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Determination of amantadine in human plasma by capillary gas chromatography using electron-capture detection following derivatization with pentafluorobenzoyl chloride

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Abstract: A specific, sensitive, and accurate capillary gas chromatographic method for the quantitation of amantadine in human plasma is described. Amantadine and the internal standard, rimantadine were extracted from plasma under alkaline conditions into toluene. Both compounds were derivatized with pentafluorobenzoyl chloride. The derivatives were separated on a HP-1 capillary column at 180°C and detected using a ^{63}Ni electron-capture detector. The minimum quantifiable limit of the assay is 2.3 ng ml^{-1} of amantadine base using 1 ml of plasma. The method was used to evaluate the bioequivalence of two different formulations of amantadine hydrochloride.

Keywords: *Amantadine; pentafluorobenzoyl chloride derivative; gas chromatography; electron-capture detection.*

Introduction

Amantadine hydrochloride (1-adamantanamine hydrochloride, Fig. 1), an antiviral agent, is used for the treatments of influenza [1] and Parkinsonism [2]. When dosed orally amantadine shows relatively low plasma concentrations making it necessary to devise a very sensitive assay for its quantitation in plasma and other biological specimens.

Since the early 1960s several different gas–liquid chromatographic methods have been proven useful. These include both flame ionization [3–5] and electron-capture [6, 7] detection. The flame ionization procedures lacked the required sensitivity for measuring amantadine in plasma and was used mostly to quantify amantadine in urine. Biandrate *et al.* [6] and Sioufi and Pommier [7] both prepared an electron capturing derivative of amantadine with trichloroacetyl chloride to enhance sensitivity. The linearity of the Biandrate method [6] ranged between 25 and 1000 ng ml^{-1} in human plasma, while Sioufi and Pommier [7] enhanced the sensitivity of the method to 10 ng ml^{-1}.

This paper describes a capillary gas chromatographic system with electron-capture detection using pentafluorobenzoyl chloride for derivatization of amantadine and rimantadine, an internal standard. This method is less laborious, requiring minimal sample clean-up and is more sensitive than previous procedures. Using this method, as little as 2.3 ng in 1 ml of human plasma can be analysed with good reproducibility.

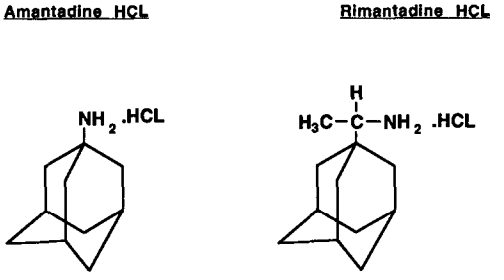

Figure 1
Structures of amantadine hydrochloride and rimantadine hydrochloride.

Materials and Methods

Chemicals, reagents and control plasma

Amantadine hydrochloride and rimantadine hydrochloride were synthesized by Medicinal Chemistry Section of The Du Pont Merck Pharmaceutical Co. (Wilmington, DE, USA). Both compounds were >99% pure as evaluated by GC. Toluene was purchased from American Scientific Products (Muskegon, MI, USA). Sodium hydroxide (1 N), Dilut-It® (analytical concentrate) was purchased from

J.T. Baker (Phillipsburg, NJ, USA). Pentafluorobenzoyl chloride was purchased from Aldrich (Milwaukee, WI, USA). Control human plasma was purchased from Biological Specialities (Lansdale, PA, USA).

Chromatography system

The gas chromatographic measurements were made using a Hewlett–Packard system (Model 5890A, Hewlett–Packard, Avondale, PA, USA), equipped with a ^{63}Ni electron-capture detector, a dedicated on-column capillary inlet, automatic sample injector (Model 7673A) and an integrator (Model 3396A). The column was an HP-1 (Hewlett–Packard) fused-silica (10 m × 0.53 mm i.d.) megabore capillary with a 2.65 μm film thickness.

Chromatography conditions

The carrier gas was helium at 20 ml min^{-1} and the auxiliary gas was argon–methane (95:5) at 40 ml min^{-1}. The gas chromatograph oven temperature was isothermal at 180°C. The detector temperature was 300°C.

