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Quantitative Determination of Rimantadine in Human Plasma and Urine by GC–MS

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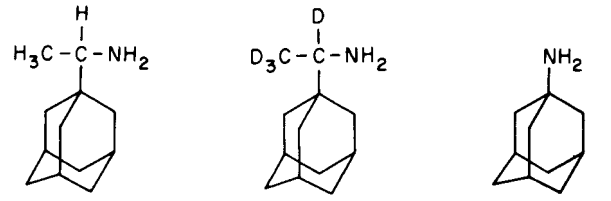
A GC–MS procedure has been developed for the quantitation in plasma and urine of rimantadine, an antiviral drug effective against type A influenza. The assay utilizes selective ion monitoring, methane negative ion chemical ionization (NCI) and stable isotope dilution. Sensitivity to NCI is effected by derivation of rimantadine with pentafluorobenzoyl chloride. The method has been used to quantitate plasma concentrations of rimantadine over a range from 4.2 ng/ml to 416 ng/ml, and urinary concentrations of rimantadine over a range of 21 ng/ml to 2077 ng/ml.

INTRODUCTION

Rimantadine is an agent effective against influenza A infections.[1] It is closely related to amantadine in structure; and while as efficacious as amantadine,[1] it is much less toxic to patients at equivalent doses.[2] The mode of action of rimantadine is not understood, but it may act after the virus penetrates the host cell.[3]



Rimantadine-D_0 Rimantadine-D_4 Amantadine

An EC-GC assay for rimantadine·HCl with a limit of quantitation of only 25 ng/ml has been described.[2] In addition, the analysis time of the EC-GC method is approximately 20 min, a significant consideration given the thousands of clinical and preclinical samples which must be analyzed to bring a new drug to market.

This paper reports a GC-MS assay for rimantadine in human plasma and urine. The assay was developed to provide a more sensitive and rapid procedure for rimantadine than the EC-GC method.

EXPERIMENTAL SECTION

Materials

Rimantadine was obtained from the Quality Control Department of Hoffmann-La Roche, Inc. Rimantadine-D_4 was synthesized by Dr P. Muccino of the Isotope Synthesis Group at Hoffmann-La Roche, Inc. All reagents were of analytical grade, except methanol (Burdick and Jackson, Muskegon, MI) and toluene (Fisher Scientific Co., Fair Lawn, NJ) which were UV grade. Sodium hydroxide was from Fisher Scientific Co., and anhydrous dibasic sodium phosphate was from Mallinckrodt, Inc. (St. Louis, MO). Pentafluorobenzoyl chloride was from Fluka Chemical Corp. (Ronkonkoma, NY), or Aldrich Chemical Co. (Milwaukee, WI). Bond-Elut® CN columns (Part No. 624203, 200 mg cyanopropyl packing, 2.8 ml capacity) were supplied by Analytichem International (Harbor City, CA). The disposable borosilicate culture tubes (10×75 mm) and polypropylene containers (Cat. No. MS505) were obtained from Fisher Vacutainers® (10 ml, Cat. No. 6527 were obtained from Becton-Dickinson (Rutherford, NS).

Stock and calibration solutions

A stock solution was prepared by dissolving 10.0 mg of rimantadine·HCl in 10.0 ml of methanol in a volumetric flask. An intermediate stock solution containing 2000 ng/50 μl of rimantadine·HCl 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 416, 166, 41.5, 16.6 and 4.2 ng/50 μl of rimantadine (free base) in methanol.

The internal standard solution was prepared by diluting 0.2 ml of a 1.0 mg/ml methanolic stock solution of tetradeuterated rimantadine hydrochloride with 100 ml of methanol. All solutions were stored at 4 °C.

Plasma samples

Blood was drawn into heparinized vacutainers, centrifuged, and the plasma was transferred into siliconized glass scintillation vials. The sample was stored at −17 °C until analyzed.

A Q.A. (quality assurance) sample was prepared by pooling aliquots from experimental samples. Enough plasma was obtained so that duplicate aliquots could be analyzed along with each set of samples over the course of all the analyses. The Q.A. sample was also used to determine long-term stability of the drug in plasma and to check the reproducibility of the assay.

Urine samples

Urine samples were collected in amberized glass containers without any preservative. An aliquot of this

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sample was transferred into a polypropylene container (Fisher, Cat. No. MS-50S) and stored at −17 °C until analyzed.

