Determination of Memantine in Plasma and Vitreous Humour by HPLC with Precolumn Derivatization and Fluorescence Detection

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Abstract

A new HPLC procedure with precolumn derivatization and rimantadine as the internal standard for determining memantine, a candidate agent for the treatment of glaucoma in plasma and vitreous humour, has been developed and validated. Precolumn derivatization was performed with 9-fluorenylmethyl-chloroformate-chloride (FMOC-Cl) as the derivatization reagent and followed by a liquid–liquid extraction with *n*-hexane. Optimal conditions for derivatization were an FMOC-Cl concentration of 1.5 mM, a reaction time of 20 min, the temperature at 30°C, the borate buffer pH 8.5, and a borate buffer–acetonitrile ratio of 1:1. The derivatives were analyzed by isocratic HPLC with the fluorescence detector λex 260 nm λem 315 nm on a Novapack C_{18} reversed-phase column with a mobile phase of acetonitrile–water (73:27, v/v), 40°C, and a flow rate of 1.2 mL/min. The linear range was 10–1000 ng/mL with a quantification limit of ~ 10 ng/mL for both types of samples. This analytical method may be suitable for using in ocular availability studies.

Introduction

Memantine (1-amino-3, 5-dimethyladamantane hydrochloride) is a novel, low-to-moderate affinity, noncompetitive *N*-methyl-D-aspartate receptor antagonist of the open channel blocker type (Figure 1). Memantine is used in the treatment of Parkinson's disease, some types of movement disorders (1), some dementia syndromes (2), and has recently been described as a potential treatment for glaucoma (3).

Memantine may rescue neurons by blocking excessive glutamate receptor activation and has been tested as a treatment for glaucoma (4–6).

Analysis of memantine concentrations in tissues is complicated because the molecule has no ultraviolet, visible, or fluorescent absorption, and cannot be directly quantified by chromatographic methods using spectrophotometric or fluorescence detection.

Methods to determine the concentrations of memantine-related compounds (amantadine, rimantadine, aminoacids, and polyamines) in biological samples include extraction and derivatization techniques coupled with gas chromatography (GC) or high-performance liquid chromatography (HPLC). A number of different derivatization reagents have been used in most of these determinations: pentafluorobenzoyl chloride (7), trichloroacetyl chloride (8, 9), 9-fluorene acetate (10), 1-fluoro-2, 4-dinitrobenzene (11), *o*-phthaldiadehyde and 1-thio-β-glucose (12), dansyl chloride (13), and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (14). A new HPLC method for the determination of amantadine and its analogues in rat plasma derivatized with anthraquinone-2-sulfonyl chloride (ASC) with UV detection has recently been described (15). A number of methods to measure memantine concentrations in plasma have been developed using LC methods with fluorescence detection and dansyl chloride as the derivatization agent (16) or, alternatively, using (2-naphthoxy) acetyl chloride as the fluorescent reagent (17). These methods have shown high quantification limits (LOQ) for biological samples and pharmaceutical formulations (17) although none of them have been used to assay vitreous humour samples. Other studies, in which memantine concentrations were quantified in plasma, cerebrospinal fluid, urine, blood, and other tissues, used instruments that are not always readily available in laboratories, such as gas and liquid chromatography-mass spectrometry (18–22).

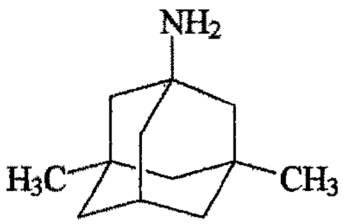

Figure 1. The structure of memantine.

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FMOC-Cl (9-fluorenylmethyl chloroformate chloride) has been shown to be a suitable reagent for fluorescence labelling of polyamines and primary and secondary amines (23–25). FMOC-Cl has been used for the analysis of amino acids (26–28) and for assays of aminoglycoside antibiotics and other compounds containing amino groups (29–34). A method has been described by Hare et al. (6) for the quantification of memantine in serum and vitreous humor by HPLC and derivatization with FMOC-Cl in a study of glaucoma in monkeys. This study did not optimize the analytical conditions and no information was given on the internal standard (IS) used or the LOQ. As memantine was eluted at 40 min, it is assumed that the LOQ were high.

The aim of the present study was, firstly, to develop and validate a simple, specific, and rapid method of determining memantine concentrations in the vitreous humour and plasma by HPLC with fluorescence detection using FMOC-Cl as a derivatizing reagent, and, secondly, to test this method after subtenon (periocular) administration of a saline formulation of memantine.

