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QUANTITATION OF THE ENANTIOMERS OF RIMANTADINE IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

BARBARA J. MIWA, NADIA CHOMA, SHARON Y. BROWN, NANCY KEIGHER, WILLIAM A. GARLAND and ELAINE K. FUKUDA*

Department of Drug Metabolism, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

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SUMMARY

A gas chromatographic–mass spectrometric procedure has been developed for the quantitation in plasma and urine of the enantiomers of rimantadine, an antiviral drug effective against type A influenza. The assay utilizes derivatization with an optically active reagent, selective ion monitoring, methane negative-ion chemical ionization (NICI) mass spectrometry and stable isotope dilution. The method has been used to measure concentrations of each rimantadine enantiomer over a range of 2.5–250 and 12.5–1250 ng/ml in the plasma and urine, respectively, of four male volunteers administered rimantadine. In plasma and urine, no differences were observed in the disposition of the unconjugated enantiomers. In urine, one enantiomer, but not both, was released following enzymatic hydrolysis.

INTRODUCTION

It is well recognized [1,2] that stereochemical considerations are significant factors in the disposition of drugs. Drug effects, toxic or efficacious, are influenced by the stereochemistry of drug absorption, distribution, metabolism and excretion. The reason for this is that the binding of a drug molecule to an optically active receptor or metabolizing enzyme results in the formation of diastereometric complexes with different chemical and physical properties. As a result, the rate or the nature of the reaction at the receptor or metabolizing enzyme might be different for the different enantiomers. In spite of this, most drugs having chiral centers are administered as racemates with no consideration given to potential differences in the efficacy and/or toxicity of the enantiomers.

This paper reports a gas chromatographic–mass spectrometric (GC–MS) assay which was developed to determine whether there are differences in the pharmacokinetics and metabolism of the enantiomers of rimantadine, an antiviral

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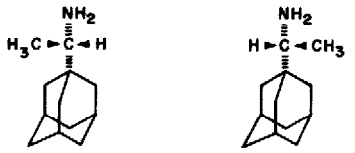

S-RIMANTADINE R-RIMANTADINE

Fig. 1. Structures of *R*-rimantadine and *S*-rimantadine.

drug effective against type A influenza. Rimantadine has a chiral center and is clinically administered as a racemate (Fig. 1). The method utilizes solid-phase extraction, derivatization with an optically active reagent to form diastereomers, methane negative-ion chemical ionization (NICI) MS, selective ion monitoring and stable isotope dilution. The optically active reagent, (+)-α-methyl-α-methoxy (pentafluorophenyl) acetic acid (MMPA) [3], imparts NICI sensitivity to rimantadine.

EXPERIMENTAL

Materials

Rimantadine hydrochloride was obtained from the Quality Control Department of Hoffmann-La Roche. Tetradeuterated rimantadine hydrochloride (deuteriums replacing the methyl hydrogens and the hydrogen attached at the chiral center) was synthesized by Dr. R. Muccino of the Isotope Synthesis Group at Hoffmann-La Roche. All reagents were of analytical grade (Fisher Scientific, Fairlawn, NJ, U.S.A.) except methanol (Burdick and Jackson, Muskegon, IL, U.S.A.) which was of UV grade. Sodium hydroxide was obtained from Fisher Scientific and anhydrous dibasic sodium phosphate was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). 1,3-Dicyclohexylcarbodiimide (DCC) was purchased from Aldrich (Milwaukee, WI, U.S.A.) as was 1-hydroxybenzotriazole hydrate (HOBT). The quinine salt of MMPA [3] was a gift from Dr. W.F. Trager of the University of Washington. Bond-Elut® CN columns (Part No. 624203, 200 mg cyanopropyl packing, 2.8 ml capacity) were supplied by Analytichem International (Harbor City, CA, U.S.A.). The disposable borosilicate culture tubes (12×75 mm) were obtained from Fisher Scientific.