A Q.A. (quality assurance) sample was prepared by pooling aliquots from experimental samples. Enough urine was obtained so that duplicate aliquots could be analyzed along with each set of samples over the course of all the analyses. The Q.A. sample was also used to determine long-term stability of the drug in urine, and to check the reproducibility of the assay.

Plasma collection device

The plasma collection device experiments were performed using human blood as follows. A volume of 30 ml of blood was collected in heparinized vacutainers The blood was combined, and was fortified with rimantadine to give a concentration of about 200 ng/ml; then 5 ml aliquots of the fortified blood were transferred to three vacutainers and three siliconized glass tubes. All the tubes were shaken gently for 30 min, and aliquots of 1 ml of sample from each tube were fortified with internal standard and analyzed. A blood calibration curve was generated using the same method as for plasma except using 1 ml of whole blood to calculate the concentration of rimantadine in the fortified blood. The remaining fortified blood was centrifuged to separate the plasma. One ml of plasma from each tube was analyzed the same way as the blood above using a separate plasma calibration curve.

Extraction and derivatization method

The plasma sample was thawed and 1.0 ml was transferred into a 10 × 75 mm glass disposable culture tube. Fifty μl of the internal standard solution was added and the mixture was vortexed. One ml of sodium phosphate buffer (0.1 M, pH 9.1) was added and the solution was vortexed again. The Bond-Elut® CN column was activated by drawing 1 ml of methanol twice through the column by suction (1 Torr). A Vac-Elut® having a column capacity of 10 columns was used. After washing the column twice with 1 ml of distilled water, the sample was added to the column and the columns were washed twice with 1 ml of distilled water. The compound of interest was extracted from the column by washing twice with 1 ml of methanol, and collecting the eluent into a clean disposable culture tube. The solvent was evaporated in an N-Evap water bath (Organomation Associates, Inc., South Berlin, MA) at 40–45 °C with a stream of nitrogen. The residue was dissolved in 50 μl of toluene, and derivatized with 5 μl of pentafluorobenzoyl chloride at room temperature for 30 min. Subsequently, an additional 400 μl of toluene was added, and the excess derivatizing reagent was neutralized with 200 μl of 5N sodium hydroxide. The mixture was vortexed, centrifuged at 2000 rpm at 10 °C for 3 min, and approximately 350 μl of the toluene layer was transferred into a clean disposable culture tube and evaporated to dryness with a stream of nitrogen. The residue was dissolved in 50 μl of toluene, transferred into a 200 μl glass insert in a Chrompak® (Chrompak International, Netherlands) crimp vial, and 1.0 μl was analyzed by GC/NCIMS.

Urine samples were analyzed in an identical fashion to the plasma samples, except 0.2 ml instead of 1 ml was analyzed.

Calibration standards

Five calibration standards and the quality assurance sample were analyzed in duplicate along with each set of experimental samples. The calibration standards contained 4.2, 16.6, 41.5, 166, or 416 ng of rimantadine in either 1 ml of drug-free plasma or in 0.2 ml of drug-free urine.

Recovery experiments

Since no radio-labelled rimantadine was available, the GC–MS assay was used to determine the percent recovery from both human plasma and urine.

The method involved preparing twelve samples (Nos. 1–12) using the appropriate drug-free matrix. Samples Nos. 1–6 were spiked with rimantadine to give a concentration of approximately 200 ng/ml. All samples 1–12 were spiked with tetradeuterated rimantadine and were extracted and processed up to the derivatization step. Samples Nos. 7–12 were spiked with the same amount of rimantadine as was previously added to samples Nos. 1–6.

The recovery was calculated by comparing the mean absolute responses from m/z 353 (from derivatized rimantadine) for samples Nos. 1–6 and samples Nos. 7–12.

Stability experiments

Bench top stability was determined by comparing the concentrations of experimental samples allowed to set at room temperature for 0, 3 and 6 h. The experimental samples were aliquots from the Q.A. sample stored at −20 °C. The long-term stability at −20 °C was determined by duplicate determinations of the same Q.A. sample over a period of 5 months (plasma) or 3 months (urine).

Instrumentation

Gas chromatograph. A Carlo Erba GC was equipped with a capillary column, Chrompack® CP Sil 8 CB (25 m × 0.33 mm I.D., film thickness 1.25 μm). The column was maintained at 265 °C with methane as the carrier gas. The flow was set to give 1×10^{-4} Torr ion source gauge reading. The injector, column and interface/transfer line were set to 300, 265 and 300 °C, respectively. Under these conditions the retention time of rimantadine was 2.8 min.

A Hewlett-Packard Model 7672A automatic liquid sampler was used to inject samples. This autosampler has a sample capacity of 99 samples.