Experimental

Reagents and standard solutions

Memantine hydrochloride, amantadine hydrochloride, rimantadine hydrochloride, and neomycin hydrochloride were purchased from Sigma (St. Louis, MO). Apramycin hydrochloride and glycine were obtained from Lab Maymo (Barcelona, Spain) and Serva (Barcelona, Spain), respectively. The derivatizing agent, FMOC-Cl, was supplied by Sigma. Acetonitrile, methanol, and other solvents used were HPLC-grade and purchased from Scharlau (Barcelona, Spain). Water was purified using a Milli-Q system (Millipore, MA).

Stock solutions of memantine, the IS (100 µg/mL), and glycine (0.1 M) were prepared in purified water, aliquoted to eppendorfs, and stored at –20°C to minimize their potential degradation. Working solutions were obtained daily by diluting these stock solutions with purified water. Memantine working solutions were prepared in the range of concentrations of 0.2–20 µg/mL and IS was prepared at 1 µg/mL. FMOC-Cl solution (90 mM) was prepared in acetonitrile protected from light with aluminum foil and kept at 4°C until used.

Chromatographic instrumentation and conditions

The analysis was performed with a Waters (Barcelona, Spain) HPLC system equipped with two 515 HPLC pumps, a Gecko-2000 column heater (CIL Cluzeau Info Labo, Paris, France), a M-717 autosampler with the injection volume set to 10 µL, and a 474 fluorescence detector (λex/em: 260/315 nm). The chromatograms were processed by Millennium Integration System 2000 from Waters Corporation (Milford, MA). Two types of columns from Waters were assayed: a Nova-Pak C_{18} reversed-phase column (150 mm × 3.9 mm i.d., 4 µm) protected by a guard column cartridge (20 mm × 3.9 mm i.d.) of the same material; and a Spherisorb ODS (250 mm × 4.6 mm i.d., 5 µm). Both columns were heated at 25°C, 30°C, and 40°C during the analysis. Likewise, different mobile phases were assayed, starting in isocratic mode with diverse proportions of an acetonitrile–water mixture (90:10, 80:20, 75:25, 73:27, and 65:35, v/v), as well as using gradient mode in a range of concentrations from 65:35 to 90:10 at different times. In addition, a methanol–water mixture was assayed in an isocratic mode at different proportions (90:10, 85:15, and 83:17, v/v). The mobile phase was filtered, degassed, and pumped at a flow rate of 1.0 and 1.2 mL/min. The IS was chosen by testing different compounds with physico-chemical characteristics similar to those of memantine hydrochloride, such as amantadine hydrochloride, neomycin hydrochloride, apramycin hydrochloride, and rimantadine hydrochloride.

Optimization derivatization and extraction conditions

The reaction of FMOC-Cl with amines is shown in Figure 2. This reaction of memantine with FMOC-Cl was optimized using a working solution of memantine at 1 µg/mL as a model. Several parameters were assayed: the FMOC-Cl concentration (0.1–5 mM); the borate buffer–acetonitrile ratios (1:1–2:1, pH 5–10); the borate buffer concentration (0.025–0.2 M); temperature (25–55°C); and reaction time (5–50 min). The FMOC-Cl reaction was stopped using different methods (glycine, methanol, or rapidly lowering temperature to 4°C).

Plasma and vitreous humour samples were frozen at –20°C until used. All samples were thawed to ambient temperature and centrifuged at 4000 rpm for 10 min. The upper layer was collected, filtered through a 0.2 µm pore nylon membrane, and aliquots (250 µL) with alkaline solution to control the amino group protonation were obtained to be derivatizated, extracted, and analyzed later.

Different solvents were assayed by liquid–liquid extraction [e.g., dichloromethane, diethyl ether-chloroform mixture (7:3, v/v), and *n*-hexane]. Other variations assayed were different proportions of extraction solvent: 1:1, 1:2, 1:3, and 1:5 (v/v); extraction in one or two stages; and verification of extraction before or after the derivatization reaction.

After the derivatization reaction, the samples were extracted and agitated for 60 s on a vortex mixer. The samples were shaken at 224 oscillations/min for 30 min and centrifuged at 4000 rpm for 10 min. The organic phase then was removed and evaporated to dryness in a thermostatic bath (40°C) under nitrogen stream. The residue was reconstituted with 50 µL of acetonitrile and agitated in a vortex for 2 min; 10 µL of the solution was used for HPLC analysis.