Stock solutions

Primary stock solutions containing 1 mg ml^{-1} of amantadine hydrochloride and the internal standard, rimantadine hydrochloride were prepared by dissolving 100 mg of each compound in 100 ml of nanopure water. Amantadine hydrochloride working stock solutions 0.03, 0.05, 0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 μg ml^{-1} were prepared by diluting the appropriate volumes of the primary stock solution in nanopure water. An aliquot of the rimantadine hydrochloride primary stock solution was diluted in nanopure water to prepare a working stock solution of 1.0 μg ml^{-1}.

Preparation of plasma standards and samples and extraction procedure

Plasma standards were prepared by adding 100 μl of each amantadine hydrochloride working stock solution (0.03, 0.05, 0.10, 0.20, 0.50, 1.0, 2.0 and 5.0 μg ml^{-1}) and 100 μl of the rimantadine hydrochloride working stock solution (1.0 μg ml^{-1}) to 8-ml screw-cap test tubes (13 × 100 mm) containing 1 ml of control (blank) human plasma. The final concentrations of amantadine base were 2.3, 4.0, 8.2, 16.1, 40.3, 80.6, 161.2 and 402.9 ng ml^{-1}. Plasma samples were prepared by adding 100 μl of the rimantadine hydrochloride working stock solution (1.0 μg ml^{-1}) to 1 ml of plasma. One millilitre of 1 N NaOH and 2 ml of toluene were added to each plasma standard and plasma sample, the tubes were capped and shaken for 30 min on a slow rocking wrist-action shaker. After centrifuging (1500 rpm) for 10 min, an aliquot of the toluene layer (~1.5 ml) was aspirated into a clean 13 × 100 mm screw-cap test tube.

Derivatization procedure

Twenty microlitres of 0.04% by volume solution of pentafluorobenzoyl chloride in toluene was added to each tube. The tubes were capped, mixed well and placed in a water bath at 60°C for 30 min. After completion of the reaction, 1 ml of 1 N NaOH was added to each tube and the tubes were shaken for 30 min to hydrolyse unreacted pentafluorobenzoyl chloride. After centrifugation, an aliquot (~1 ml) of the toluene was aspirated into a 11 × 32 mm injection vial. The caps on the vials were crimped and each vial was loaded into the automatic sample injector. One microlitre of each sample was injected into the chromatographic column and chromatographed.

Data analyses

Peak areas were measured by the Hewlett–Packard 3396A integrator. The peak area ratio of amantadine base to the internal standard was determined. Two power curves were used to determine slopes, intercepts and correlation coefficients for the standards. One was used to evaluate standard amantadine base concentrations ranging from 2.3 to 40.3 ng ml^{-1} (low standard curve) while the second was used to evaluate amantadine base concentrations ranging from 40.3 to 402.9 ng ml^{-1} (high standard curve).

Assay validation

The intra- and inter-day variability of the method were evaluated by repetitive analysis of spiked human plasma at different amantadine base concentrations. For the inter-day variability, spiked human plasma samples were derivatized and analysed on three different days. Accuracy of the assay was determined by analysing spiked plasma samples within the range of the standards that were prepared by a person other than the analyst. The extraction recovery of amantadine was determined by comparing the peak area ratio from extracted samples to those of unextracted samples.

Human pharmacokinetics and analysis procedure

Amantadine hydrochloride was given as a single oral dose (100 mg) to fasted male (19–40 years old) volunteers (n = 24). The study was an open-label, randomized, two-way, two-period crossover study performed at a single clinical site. The subjects received a single oral dose of each formulation of amantadine hydrochloride. Each dose was separated by a 2-week washout period. Venous blood samples were collected after each dose at prior to dosing, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 15, 24, 48, 72, 96 and 120 h after dosing. Plasma samples were stored at −20°C until analysed. On each assay day, a series of standards containing amantadine base concentrations of 0, 2.3, 4.0, 8.1, 16.1, 40.3, 80.6, 161.2 and 402.9 ng ml^{-1} and a series of quality control samples containing 2.4, 12.9, 28.2, 48.3, 112.8 and 201.4 ng ml^{-1} were derivatized and analysed with each set of clinical samples which consisted of all plasma samples from one to five subjects.