GC–MS and data acquisition. A Kratos MS-50 magnetic sector mass spectrometer was tuned to give the maximum response consistent with reasonable ion peak shape and a resolution of about 7000. Methane was used as the

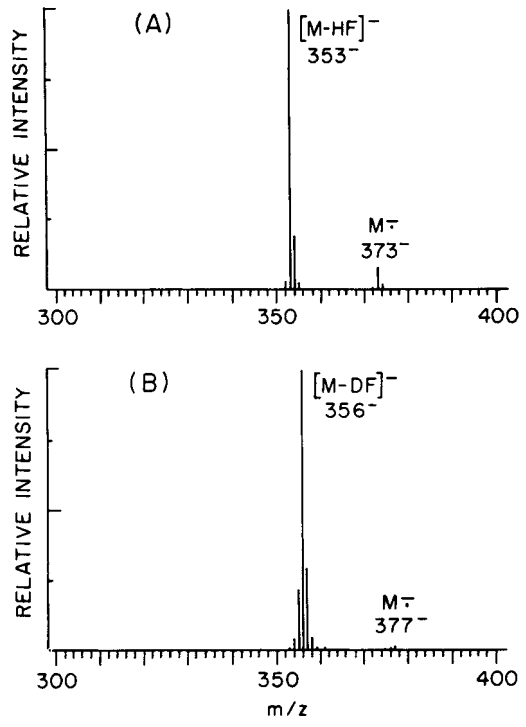

Figure 1. Methane NCI mass spectra of pentafluorobenzoyl rimantadine (A) and tetradeuterated pentafluorobenzoyl rimantadine (B). The base peaks in the mass spectra (A) and (B) are m/z 353 and m/z 356, respectively.

negative chemical ionization reagent gas. The unlabelled and deuterium-labelled ions were monitored by a VG (Vacuum Generators) MID (multiple ion detection) system. These ions were monitored relative to an external lock mass of $C_7F_{14}^-$ (m/z 350) from perfluorotributyl amine. The actual ions monitored were the $[M-HF]^-$ (m/z 353) of the unlabelled analyte, and the $[M-DF]^-$ ion (m/z 356) of the tetradeuterated-labelled internal standard.

QSIMPS (Quantitative Selective Ion Monitoring Processing System)[4] was used to control the auto sampler and GC-MS divert valve and to collect and process the selected ion current profile data.

The ratio of the peak heights[4] and concentrations (x) from the duplicate analysis of the calibration standards were fitted, using weighted ($1/y$) nonlinear regression, to the equation:

$$R = \frac{a + x}{bx + c}$$

where R is the ion ratio ((m/z 353)/(m/z 356)), x is the analyte concentration and a, b and c are parameters adjusted to give the best fit to the calibration data.[5] For this study, the value of b was in general small so that the calibration curve was virtually linear. Given an ion ratio in an experimental sample and the calculated values for a, b and c, a value for x can be calculated.

Peak heights were calculated by fitting the top 80% of the peak profile to the EMG (extended modified Gaussian) peak model,[4] 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 from the average intensity value for ten scans before and ten scans after the peak.

RESULTS AND DISCUSSION

Figure 1 shows the methane NCI mass spectra of the derivatized analyte and the derivatized deuterated internal standard. The base peak represents the loss of HF (m/z 353) from the derivatized analyte and the loss of DF (m/z 356) from the deuterated compound. If air (O_2) is not excluded from the mass spectrometer, these ions are no longer the base peaks; rather, additional losses of H and D, respectively, are observed and the calibration curves become nonlinear.

Figures 2 and 3 are examples of selected ion current profiles from the assay. Figure 2 shows the profiles from a 4.2 ng/ml rimantadine plasma standard fortified with 83 ng/ml of internal standard. This demonstrates the good sensitivity of the technique at the lowest concentration used for quantitation. Figure 3 shows the profile from a plasma sample from a renal failure patient, fortified with 83 ng/ml of internal standards. The concentration of rimantadine in this sample was 18 ng/ml. Due to the high degree of specificity of this assay, no interferences from other plasma components were observed.