The recovery of memantine was determined by comparing peak heights ratios obtained from the spiked vitreous humour and plasma samples and the standard aqueous samples. The assay was determined at seven concentration levels in all dynamic range (10–1000 ng/mL) using three replicates per concentration.

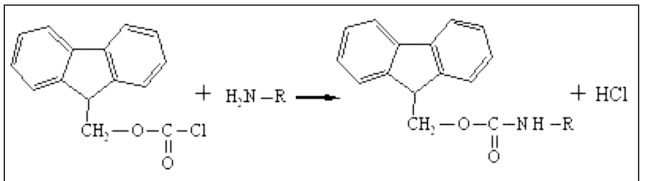

Figure 2. Simplified reaction scheme for the derivatization of memantine with 9-fluoroenylmethyl chloroformate chloride (FMOC-Cl).
R = C12H19 (Tricycle of memantine).

Analytical method validation

Calibration

The calibration curves of memantine at seven levels of concentration for triplicate (10–1000 ng/mL) were prepared by analysis of blank plasma and vitreous humour samples spiked with appropriate volumes of memantine working solutions. Rimantadine hydrochloride, neomycin hydrochloride, apramycin hydrochloride and amantadine hydrochloride were assayed at 50 ng/mL as IS. Samples were derivatised, extracted, and analyzed by liquid chromatography. Calibration curves were obtained by the linear least-squares method, characterized using the regression coefficient, slope and intercept, and used to determine the detection limits (LOD) (35,36). The calibration curves were based on the peak height ratios of memantine with different reagents that were used as the IS.

The matrix effect

It was verified by comparing the slopes obtained in the addition samples (plasma or vitreous humour) with the slopes obtained in the analysis of aqueous samples. The t-test was used ($p < 0.05$).

Precision and accuracy

Precision and accuracy were calculated according to VICH GL2 ("Guideline on Validation of Analytical Procedures: Methodology"). The intra- and inter-assay precision was evaluated at five concentration levels using six replicates per concentration in one day and for three different days, respectively, expressing it as the coefficient of variation (CV).

The accuracy values were determined for each concentration level by comparing the concentration of memantine obtained with the method with the nominal amount of memantine spiked (EU Council Directive 96/23/EC [2002/657/EC]).

Ruggedness study (minor changes)

The ruggedness study of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in several parameters. Ruggedness provides an indication of the reliability of an analytical procedure during normal usage. The Youden methodology was used (37). The parameters that could have influence on the results were chosen, such as the storage time of the borate buffer, acetonitrile lot or batch, operator, centrifuging time, type of membrane filter, and small variations in the mobile phase, such as flow rate and percentage of acetonitrile. This study was made using eight standards of aqueous memantine at 500 ng/mL, (Table III). The differences (D_i) and standard deviation of differences (S_{Di}) were calculated for detecting if some parameters can have an influence on the results.

$$D_i = \sum(F_i)/4 - \Sigma(f_i)/4$$

$$S_{Di} = \sqrt{\sum[2 \times \Sigma\,(D_i^2/7)]}$$

Stability

The stability of memantine and the IS in water, plasma, and vitreous humour, before and after derivatization, was studied. Each batch was prepared with six control samples of memantine and rimantadine (500 ng/mL). All samples were frozen at –20°C for 100 days for the aqueous samples and for 15 days for plasma and vitreous humour. In addition, the stability of analytes in the samples before derivatization was also evaluated by analysis after 24 h of storage at 4°C. The stability of memantine and the rimantadine derivate were studied over a 4 days storage period at –20°C. In all the experiments, the mean values at the baseline were compared with each time and a t-test and F-test were carried out.

Application of the method

All procedures were carried out under Project License PI 07/07 and approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza. The care and use of animals was compliant with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Fifteen New Zealand white rabbits (body weights up to 2.5–3 kg) were used in the bioavailability study. A volume of 0.3 mL of memantine saline solution (15 mg/mL) was administered periocularly by subtenon injection; the injection was performed under microscopic control, using topical lidocaine 1% drops, and the drug was injected into the tenon capsule using a 22 or 23-gauge trocar. Plasma (2 mL) and vitreous humour (1 mL) samples were obtained at 10, 60, 120, 240, and 1440 min. To that end, plasma collection was carried out using a catheter that was placed in the central ear artery. After that, the animals were sacrificed at these times after intravenous injection of sodium pentobarbital (30 mg/kg), the eyes were removed and cut at the equator, and the vitreous was extracted. The samples were stored at –20°C until analysis.

