractive pentafluorobenzoylation at alkaline pH with cyclohexane saturated with triethanolamine–chloroform (2:1) containing pentafluorobenzoyl chloride, selective ion monitoring, methane negative ion chemical ionization mass spectrometry and stable isotope dilution. The method has been used to measure plasma concentrations of rimantadine, *m*-hydroxyrimantadine and the two epimers of *p*-hydroxyrimantadine between 5–250, 5–100 and 2.5–50 ng/ml, respectively. Similarly, the urine concentrations of these analytes measured were between 25–1250, 25–500 and 12.5–250 ng/ml, respectively.

INTRODUCTION

Rimantadine[a] is a chemotherapeutic agent used for the prophylaxis and treatment of influenza A viral infections [1]. The structure of rimantadine and its *meta-* and *para*-hydroxylated metabolites are shown in Fig. 1.

Gas chromatography–electron-capture detection [2] and gas chromatography–mass spectrometry (GC–MS) [3] methods for the analysis of only rimantadine in plasma and urine have been reported previously. Recently, we

[a]The proper nomenclature (IUPAC name) for all compounds listed in this paper is as follows: rimantadine, α-methyltricyclo[3.3.1.1^{3,7}]decan-1-β-methanamine; *m*-hydroxyrimantadine, 3α-(1-aminoethyl)tricyclo[3.3.1.1^{3,7}]decan-1β-ol; *p*-hydroxyrimantadine (equatorial epimer), 1β-(1-aminoethyl)tricyclo[3.3.1.1^{3,7}]decan-4α-ol; *p*-hydroxyrimantadine (axial epimer), 1β-(1-aminoethyl)tricyclo[3.3.1.1^{3,7}]decan-4β-ol.
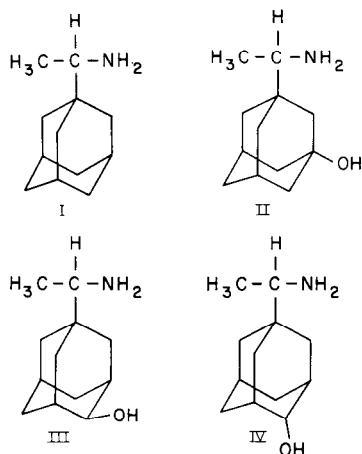
Fig. 1. Structures of rimantadine (I), *m*-hydroxyrimantadine (II) and the equatorial (III) and axial (IV) epimers of *p*-hydroxyrimantadine.

reported the urinary metabolites of rimantadine as conjugated rimantadine, conjugated and free *m*-hydroxyrimantadine and conjugated and free *p*-hydroxyrimantadine (both epimers) [4]. In this paper, we report an assay to quantitate rimantadine and its hydroxylated metabolites using GC–negative ion chemical ionization (NICI) MS.

EXPERIMENTAL

Materials

Rimantadine hydrochloride was obtained from the Quality Control Department of Hoffmann-La Roche. Both *m*-hydroxy- and *p*-hydroxyrimantadine were synthesized by Dr. P. Manchand of Hoffmann-La Roche. [^{2}H$_4$]Rimantadine hydrochloride was synthesized by Dr. A. Liebman of the Isotope Synthesis Group at Hoffmann-La Roche. Both ^{2}H$_4$ stable isotope analogues of *m*-hydroxy- and *p*-hydroxyrimantadine were synthesized by Dr. S. Choudhry of the Isotope Synthesis Group at Hoffmann-La Roche. For all deuterated compounds, the deuteriums replaced the three hydrogens located on the side-chain methyl and one hydrogen on the carbon attached to the adamantane ring.

All reagents were of analytical or UV grade. Culture tubes (15 ml; Pyrex 9826) with Teflon®-lined screw caps were treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.) if new. Disposable borosilicate culture tubes (Fisher Scientific, Fairlawn, NJ, U.S.A.; 100×16 and 150×16 mm) were used as is. All glassware was rinsed with methanol before use.

The following solvent reagents were prepared.

Triethanolamine (TEA)–cyclohexane–chloroform reagent. TEA (0.5 ml) was

mixed with 500 ml of cyclohexane for 90 min. After allowing the mixture to stand at room temperature overnight, two volumes of this solution were combined with one volume of chloroform.

2% Pentafluorobenzoyl chloride (PFBCl). Neat PFBCl (100 μl) was diluted with 5 ml of cyclohexane.