Extraction and derivatization method

The extraction procedure has been described [4]. The derivatization procedure was based on the well established use of HOBT and DCC as catalysts to couple amino acids or peptides without racemization [5]. The MMPA reagent solution was prepared as follows: 3.65 mg of the MMPA quinine salt were dissolved in 0.5 ml of 0.1 *M* hydrochloric acid and 0.5 ml of water. The mixture was shaken with 1 ml of dichloromethane, centrifuged, and the dichloromethane phase was transferred to another tube. The DCC reagent solution was prepared by dissolving 1.27 mg in 1 ml of dichloromethane. The HOBT reagent was prepared by dissolving 0.94 mg in 1 ml of dimethylformamide.

Sufficient amounts of the MMPA and HOBT reagent solutions to analyze the available samples were combined, vortexed and left at room temperature for 5 min. A 50-μl volume of the MMPA–HOBT reagent solution was added to each extract, and the tubes were vortexed. A 25-μl volume of the DCC reagent solution was added to each tube; the tubes were capped tightly, placed in a shaker (20 strokes/min) and allowed to react for 1 h at room temperature.

To remove excess reagents, a base extraction was carried out. A 0.5-ml volume of 0.1 *M* sodium hydroxide and 2 ml of dichloromethane were added to each tube which was vortexed for about 10 s and centrifuged. The aqueous phase was aspirated, and the dichloromethane layer was transferred to another tube. This solution was evaporated under dry nitrogen, the residue was redissolved in 50 μl of ethyl acetate and the reconstituted residue was analyzed by GC–MS.

Glusulase® treatment of urine samples

To hydrolyze conjugated rimantadine, a 5-ml aliquot of urine was adjusted to pH 5.5 with either 2 *M* hydrochloric acid or 2 *M* sodium hydroxide, and the solution was incubated for 18 h at 37°C with 50 μl of Glusulase (DuPont Pharmaceuticals, Wilmington, DE, U.S.A.).

Calibration standards

The stock solution was prepared by dissolving 1 mg of the free base equivalent of rimantadine hydrochloride in 1 ml of methanol. An intermediate stock solution containing 2000 ng per 50 μl of rimantadine was prepared by diluting 1.0 ml of the stock solution described above with 25.0 ml of methanol. This intermediate stock solution was used to prepare calibration solutions containing 500, 200, 50, 20 and 5 ng of rimantadine per 50 μl of methanol. Five calibration standards and a quality assurance sample (prepared by combining previously analyzed plasma or urine samples) were analyzed in duplicate along with each set of experimental samples. The calibration standards contained 5, 20, 50, 200 and 500 ng of racemic rimantadine in either 1 ml of drug-free plasma or in 0.2 ml of drug-free urine.

Instrumentation

Gas chromatograph. A Carlo Erba Model 4200 gas chromatograph (Kratos Analytical, Ramsey, NJ, U.S.A.) was interfaced to a Kratos MS-50 mass spectrometer (described below) and equipped with a DB-1 capillary column (J & W, Folsom, CA, U.S.A.) (30 m×0.32 mm I.D., film thickness of 0.25 μm). The column oven was programmed from 164 to 264°C at 10°C/min with a 1-min cooling period and a 1-min temperature stabilizing period before the next injection. Hydrogen was used as the carrier gas at a pressure of about 5 kPa. The injector and transfer line temperature were set to 300 and 190°C, respectively.

Similarly, a VISTA 6000 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) was interfaced to a Nermag R10-10C mass spectrometer and used in an identical fashion.

GC–MS and data acquisition. Full mass spectra were acquired using a Nermag R10-10C mass spectrometer. All quantitative work was carried out using a Kratos MS-50 magnetic sector mass spectrometer (Kratos Analytical) tuned to give the

maximum response consistent with reasonable ion peak shape and a resolution of about 5000. Methane was used as the NICI reagent gas. The unlabelled and deuterium-labelled ions were monitored by a Vacuum Generators (Altrincham, U.K.) multiple ion detection system. The actual ions monitored were m/z 379 ($[M-HF-CH_3OH]^-$) from the unlabelled analyte and the corresponding fragment ion at m/z 383 from tetradeuterated rimantadine. These ions were monitored relative to an external lock mass of $C_8F_{14}N^-$ (m/z 376) from perfluorotributylamine.

QSIMPS (quantitative selective ion monitoring processing system) was used to control the autosampler and the GC–MS divert valve and to collect and process the selected ion current profile data [6].