Results and Discussion

Memantine concentrations in vitreous humour and plasma were quantified using HPLC coupled with fluorescence detection, following precolumn derivatization with FMOC-Cl, a reagent that reacts with both primary and secondary amines. Different authors have described various analytical methods for determining memantine concentrations in different tissues (14, 16,17,21,22,38), some of which required complicated and expensive analytical systems such as LC–MS and CG–MS that are not readily available in some laboratories.

Several different derivatization reagents have also been used, such as FMOC-Cl (6), (2-naphthoxy) acetyl chloride (17), dansyl chloride, or o-phthaldialehyde (17); most studies reported that analytical assays used to measure adamantine, amantadine, or rimantadine could likely be adapted and used to measure memantine (16).

Optimization derivatization and extraction conditions

The derivatization reaction of memantine (Figure 2) with FMOC-Cl appeared to be dependent on the FMOC-Cl concentration, reaction time, pH, borate buffer concentration, and the volume ratio between the borate buffer and acetonitrile (Figure 3).

As can be seen, the optimal conditions for the derivatization reaction were: 1.5 mM FMOC-Cl, reaction time 20 min, borate buffer 0.1 M (pH 8.5), reaction temperature 25–40°C, borate buffer–acetonitrile ratio of 1:1–1:4.

The derivatization reaction was stopped by the addition of 5 mM glycine, due mainly to the fact that the chromatographic signal of its derivatization product did not interfere with analyte signals, and excess FMOC-Cl was eluted in the void volume.

The effect of FMOC-Cl concentration was evaluated in the interval of 0.1–5 mM. The peak height of the memantine analytes formed rose quickly when the FMOC-Cl concentration was increased from 0.1 to 1 mM and further increases did not result in any significant improvement. In practice, FMOC-Cl concentration was limited to 1.5 mM because higher concentrations increased reagent excess and had a deleterious effect on the quantification of low analyte concentrations. The effect of the reaction time was evaluated in the interval of 5–50 min. Conversion yields increased when the reaction time was extended from 5 to 20 min, but no further derivatization took place after 20 min of reaction. The effect of the pH was evaluated by using borate buffers with a pH ranging from 5 to 10. The reaction progressed very rapidly, and the highest analyte responses were reached at pH 8.5. At this pH, the reactions of the amino groups of memantine and the IS with FMOC-Cl were well developed, and all peaks were clearly resolved. The effect of the reaction temperature (25–55°C) was evaluated, but no significant improvement was observed; the appropriate reaction temperature was found to be 30°C. The effect of buffer–acetonitrile ratios from 1:1 to 2:1 and buffer concentrations of 0.025 to 0.2 M were studied. The results fell abruptly at a buffer–acetonitrile ratio of 1:4, and there were no significant differences with respect to the previous proportions. The derivatization reaction product is reduced at higher buffer–acetonitrile ratios. The results showed a precipitate using a buffer concentration of 0.2 M to the buffer–acetonitrile ratios of 1:4 and 1:5, probably due to precipitation of FMOC-Cl in the reaction media. Taking the buffer–acetonitrile ratio of 1:1 as optimal, there were no significant differences in the influence of the buffer concentration. Consequently, a buffer–acetonitrile ratio of 1:1 and a borate buffer concentration of 0.1 M were considered optimal.

To avoid problems caused by the interference of excess FMOC-Cl and the products of FMOC-Cl hydrolysis, which are highly fluorescent, several assays were performed to stop the derivatization reaction by hydrophilic reagents, such as glycine or methanol or even low temperature (i.e., rapid cooling) (15,17,24,31). The ability of hydrophilic reagents to improve the chromatographic separation of hydrophobic polyamine derivatives has been described previously (39,40). Furthermore, glycine reacts with excess FMOC-Cl, thus, eliminating extraction steps and the long incubation times often used in the derivatization reaction (41,42).