Results and Discussion

Derivatization, chromatography and specificity

The reaction conditions between amantadine, rimatadine and pentafluorobenzoyl chloride were studied. It was found that the derivatization proceeded rapidly, altering the length of reaction from 10 min to 1 h at 37°C and reaction temperature from 37 to 80°C (at 30 min reaction time) did not have any effect on the formation of the derivatives. Thirty minutes at 60°C was selected to ensure complete formation of the derivatives. Sample chromatograms for the control human plasma, plasma spiked with amantadine and plasma from a subject 72 h after a 100-mg dose of amantadine hydrochloride are shown in Fig. 2. Amantadine and the internal standard were well separated with retention times of approximately 7 and 13 min, respectively. No interfering peaks from endogenous substances and the derivatizing reagent was observed. Correlation coefficients for the standard curves usually exceeded 0.99.

Assay validation

Results for the intra-day and inter-day assays are shown in Table 1. Quadruplicate plasma samples spiked with various amantadine concentrations were assayed on the same day. Triplicate sets of plasma samples spiked with various amantadine base concentrations were prepared and each set was analysed on separate days. The intra-day and inter-day relative standard deviations ranged from 1.64 to 7.52% and 3.00 to 16.36% over an amantadine base concentration range of 2.3–402.9 ng ml^{-1}, respectively. The accuracy results for the assay are shown in Table 2. The found concentrations of the accuracy samples were compared to the spiked concentrations. The mean differences ranged from −17.2 to 6.2% for concentrations of 2.4, 12.9, 28.2, 48.3, 112.8 and 201.4 ng ml^{-1}. The extraction recovery was determined by comparing the absolute peak area ratios of amanatadine from

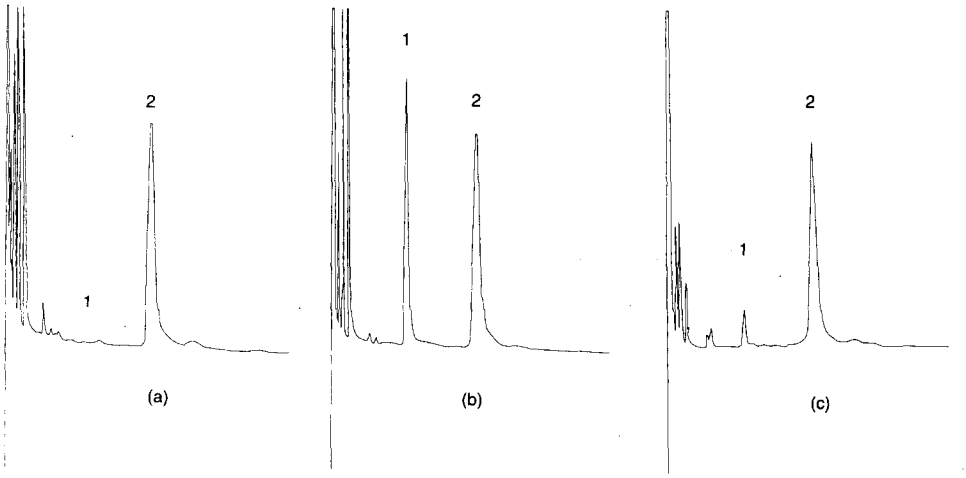

Figure 2
Typical chromatograms of human plasma extracts derivatized with pentafluorobenzoyl chloride: (a) 1 ml of control plasma, (b) 1 ml of control plasma spiked with 40.3 ng of amantadine and (c) 1 ml of human plasma at 72 h after receiving a single 100-mg oral dose of amantadine hydrochloride (1 = amantadine, 2 = rimantadine).

Table 1
Intra-day and inter-day reproducibility results of amantadine in human plasma

Spiked conc. (ng ml^{-1})	Intra-day (n = 4) Found conc. (ng ml^{-1})	RSD (%)	Inter-day (n = 3) Found conc. (ng ml^{-1})	RSD (%)
2.3	2.2 ± 0.2	7.52	2.0 ± 0.2	7.35
4.0	4.3 ± 0.3	6.70	3.6 ± 0.6	16.36
8.1	9.1 ± 0.4	4.29	7.8 ± 1.1	14.49
16.1	16.1 ± 1.0	6.20	15.4 ± 1.0	4.19
40.3	32.9 ± 1.9	5.78	37.1 ± 4.5	7.83
80.6	76.5 ± 5.0	6.52	78.2 ± 2.9	3.00
161.2	163.5 ± 7.0	4.28	155.7 ± 8.5	8.99
402.9	422.2 ± 6.9	1.64	387.9 ± 29.7	3.72