Stock and calibration solutions

Stock solutions of each analyte (rimantadine, *m*-hydroxyrimantadine and *p*-hydroxyrimantadine) and each of their respective reference standards were prepared separately at a concentration of 1 mg/ml in methanol. The appropriate weighing correction was made for any compound which is a hydrochloride salt. All concentrations stated herein refer to the free-base equivalent, not the hydrochloride salt.

Three intermediate analyte stock solutions were prepared at a concentration of 2000 ng per 50 μl of methanol. These stock solutions were used to prepare combination spiking solutions (in methanol) shown in Table I.

Plasma samples

Blood was drawn into Vacutainer® tubes (Becton-Dickinson, Type 6527) containing heparin as the anticoagulant. The tubes were then centrifuged, and the plasma was transferred into siliconized glass scintillation vials. The samples were stored at $-20\,°C$ until analyzed.

The experimental plasma analyzed were from subjects dosed according to protocol N3069B (A. Dunton, investigator) and N3028A (M. Izard, investigator).

TABLE I

COMBINATION CALIBRATION SPIKING SOLUTIONS

Solution	Components[a]	Concentration of each component (ng per 50 μl)
1	A	250
	B, C	100
2	A	100
	B, C	50
3	A	50
	B, C	20
4	A	25
	B, C	10
5	A, B, C	5
6	D	50
	E, F	100

[a]A = rimantadine; B = *m*-hydroxyrimantadine; C = *p*-hydroxyrimantadine; D = [^{2}H$_4$]rimantadine; E = [^{2}H$_4$]*m*-hydroxyrimantadine; F = [^{2}H$_4$]*p*-hydroxyrimantadine.

Low and medium quality assurance (QA) samples were prepared by fortifying drug-free plasma with rimantadine, *m*-hydroxy- and *p*-hydroxyrimantadine to concentrations of 100, 50 and 50 ng/ml (medium QA) and 25, 10 and 10 ng/ml (low QA), respectively. Enough QA sample was prepared so that duplicate 1.0-ml aliquots could be analyzed along with each set of experimental samples over the course of all the analyses. The QA samples were also used to determine long-term stability of the drug in plasma and to check the reproducibility of the assay. Both QA samples were stored at $-20°C$.

Urine samples

Urine was collected in polypropylene containers and the volume was measured. An aliquot of urine was transferred to a glass scintillation vial and stored at $-20°C$ until analysed.

Low and medium QA samples were prepared by fortifying drug-free urine with rimantadine, *m*-hydroxy- and *p*-hydroxyrimantadine to concentrations of 500, 250 and 250 ng/ml (low QA) and 1000, 1000 and 1000 ng/ml (high QA), respectively. Enough QA sample was prepared so that duplicate 0.2-ml aliquots could be analyzed along with each set of experimental samples over the course of all the analyses. These QA samples were also used to determine long-term stability of the analytes in urine and to check the reproducibility of the assay. Both QA samples were stored at $-20°C$.

Calibration standards

Five calibration standards and both QA samples were analyzed in duplicate along with each set of experimental samples. The calibration standards were prepared by fortifying 1.0 ml of drug-free plasma or 0.2 ml of drug-free urine with 50 μl of either solution 1, 2, 3, 4 or 5 (Table I). All samples were also fortified with 50 μl of solution 6 (Table I).

Extraction/derivatization method

The drug-free, QA or experimental plasma sample was thawed and 1.0 ml was transferred into a 15-ml culture tube. The drug-free plasma samples used for calibration standards were fortified as described in the previous section. The appropriate reference standards (solution 6, Table I) were added to all samples and the samples were vortex-mixed for 10 s. After adding 1.0 ml of 0.1 *M* sodium hydroxide, the samples were mixed again. A 4-ml volume of the TEA–cyclohexane–chloroform reagent and 50 μl of the 2% PFBCl reagent were added to each tube. The tubes were stoppered and shaken at ~ 30 strokes/min for 20 min. Then, the samples were centrifuged at 1500 *g* for 10 min. Finally, the organic layer was transferred to another tube and the solvent was evaporated under a stream of dry nitrogen. The extracts were reconstituted in 300 μl of toluene.

For urine samples, the same procedure was followed using an 0.2-ml aliquot of urine.