The efficiency of different solvents, including *n*-hexane, diethyl ether-chloroform mixture (7:3, v/v), and dichloro-

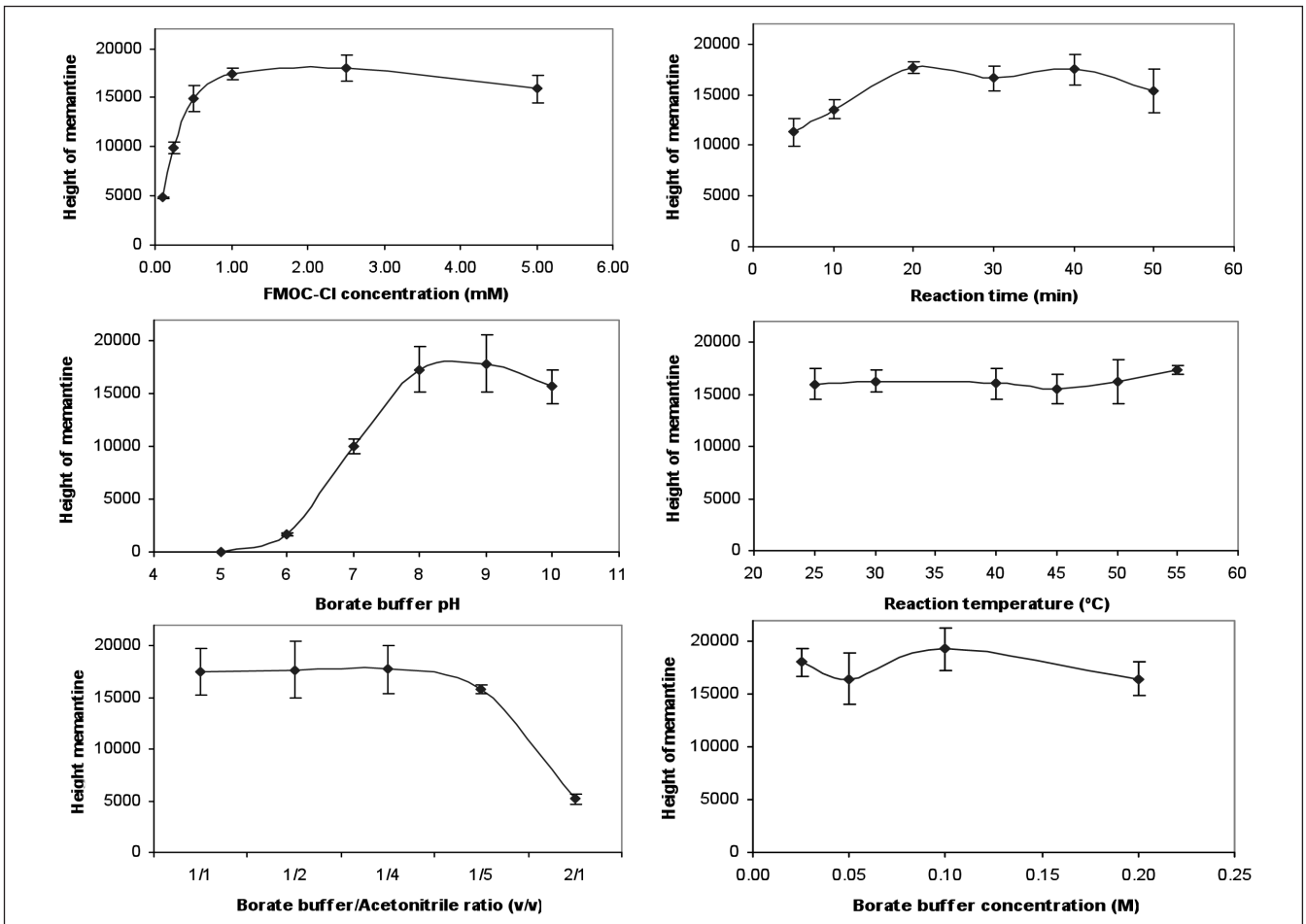

Figure 3. Effects of FMOC-Cl concentration, reaction time, borate buffer pH, reaction temperature, buffer/acetonitrile ratio, and borate buffer concentration.

methane, were assayed. High background noise, low sensitivity, and several interfering peaks due to the internal standard were obtained when using the diethyl ether–chloroform mixture, and dichloromethane was used in the assay; *n*-hexane was the most efficient extraction solvent with a 1:3 ratio (v/v). No significant differences were obtained to the 1:5 ratio and when the extraction was carried out using two stages.