Peak heights for m/z 379 and m/z 383 were calculated by fitting the top 80% of the peak profile to the EMG (extended modified gaussian) peak model [6], and then by using the parameters from the fit along with the ion intensity data to generate the value. The baseline was obtained by linear extrapolation between the average intensity value for ten scans before and ten scans after the peak.

The ratio of m/z 379 to m/z 383 and the concentration value from the duplicate analysis of the calibration standards were fitted, using weighted ($1/R^2$) non-linear regression, to the equation $R=(a+x)/(bx+c)$. In this equation, R is the ion ratio of m/z 379 to m/z 383, x is the analyte concentration and a, b and c are parameters adjusted to give the best fit to the calibration data [7]. For this study, the value of b was small, i.e., the calibration curve was virtually linear. Given an ion ratio from an experimental sample and the calculated values for a, b and c, a value of x can easily be calculated.

Biological samples

The plasma and urine samples analyzed were selected from a larger study on the disposition of rimantadine in normal male volunteers [8]. The ages of subjects 1–4 were 33, 19, 26 and 24 years, respectively. The weights of subjects 1–4 were 62, 82, 95 and 92 kg, respectively. Each subject was given two capsules each containing 100 mg of rimantadine hydrochloride and 25 μCi of [^{14}C]rimantadine.

Blood samples (7 ml) were collected at specified times using a Model 6527 Vacutainer (Becton-Dickinson, Rutherford, NJ, U.S.A.) and the plasma samples were harvested following centrifugation at 4°C. The plasma samples were stored at −20°C for several months, analyzed for rimantadine using a published procedure [4] and, after another several months of storage at −20°C, analyzed using the procedure reported in this paper. Values for the sum of the individual enantiomers were all within 20% of the values measured by the first assay.

Pharmacokinetic analysis

Plasma concentration–time data for each subject were fit to a one-compartment open model with first-order absorption and elimination [9] using the NONLIN package available with QSIMPS [6].

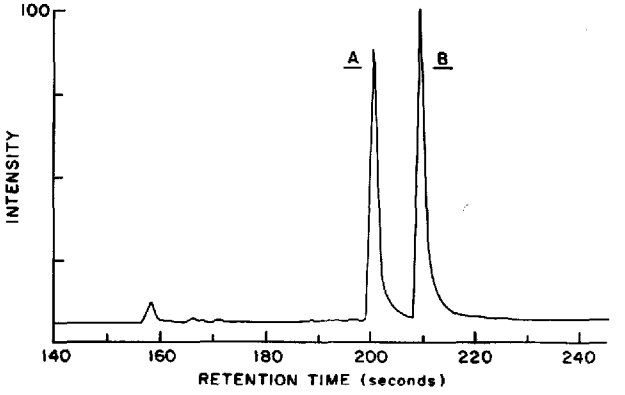

Fig. 2. Total ion current chromatogram from derivatized rimantadine. The mass spectrometer was set to scan from m/z 100 to m/z 500 with the GC temperature programmed in a manner similar to that described in the experimental section.

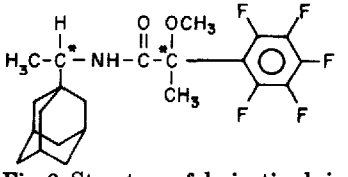

Fig. 3. Structure of derivatized rimantadine.

RESULTS AND DISCUSSION

Fig. 2 shows the total ion current chromatogram from the analysis of derivatized rimantadine (shown in Fig. 3 with the asterisks denoting the two chiral centers) by GC–MS. The peaks labelled A and B are the two diastereomers. Both A and B show a similar mass spectrum (Fig. 4). The major ion for both diastereomers is $[M-HF-CH_3OH]^{\bullet}$ at m/z 379. This ion could be formed via electron capture to form the molecular ion (shown as a hyperconjugated specie) with subsequent loss of HF and methanol to yield a resonance-stabilized radical anion as shown in Fig. 5. This mechanism is supported by the fact that tetradeuterated rimantadine gives a $[M-HF-CH_3OH]^{\bullet}$ ion at m/z 383 with no loss of deuteriums.