Instrumentation

Gas chromatograph. A Delsi gas chromatograph (Delsi-Nermag, Houston, TX, U.S.A.) was equipped with a DB-5 capillary column (J & W, Folsom, CA, U.S.A.; 30 m × 0.32 mm I.D., film thickness of 0.25 μm). The injection was split after 30 s. The column oven was programmed from 175 to 270°C at 25°C/min, with a 1-min cooling period and a 1-min temperature stabilizing period prior to the following injection. Helium was used as the carrier gas at a pressure of about 1.25 bar. The injector and transfer line temperatures were both set at 270°C. Under these conditions, the retention times for rimantadine, *m*-hydroxyrimantadine, *p*-hydroxyrimantadine (equatorial epimer) and *p*-hydroxyrimantadine (axial epimer) were about 7.0, 8.3, 9.1 and 9.3 min, respectively; this allowed reasonable separation of the two *p*-hydroxyrimantadine epimers.

Mass spectrometer. A Nermag R10-10C mass spectrometer (Delsi-Nermag) was tuned to give maximum response consistent with reasonable ion peak shape and near unit resolution. Methane was used as the reagent gas for NICI. The ions used for quantitation were m/z 353 (the $[M-HF]^-$ ion from rimantadine), m/z 369 (the $[M-HF]^-$ ion from *m*- and *p*-hydroxyrimantadine), m/z 356 (the corresponding $[M-{}^2HF]^-$ ion from [${}^2H_4$]rimantadine) and m/z 372 (the corresponding $[M-{}^2HF]^-$ ion from [${}^2H_4$]*m*- and *-p*-hydroxyrimantadine).

Data collection and analysis. The SIDAR (V.3.0) data system was used to acquire selected ion monitoring data. QSIMPS (quantitative selective ion monitoring processing system) was used to calculate concentrations and print out final reports [5].

Recovery experiments

The percentage recovery of rimantadine and its hydroxylated metabolites from plasma was determined by the following method. Six aliquots of 1.0 ml drug-free plasma were prepared and each sample was fortified with rimantadine, *m*-hydroxyrimantadine and *p*-hydroxyrimantadine to give a concentration of 50 ng/ml each. All six samples were fortified with the appropriate ${}^2H_4$-labelled reference standards at concentrations of 50, 100 and 100 ng/ml, respectively. Six external samples were prepared at the same concentrations and reacted with 2% PFBCl. The six fortified plasma samples were extracted and derivatized using the method described in a previous section.

The percentage recovery of rimantadine and its hydroxylated metabolites from urine was determined in the same manner.

All samples were analyzed using GC–MS, and the recovery was calculated by comparing the mean concentrations for the extracted samples to those of the external standards.

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Stability experiments

The benchtop stability of the metabolites of rimantadine in human plasma was determined by comparing the concentrations of three sets of experimental samples allowed to stand at room temperature for 0, 3 and 6 h. Each set included six medium QA samples stored at $-20°C$. The long-term stability at $-20°C$ was determined by duplicate determinations of the same QA sample over a period of three months.

Similar experiments were carried out using fortified (medium QA) urine samples.

RESULTS AND DISCUSSION

Fig. 2 shows the methane NICI selected ion chromatograms for m/z 353, 356, 369 and 372 from an extracted plasma calibration standard fortified with rimantadine and its two hydroxylated metabolites. This standard contained 250 ng of rimantadine, 100 ng of m-hydroxyrimantadine and 100 ng of p-hydroxyrimantadine as well as 50, 100 and 100 ng of the respective $^2H_4$-labelled reference standards. The first eluting peak is derivatized rimantadine, followed by derivatized m-hydroxyrimantadine and the two derivatized p-hydroxy epimers (equatorial and axial, respectively).

Fig. 3 shows the methane NICI mass spectrum of all analytes. The base peak
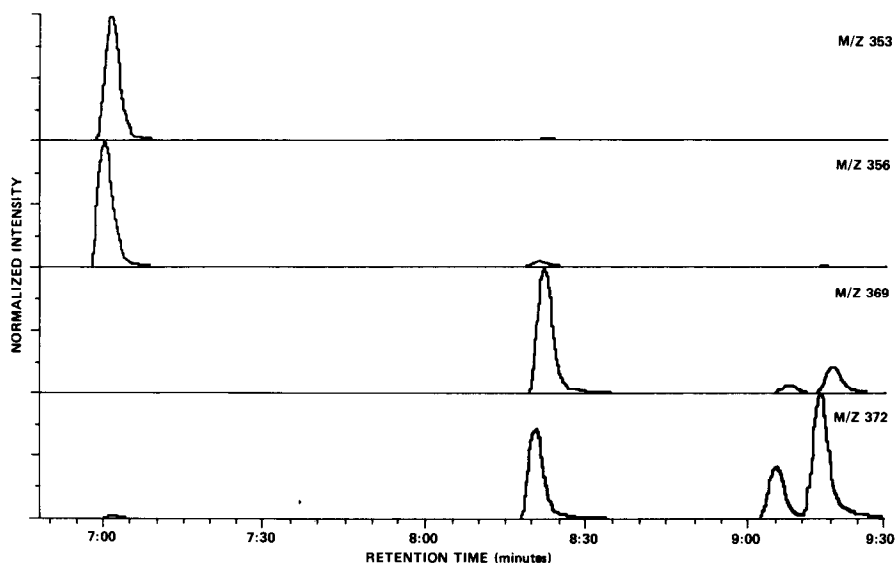