Several extraction procedures were investigated; however, due to the low molecular weight of memantine, better analyte recoveries were achieved when derivatization was followed by extraction; this was accompanied by peaks from the derivatization of endogenous matrix amines with the derivatizing agent FMOC-Cl. These peaks did not interfere with the analyte or internal standard.

In conclusion, extraction in one stage using *n*-hexane (1:3, v/v), after derivatization, was determined to be the best conditions for the assay.

The recoveries for the vitreous and plasma samples were 93.6 and 86.1%, respectively. The assay was determined at all dynamic ranges of concentrations (10–1000 ng/mL), and the Cohran test was used to corroborate that no different recoveries were found depending upon the concentration level.

Analytical results and validation

Although several chromatographic methods have been developed for the determination of memantine and similar compounds by derivatization, these studies are limited by several inadequacies, such as the lack of an internal standard (6), time-consuming extraction procedures, low extraction yields (16), poor sensitivity or prolonged elution times (6,13,17), and a high cost (18,19,21,22). Finally, no validation data or related chromatograms for memantine analysis in vitreous humour are available (6).

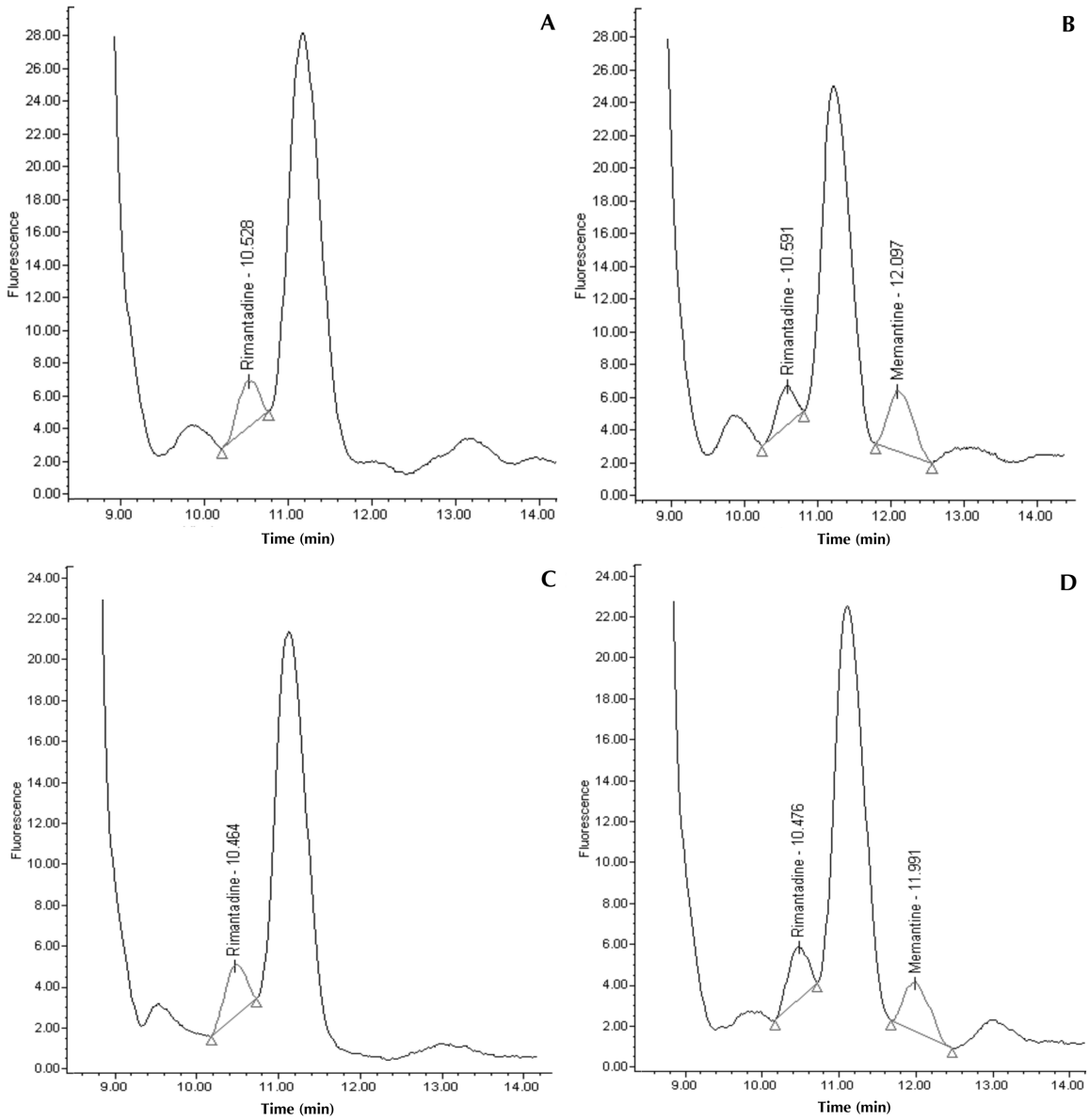

Figure 4. Chromatograms obtained from an extract of (A) rabbit blank plasma spiked with rimantadine as the IS (50 ng/mL), (B) rabbit blank plasma spiked with 50 ng/mL memantine and the IS (50 ng/mL), (C) rabbit blank vitreous humour spiked with rimantadine as the IS (50 ng/mL), (D) rabbit blank vitreous humor spiked with 50 ng/mL memantine and the IS (50 ng/mL).

Memantine was analyzed in plasma and vitreous humour using HPLC coupled with fluorescence detection and precolumn derivatization with a fluorescent agent. Rimantadine was selected as the internal standard because of its suitable retention times and the stability of the derivatized product.

The Novapack C_{18} HPLC reversed-phase column packed with 4 µm particle size, preceded by a guard column cartridge of the same material, was selected as the most efficient setup. In the present study, the optimal mobile phase was a mixture of acetonitrile–water (73:27, v/v) with a flow rate of 1.2 mL/min at 40°C and, for vitreous humour and plasma samples. When the proportion of water to acetonitrile was increased in the mobile phase, separation improved but sensitivity decreased. When the proportion of acetonitrile to water was increased, the retention time decreased and sensitivity increased; however, an interference peak was detected near the memantine peak in both vitreous humour and plasma samples. The use of gradient elution did not improve the separation. When methanol was included in the mobile phase, interference peaks were eluted after the compounds of interest. This substantially prolonged the chromatograms, making it impractical for a large number of samples because of the loss of sample stability over time.