For every experimental calibration curve each concentration was analyzed in duplicate. For calibration curves used in several studies involving the assay of over 3000 plasma samples, the mean inter-assay precision was 5.8% and the mean intra-assay precision was 9.9%. For calibration curves used in several studies involving the assay of over 500 urine samples, the overall inter-assay precision was 6.0% and the overall intra-assay precision was 9.9%. At 4.2 ng/ml, the lower limit of quantitation for plasma, the inter-assay and intra-assay were 10.1% and 13.6%, respectively. At 20.7 ng/ml, the

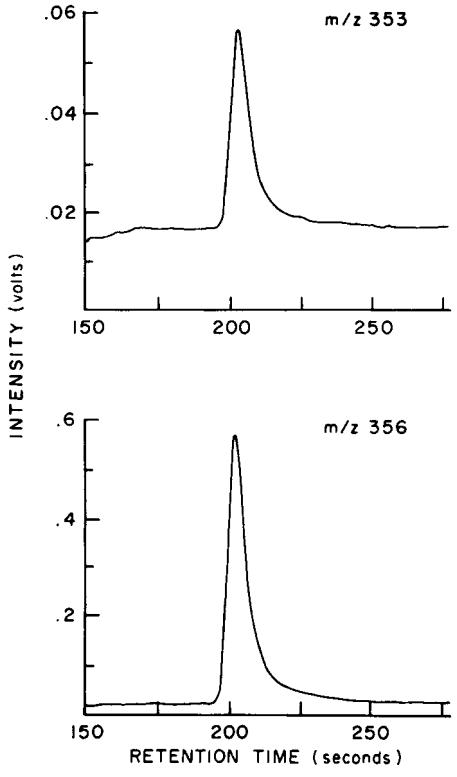

Figure 2. Selected ion current profile from a drug-free plasma extract fortified with rimantadine (4.2 ng/ml concentration) and its tetradeuterated internal standard (83 ng/ml concentration).

Table 1. Plasma collection device experiments

Sample		Rimantadine concentration (ng/ml)[a] Vacutainer	Glass tube
Human blood	Mean ± SD[b]	194 ± 3.0	194 ± 0.5
	RSD[c]	1.5	0.3
Human plasma	Mean ± SD	119 ± 15	125 ± 4.5
	RSD	12.6	3.6

[a] One ml aliquot of the sample was analyzed from each tube.
[b] Based on three determinations.
[c] Relative standard deviation.

Table 2. Percentage of recovery of rimantadine from human plasma and urine

Plasma sample	Peak height (volts) of *m/z* 353[a]	
1	2.08	
2	4.15	
3	3.21	
4	2.79	
5	2.92	
6	2.80	
7	7.43	
8	5.51	
9	7.18	
10	10.70	
11	7.16	
12	8.80	Average % recovery = 38.4%
Urine sample		
1	4.81	
2	3.84	
3	3.79	
4	4.15	
5	3.14	
6	2.69	
7	5.69	
8	8.80	
9	7.77	
10	4.61	
11	3.88	
12	5.90	Average % recovery = 61.1%

[a] This refers to the SIM chromatographic peak height of *m/z* 353.

Table 3. Benchtop and long-term stability of rimantadine in human plasma

Benchtop stability

Time (h)	Rimantadine concentration (ng/ml) ± SD[a] (RSD)[b]
0	100.4 ± 3.0 (3.0)
3	103.4 ± 1.6 (1.6)
6	104.5 ± 2.4 (2.3)

Long-termm stability

Assay date	Rimantadine (ng/ml) ± SD (RSD)	Number of determinations
2/86	122.7 ± 9.2 (7.5)	30
3/86	116.7 ± 1.5 (1.3)	6
4/86	121.0 ± 10.4 (8.6)	8
5/86	110.0 ± 10.6 (9.6)	30
6/86	105.8 ± 4.1 (3.9)	16

[a] Based on six determinations.
[b] Relative standard deviation.

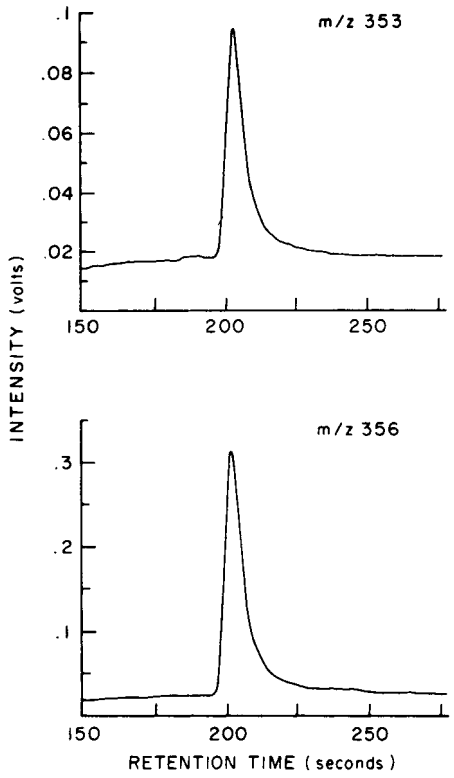

Figure 3. Selected ion current profile from an extract of a plasma sample from a renal failure patient given 200 mg of rimantadine hydrochloride. The sample was fortified with tetradeuterated rimantadine to give a final concentration of 83 ng/ml. The measured concentration of rimantadine in this sample was 18 ng/ml.

lower limit of quantitation for urine, the inter-assay and intra-assay precisions were 8.1% and 27.2%, respectively.