Fig. 2. Selected ion current profiles from a derivatized extract of a 1.0-ml drug-free plasma standard fortified with 250, 100 and 100 ng of rimantadine and its two hydroxylated metabolites. This sample was also fortified with 50, 100 and 100 ng of the respective $^2H_4$-labelled reference standards.
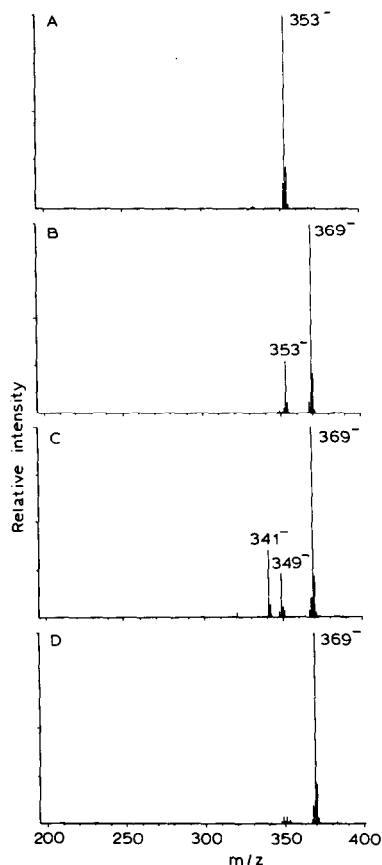
Fig. 3. NICI mass spectra of the pentafluorobenzoyl derivatives of (A) rimantadine, (B) *m*-hydroxyrimantadine, (C) *p*-hydroxyrimantadine (equatorial epimer) and (D) *p*-hydroxyrimantadine (axial epimer).

for pentafluorobenzoyl rimantadine is m/z 353 which is the $[M-HF]^-$ ion. Similarly, the pentafluorobenzoylhydroxylated metabolites of rimantadine lose HF under methane NICI conditions, giving base peaks at m/z 369.

Fig. 4 shows selected ion current profiles from an extract of a 6-h post-dose 1.0-ml plasma sample from a subject given a 200-mg oral dose of rimantadine hydrochloride. The GC conditions for the analysis of this sample were modified from the conditions described in the Experimental section, so that a shorter run time could be achieved. The concentrations of rimantadine, *m*-hydroxyrimantadine, *p*-hydroxyrimantadine (equatorial epimer) and *p*-hydroxyrimantadine (axial epimer) measured were 230, 17, 48 and 13 ng/ml. No interferences from other endogenous plasma components were observed.

With each set experimental samples, calibration and both QA samples were analyzed in duplicate. The overall inter- and intra-assay precisions obtained
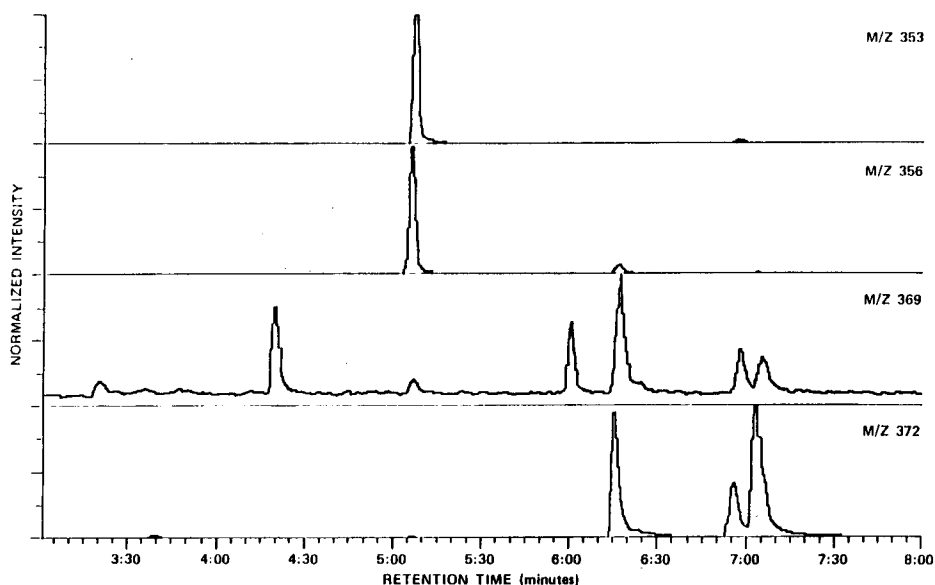
Fig. 4. Selected ion current profiles from an extract of a 6-h post-dose plasma sample taken from a healthy subject given a 200-mg dose of rimantadine hydrochloride.