The injection volume was 10 µL; FMOC-memantine and FMOC-rimantadine were detected at λex 260 nm and λem 315 nm.

Under the chromatographic conditions described herein, the retention times of memantine and rimantadine were approximately 10.5 and 12.0 min, respectively. No interference peaks or baseline aberrations were observed at these retention times for plasma and vitreous humour (Figure 4A and 4C). Chromatograms (Figure 4B and 4D) showed separation of the FMOC-Cl derivatives of memantine (50 ng/mL) and rimantadine (50 ng/mL) for plasma and vitreous humour. The total analysis time did not exceed 15 min, and all the compounds eluted as distinct symmetrical peaks. Moreover, no interference from endogenous tissue constituents was observed.

Liquid–liquid extraction with *n*-hexane (1:3, v/v), after the derivatization from the plasma and vitreous humour, produced a clean extract that was free of interfering endogenous peaks, as demonstrated by the drug-free plasma and vitreous blanks (Figure 4A, 4C).

Calibration

Calibration curves were plotted as the peak height ratio (drug/internal standard) versus the drug concentration. The assay was linear in the concentration range of 10–1000 ng/mL for memantine. The linear-regression equations obtained were $y = (0.0272 \pm 0.0003)x - (0.3475 \pm 0.1466)$, $r^2 = 0.9971$ for vitreous humour ($n = 21$), and $y = (0.0198 \pm 0.0002)x - (0.0087 \pm 0.0725)$, $r^2 = 0.9987$ for plasma (n = 21).

The existence of matrix effects was verified by adding a standard to a blank sample (Table I). A statistical comparison (*t*-test) of the slopes obtained in the addition experiment and in the analysis of synthetic samples showed that memantine was free of a matrix effect in vitreous humour (the slope of the addition experiment did not differ from the slope calculated by the analysis of water standard solutions). In contrast, in plasma there was a matrix effect, causing the slope of the addition experiment for memantine in plasma to be significantly lower than the slope obtained with water solutions.

LOQ, accuracy, precision, ruggedness, and stability

The LOD was determined as the lowest concentration with a signal-to-noise (S/N) ratio of 3. The LOD of memantine was 3 ng/mL. The LOQ, defined as S/N = 10, for plasma and vitreous humour was 10 ng/mL.