Table 2
Accuracy results for amantadine in human plasma*

Spiked conc. (ng ml^{-1})	Found conc. (ng ml^{-1})	RSD (%)	Error (%)
2.4	2.4	8.3	0.0
12.8	13.7	2.2	+6.2
28.2	28.7	4.2	+1.7
48.3	46.9	5.7	−2.9
112.8	98.3	1.3	−12.9
201.4	166.7	5.8	−17.2

*n = 6.

extracted plasma samples to those obtained from unextracted samples of the same concentration. The recoveries were 89.66 ± 6.46, 93.09 ± 1.95, 101.82 ± 7.22, 96.54 ± 3.29, 99.33 ± 1.39, 94.71 ± 8.83 and 105.52 ± 7.87% for the 4.0, 8.1, 16,1, 40.3, 80.6, 161.2 and 402.9 ng ml^{-1} samples, respectively. Judging from the reproducibility and accuracy results and using 20% as the criteria, the minimum quantifiable limit for the assay is 2.3 ng ml^{-1} of amantadine base when 1 ml of plasma was extracted. Plasma samples from the bioequivalence study were stored at −20°C and analysed on 14 different occasions during a 3-month period. Previously, Sioufi and Pommier [7] showed that amantadine was stable in frozen plasma at −20°C for at least 6 months, therefore based on their results, the plasma samples were assumed to be stable during the analysis period. The precision and accuracy of the analysis was evaluated by analysing a set of quality control samples on each assay day of the bioequivalence study. The results are presented in Table 3. Relative standard deviations for the quality control samples ranged between 1.3 and 8.8% over the concentration range of 2.4–201.4 ng ml^{-1}.

Human pharmacokinetic study

The method was used to determine amantadine base plasma concentrations in subjects after a single 100 mg dose of each of the two formulations of amantadine hydrochloride. Figure 3 depicts the amantadine plasma con-

Table 3
Results of the quality control samples analysed during the analysis of the samples from the bioequivalence study*

Spiked conc. (ng ml^{-1})	Found conc. (ng ml^{-1})	RSD (%)
2.4	2.5	8.0
12.9	13.7	8.8
28.2	28.4	3.5
48.3	48.5	3.3
112.8	114.8	1.3
201.4	200.7	2.6

*n = 14.

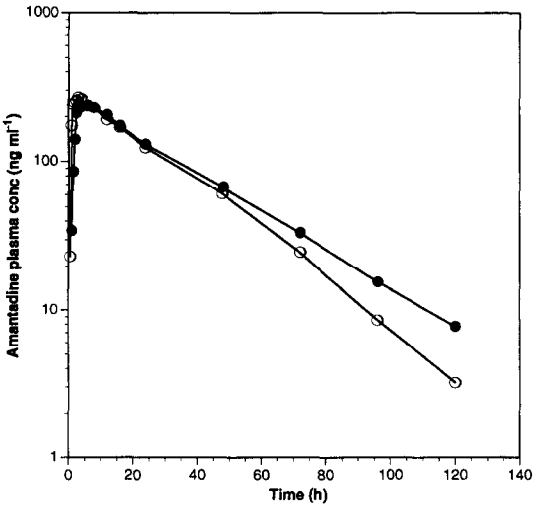

Figure 3
Plasma concentration versus time profile in a subject following a single 100 mg oral dose of the (●) A and (○) B formulations of amantadine hydrochloride.

centration–time profile for one subject. A maximum plasma concentration of 238.5 ng ml^{-1} was attained in 6 h after administration of formulation A while the maximum plasma concentration attained after formulation B was 261.5 ng ml^{-1} at 4 h. The terminal half-lives were 23.1 and 18.5 h for formulations A and B, respectively. Plasma concentrations of 7.7 and 3.2 ng ml^{-1} were detectable in the plasma 120 h after adminstration of formulations A and B, respectively. The validation results demonstrate that this method is specific, accurate and is approximately five-fold more sensitive than the previously published methods [6, 7]. The method was applicable in studying the bioequivalence of two different formulations of amantadine hydrochloride. The results of the bioequivalence study will be reported elsewhere.

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