from the analysis of plasma calibration curves used in the assay of a few hundred samples were 1.2 and 4.4% for rimantadine, 3.3 and 4.6% for m-hydroxyrimantadine, 4.3 and 5.2% for p-hydroxyrimantadine (equatorial epimer) and 1.6 and 4.8% for p-hydroxyrimantadine (axial epimer). Similar results were obtained from the analysis of urine calibration curves. The inter-assay precision was estimated from the day-to-day reproducibility of the back-calculated concentration values of the calibration curve standards. The intra-assay precision was estimated from the ratio of the duplicate analyses, including the QA samples. At the lower limit of quantitation, the inter-assay and intra-assay precision values were 3.8 and 12.6% for rimantadine, 7.1 and 2.9% for m-hydroxyrimantadine, 14.5 and 9.2% for p-hydroxyrimantadine (equatorial epimer) and 3.2 and 10% for p-hydroxyrimantadine (axial epimer).

The extraction recovery for rimantadine from 1.0 ml of human plasma and 0.2 ml of human urine was 87 and 90%, respectively. For m-hydroxyrimantadine, the recovery was 86% from plasma and 92% from urine. For p-hydroxyrimantadine the recoveries for the equatorial and axial epimers were 81 and 85% from human plasma and 90 and 93% from human urine, respectively.

Rimantadine was found to be stable in human plasma and urine on the benchtop for at least 6 h [3]. The results of benchtop stability experiments with m-hydroxy- and p-hydroxyrimantadine in human plasma and urine are shown in Tables II and III, respectively. These data demonstrated that both analytes are stable for at least 6 h.

TABLE II

BENCHTOP STABILITY OF m-HYDROXYRIMANTADINE AND p-HYDROXYRIMAN-
TADINE IN HUMAN PLASMA

The values in parentheses are relative standard deviations based on six determinations.

Time (h)	Concentration (mean ± S.D.) (ng/ml)		
	m-Hydroxyrimantadine	p-Hydroxyrimantadine	
		Equatorial epimer	Axial epimer
0	50.9 ± 2.2 (4.4)	25.2 ± 1.5 (6.0)	24.1 ± 1.3 (5.6)
3	51.0 ± 1.2 (2.4)	26.3 ± 1.7 (6.4)	24.1 ± 1.1 (4.8)
6	49.5 ± 1.5 (3.0)	26.2 ± 1.1 (4.1)	24.2 ± 1.0 (4.1)

TABLE III

BENCHTOP STABILITY OF m-HYDROXYRIMANTADINE AND p-HYDROXYRIMAN-
TADINE IN HUMAN URINE

The values in parentheses are relative standard deviations based on six determinations.

Time (h)	Concentration (mean ± S.D.) (ng/ml)		
	m-Hydroxyrimantadine	p-Hydroxyrimantadine	
		Equatorial epimer	Axial epimer
0	218.8 ± 6.5 (3.0)	130.8 ± 7.7 (5.9)	134.5 ± 5.5 (4.1)
3	215.6 ± 8.8 (4.1)	132.2 ± 5.4 (4.1)	132.9 ± 5.2 (4.0)
6	217.7 ± 10.5 (4.8)	124.4 ± 6.0 (4.8)	135.1 ± 4.8 (3.5)

Long-term stability of rimantadine in plasma and urine at $-20\,^{\circ}$C has been previously reported [3]; those results demonstrated stability (concentration change $<20\%$) over a four-month period in plasma and a two-month period in urine. Similarly, the results of long-term stability experiment with m-hydroxy- and p-hydroxyrimantadine in human plasma and urine at $-20\,^{\circ}$C are shown in Tables IV and V. These results demonstrate the long-term stability of all metabolites in plasma (concentration change $<20\%$) over a one-month period (except for the axial epimer of p-hydroxyrimantadine) and in urine over a three-month period.