Note that the heights of peaks A and B (Fig. 2) are unequal. This is a common phenomenon found, for example, for the asymmetric syntheses between α-keto esters and Grignard reagents and is referred to as asymmetric induction [10]. For a given batch of samples, the intensity ratio of A/B varied from 1:2 to close to 1:1, but generally was about 2:3. This variability did not affect the ability to quantitate, since the tetradeuterated reference standard compensated for the variation in the derivatization rates of the enantiomers.

Based on previous work [3,11], the material comprising peaks A and B can be tentatively assigned absolute configurations. The MMPA used to derivatize the rimantadine has the *S* configuration [10]. Based on the analyses of model amines with structures similar to rimantadine, the faster eluting peak A can be assigned

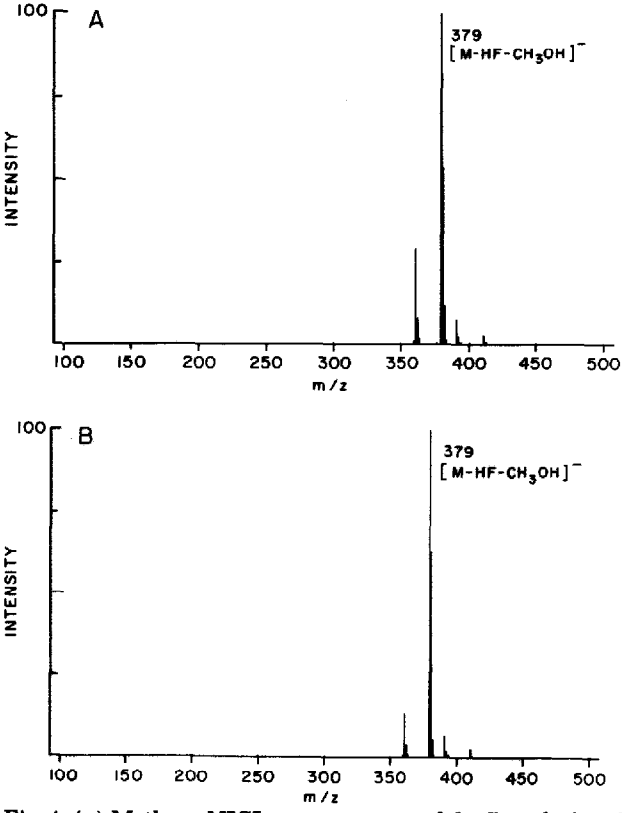

Fig. 4. (a) Methane NICI mass spectrum of the first eluting rimantadine diastereomer (labelled A in Fig. 2). (b) Methane NICI mass spectrum of the second eluting rimantadine diastereomer (labelled B in Fig. 2).

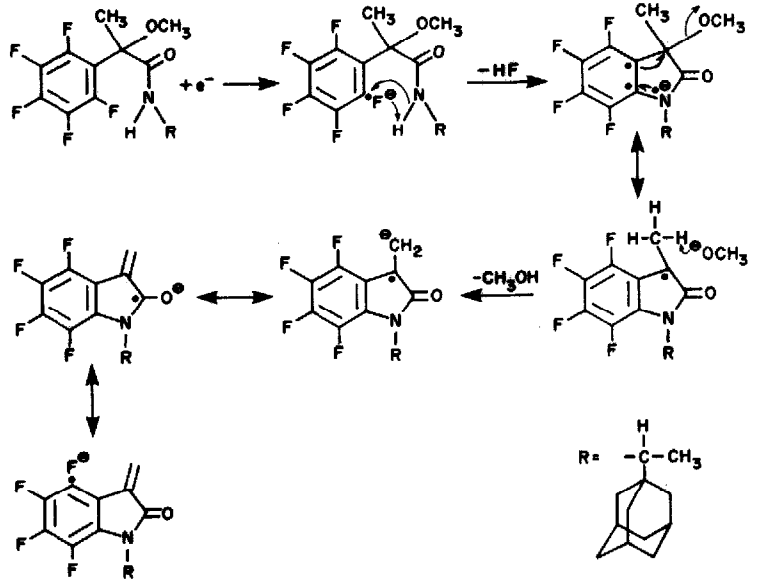

Fig. 5. Mechanism of formation of m/z 379.