The results of the plasma collection device experiments (Table 1) demonstrated that the rimantadine concentration measured in plasma was not a function of the collection device. However, comparison of blood and plasma concentrations demonstrated that a significant amount of drug is retained by the red blood cells.

The results of percent recovery experiments for rimantadine in human plasma and urine are shown in Table 2. The average percentage recovery in plasma and urine was determined to be 38.4% and 61.1%, respectively.

The results of the benchtop and long-term stability experiments demonstrated that rimantadine is stable in

Table 4. Benchtop and long-term stability of rimantadine in human urine

Benchtop stability

Time (h)	Rimantadine concentration (ng/ml) ± SD[a] (RSD)[b]
0	3874 ± 138 (3.6)
3	3776 ± 119 (3.2)
6	3853 ± 186 (4.8)

Long-term stability

Assay date	Rimantadine (ng/ml) ± SD (RSD)	Number of determinations
5/86	3796	1
6/86	3337 ± 348 (6.8)	4
7/86	3658 ± 348 (9.5)	3

[a] Based on six determinations.
[b] Relative standard deviation.

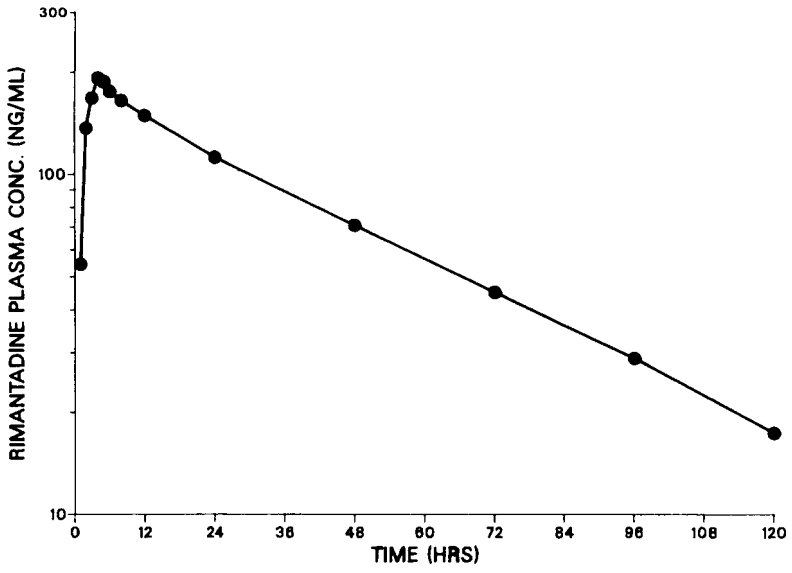

Figure 4. Plasma concentration–time curve for a healthy male subject following a single 100 mg oral dose of rimantadine hydrochloride.

plasma and urine under the stated temperature and storage device conditions (Tables 3 and 4).

Typically, about 10% of a 200 mg dose of rimantadine was found intact in the urine. Figure 4 shows the concentration of the rimantadine in the plasma of a healthy male subject following a single oral dose (100 mg) of rimantadine hydrochloride. The plasma concentration for the subject reached a maximum within 4 h, and decreased with a half-life of about 30 h.

CONCLUSIONS

A GC-MS procedure has been developed for the quantitation of rimantadine in human plasma and urine. The procedure has also been successfully used for the analysis of plasma samples from rats, mice and dogs. The assay represents an improvement to the previous EC-GC method, in terms of specificity, sensitivity (down to 4.2 ng/ml) and speed (sample analysis time on the order of 5 min). Also, it should be noted that the absolute sensitivity is quite good, especially taking into consideration the rather poor recovery (38.4%), and the fact that only 1/50 of the total extract from 1 ml of plasma was analysed.

Additionally, experiments have been done to determine for plasma and urine the effect of the collection device and storage containers, the percent recovery from plasma and urine, and the benchtop/long-term stability of rimantadine.

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