Fig. 5 shows the concentration–time profiles of rimantadine and its hydroxylated metabolites in the plasma of a healthy subject given a single oral 200-mg dose of rimantadine. All metabolites show similar time–concentration profiles, with the equatorial epimer of p-hydroxyrimantadine having the highest concentration in plasma relative to the other metabolites.

TABLE IV

LONG-TERM STABILITY OF METABOLITES OF RIMANTADINE IN HUMAN FORTI-
FIED PLASMA SAMPLES

The values in parentheses are relative standard deviations.

Assay date	Concentration (mean ± S.D.) (ng/ml)			n
	m-Hydroxyrimantadine	p-Hydroxyrimantadine		
		Equatorial epimer	Axial epimer	
Low QA samples				
1/89	9.7 ± 0.4 (4.5)	5.1 ± 0.3 (6.2)	5.0 ± 0.2 (4.0)	6
2/89	9.6 ± 0.4 (4.4)	5.0 ± 0.1 (1.7)	–	2
High QA samples				
1/89	50.6 ± 2.0 (3.9)	24.1 ± 1.2 (4.9)	24.3 ± 0.2 (4.0)	6
2/89	49.3 ± 0.8 (1.6)	26.5 ± 0.6 (2.4)	–	2

TABLE V

LONG-TERM STABILITY OF THE METABOLITES OF RIMANTADINE IN HUMAN
FORTIFIED URINE SAMPLES

The values in parentheses are relative standard deviations.

| Assay date	Concentration (mean ± S.D.) (ng/ml)			n
	m-Hydroxyrimantadine	p-Hydroxyrimantadine		
		Equatorial epimer	Axial epimer	
Low AQ samples				
10/88	244.6 ± 4.8 (2.0)	122.7 ± 10.7 (8.7)	124.9 ± 4.0 (3.2)	6
11/88	227.2 ± 6.3 (2.8)	119.2 ± 8.5 (7.1)	119.4 ± 4.0 (3.3)	2
12/88	244.5 ± 5.5 (2.2)	122.0 ± 5.7 (4.6)	126.1 ± 10.9 (8.6)	4
1/89	235.0 ± 11.0 (4.7)	120.5 ± 5.4 (4.5)	120.2 ± 1.1 (0.9)	4
High QA samples				
10/88	1007 ± 11 (1.1)	491.1 ± 15.5 (3.2)	509.8 ± 19.3 (3.8)	6
11/88	1016 ± 52 (5.1)	461.6 ± 7.7 (1.7)	538.4 ± 23.7 (4.4)	2
12/88	1045 ± 38 (3.6)	490.6 ± 4.4 (0.9)	517.0 ± 19.3 (3.7)	4
1/89	1017 ± 2 (0.2)	523.8 ± 15.5 (3.0)	525.7 ± 17.9 (3.4)	4
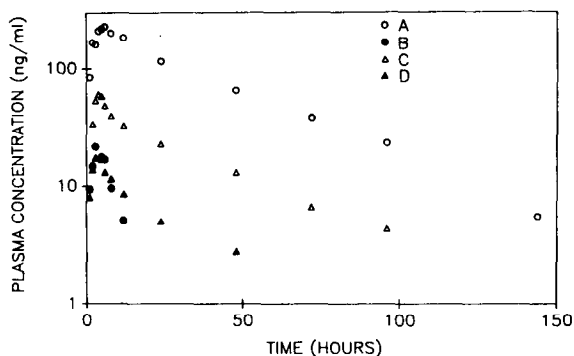
Fig. 5. Plasma concentration-time profile of rimantadine (A), *m*-hydroxyrimantadine (B), *p*-hydroxyrimantadine (equatorial epimer) (C) and *p*-hydroxyrimantadine (axial epimer) (D) for a healthy subject given a single oral 200-mg dose of rimantadine.

CONCLUSIONS

A GC–MS procedure has been developed for the quantitation of rimantadine and its hydroxylated metabolites in human plasma and urine. This assay represents an improvement over previous assays in that the metabolites are quantitated along with the parent drug.

Additionally, experiments have been carried out to determine the percentage recovery of all analytes from plasma and urine as well as their benchtop and long-term stability in both biological matrices.

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