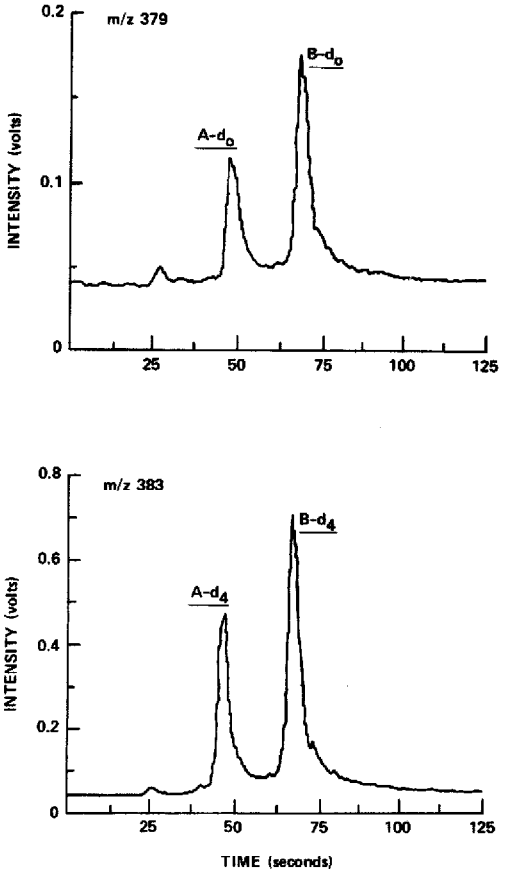

Fig. 6. Selected ion current profiles from a 72-h post-dose plasma sample from a subject given 200 mg of rimantadine. The 1.0-ml sample was fortified with 100 ng of tetradeuterated rimantadine. The chromatograms of the rimantadine-d_0 diastereomers are labelled A-d_0 and B-d_0 and those from the rimantadine-d_4 diastereomers are labelled A-d_4 and B-d_4. (The times shown are relative to the beginning of data acquisition.)

the *R* configuration (*RS* diastereomer) and peak B can be assigned the *S* configuration (*SS* diastereomer).

In order to verify that the derivatization reaction was not causing racemization, optically pure (+)-α-methylbenzylamine and (−)-α-methylbenzylamine were each derivatized using the described method. GC–MS analysis verified that in each case only a single diastereomer was formed.

Based on the fit of the data from the analysis of the plasma calibration samples to the calibration equation, the assay has an estimated overall inter-assay precision* of 5% for both diatereomers. Based on a consideration of the reproducibility of the duplicate analyses of the plasma calibration and quality assurance samples,

*Inter-assay precision was estimated from the difference between the observed value for a concentration and the concentration back-calculated from the regression line. The overall inter-assay precision was determined by averaging the results from all five calibration concentrations.

TABLE I

CONCENTRATIONS FOR THE INDIVIDUAL ENANTIOMERS IN THE PLASMA OF THE FOUR HEALTHY SUBJECTS

A and B refer to enantiomers A and B in Fig. 2. N.M. = non-measurable.

Time (h)	Concentration (ng/ml)							
	Subject 1		Subject 2		Subject 3		Subject 4	
	A	B	A	B	A	B	A	B
Pre-dose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
1	49	46	6	6	14	14	8	8
2	66	65	21	22	35	35	32	32
3	78	80	38	40	42	42	66	66
4	67	68	53	54	48	48	53	53
5	82	83	79	83	52	52	56	56
6	83	84	73	71	45	45	55	55
7	71	73	85	75	53	52	56	56
8	53	53	69	69	48	48	55	55
12	42	42	48	44	34	34	31	31
24	22	23	28	26	14	14	15	15
48	8	10	21	20	6	6	6	6
72	5	5	14	13	N.M.	N.M.	3	3
96	N.M.	N.M.	8	8	N.M.	N.M.	N.M.	N.M.
120	N.M.	N.M.	5	6	N.M.	N.M.	N.M.	N.M.

TABLE II

PARMACOKINETIC DATA FOR THE INDIVIDUAL RIMANTADINE ENANTIOMERS IN THE PLASMA OF THE FOUR HEALTHY SUBJECTS

Values are means ± standard deviations.

Kinetic parameter	Enantiomer A	Enantiomer B
C_{max} (ng/ml)	72 ± 15	73 ± 13
T_{max} (min)	375 ± 142	285 ± 76
β ($min^{-1} \times 10^3$)	0.554 ± 0.145	0.542 ± 0.141
AUC (ng·min/ml$\times 10^{-3}$)*	153 ± 65	149 ± 59
Cl (ml/min)**,***	609 ± 212	618 ± 210
V_d (l)***	1082 ± 165	1123 ± 142

*AUC values from trapezoidal rule calculations were 159000 ± 59000 ng·min/ml (enantiomer A) and 158000 ± 55000 ng·min/ml (enantiomer B).

**Clearance from dose divided by AUC from the trapezoidal rule calculation were 570 ± 177 ml/min (enantiomer A) and 571 ± 179 ml/min (enantiomer B).

***Calculation assumes the oral bioavailability is 100%.

the assay has an estimated overall intra-assay precision* of 6.3 and 7.9% for the first and second eluting diastereomers, respectively. All calibration curves had correlation coefficients greater than 0.99. The inter-assay precision of the lowest

*Intra-assay precision was estimated from the ratio of duplicate analyses. The overall intra-assay precision was determined by averaging the results from the five calibration concentrations and quality assurance samples analyzed in duplicate.

TABLE III

AMOUNT OF EACH RIMANTADINE ENANTIOMER EXCRETED IN URINE

A single sample of the 0–48 h post-dose urine pool was determined in triplicate.

Subject no.	Mean amount of rimantadine excreted (mg)			
	Unconjugated* enantiomer		Unconjugated + conjugated** enantiomer	
	A	B	A	B***
1	6.7 ± 1.3	7.0 ± 0.8	8.8 ± 0.8	15.1 ± 1.6
2	16.7 ± 1.4	18.1 ± 1.0	18.3 ± 1.2	25.0 ± 0.2
3	9.5 ± 0.4	10.2 ± 0.5	11.4 ± 0.6	21.7 ± 0.8
4	9.7 ± 0.3	10.2 ± 1.3	10.2 ± 0.9	14.0 ± 0.9

*No Glusulase treatment.
**After Glusulase treatment.
***Values for enantiomers A and B are statistically different at $p < 0.05$ by Student's t-test.

calibration standard (2.5 ng/ml for each enantiomer) was 3.4 and 2.2% for the first and second eluting diastereomers, respectively.

The excellent sensitivity of the assay is demonstrated in Fig. 6 which shows selected ion current profiles from a 72-h post-dose plasma sample from a healthy subject (subject 1) given 200 mg of rimantadine hydrochloride. Profiles from urine samples are similar.

Table I gives the plasma concentration–time profile for each rimantadine enantiomer in the plasma of four male human volunteers each administered 200 mg of racemic rimantadine hydrochloride. Table II summarizes the pharmacokinetic data for the same four subjects. The kinetic parameters listed in Table II, i.e., C_{max}, T_{max}, β, AUC, Cl and V_d, refer to the maximum concentration, the time at which the maximum concentration occurs, the elimination rate constant, the area under the concentration–time curve, clearance and volume of distribution, respectively. Each value is a mean ± standard deviation from averaging the data from all four subjects. The correlation coefficients for the fit of the concentration–time data to the one-compartment model were all greater than 0.99. No differences in the disposition of the enantiomers were observed.

Table III lists the amount of rimantadine excreted in a 0–48 h urine sample from each of the same four volunteers. As can been seen, no significant differences were observed in the relative amounts of each enantiomer of unconjugated rimantadine. However, following Glusulase treatment, the material comprising of peak B, but not the material comprising peak A, was significantly increased. A conjugation reaction at the primary amine function of a drug is unusual with relatively few examples published [12–14], and none of the examples purports a stereospecific reaction. Future research will attempt to define the exact nature and structure of the conjugate and to ascertain whether the observed stereospecificity is the result of in vivo formation or the failure of the Glusulase treatment to hydrolyze one enantiomer.

CONCLUSIONS

A GC–NICIMS procedure with excellent sensitivity and precision has been developed for the quantitation of the enantiomers of rimantadine in plasma and urine. In plasma and urine, no differences were observed in the disposition of the unconjugated individual enantiomers. In urine, one enantiomer, but not both, was generated following Glusulase treatment. The reported procedure should be applicable to other primary or secondary amines.

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