The accuracy and precision of intra- and inter-assay of memantine in tissue control samples of vitreous humour and plasma are shown in Table II. Accuracy is defined as: [(found value/added value) × 100%], which was 94.2–98.8% for vitreous humour and 96.4–105.5% for plasma, throughout the examinations for the five different concentrations. Precision is expressed as the coefficients of variation (CV). The intra- and inter-assay coefficients of variation evaluated in the standard solutions varied, respectively, between 2.0 and 8.1% and between 2.6 and 10.1% for vitreous humour, and between 2.9 and 3.8% and between 6.0 and 9.0% for plasma. The values were lower than the

Table I. Standard Addition Experiments in Vitreous Humour and Plasma from Memantine

		Memantine	
	Ref.*	Slope	R^2
Vitreous humour	94.9194	93.7825	0.9971
Plasma		84.3971†	0.9987

* Slope found in the analysis of water standard solutions.
† The differences between the standard addition and reference slopes are significant ($p < 0.05$).

Table II. Accuracy and Intra- and Inter-Assay Precision of Memantine in Vitreous Humour and Plasma

		Intra-assay ($n = 6$)			Inter-assay ($n = 18$)		
Matrix	Theorical conc. (ng/mL)	Conc. found (mean ± SD) (ng/mL)	Accuracy (%)	Precision CV (%)	Conc. found (mean ± SD) (ng/mL)	Accuracy (%)	Precision CV (%)
Vitreous humour	10	9.7 ± 0.8	96.9	8.1	9.5 ± 1.0	94.2	10.1
	50	49.4 ± 1.7	98.8	3.4	49.1 ± 3.5	98.2	7.1
	125	119.9 ± 8.4	95.9	7.0	119.6 ± 9.8	95.7	8.2
	500	477.4 ± 9.8	95.5	2.0	476.1 ± 12.5	95.2	2.6
	1000	986.7 ± 26.0	98.7	2.6	987.0 ± 40.9	98.7	4.2
Plasma	10	10.5 ± 0.3	104.6	3.2	10.0 ± 0.6	99.5	6.0
	50	50.7 ± 1.9	101.3	3.8	48.5 ± 3.1	96.5	6.3
	125	125.1 ± 4.3	100.1	3.4	120.6 ± 9.9	96.4	8.2
	500	527.3 ± 18.2	105.5	3.4	485.9 ± 33.9	97.2	7.0
	1000	1036.7 ± 30.8	103.7	2.9	973.7 ± 87.5	97.4	9.0

reference limits cited in the literature in every case (EU Council Directive 96/23/EC [2002/657/EC]).

Minor changes that may occur in the laboratory did not produce differences in the measurement results; the standard deviation of the differences, 10.87 (Table III), was not significantly larger than the standard deviation of the method in reproducibility conditions (12.5 and 33.9 for vitreous humour and plasma, respectively, Table II). None of the factors, when taken together, had any effect on the analytical results (Table III). The analytical method was sufficiently rugged in terms of the modifications selected.

The results of the stability studies show that memantine and rimantadine are stable at –20°C after 100 days in an aqueous solution and at least 15 days in plasma and vitreous humour. No degradation of the analytes before derivatization had taken place over a 24 h storage period at 4°C with the concentration of the control samples. The stability of the derivates at –20°C up to 10 h after the derivatization indicates that the derivates are sufficiently stable for the time required for the analysis.

Table III. Experiment Design for Ruggedness Study (Minor Changes)

		Combination of determination number									
Parameter	**Factor Value (F/f)***	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**D_i**	**S_{Di}**
Borate buffer storage†	1/2	1	1	1	1	2	2	2	2	3.51	
Acetonitrile branch‡	1/2	1	1	2	2	1	1	2	2	5.47	
Operator§	12	1	2	1	2	1	2	1	2	12.39	
Centrifuging time	10/8 min	10	10	8	8	8	8	10	10	–13.02	
Type of filter	0.45/0.2 mm	0.45	0.2	0.45	0.2	0.2	0.45	0.2	0.45	–0.54	
Flow rate	1.2/1 mL/min	1.2	1	1	1.2	1.2	1	1	1.2	2.01	
% acetonitrile	73/72	73	72	72	73	72	73	73	72	–6.63	
Observed result	MT (ng/mL)	464.12	456.90	476.31	459.83	480.81	459.23	453.68	449.38		10.87

* F/f: habitual factor/non-habitual factor; MT: memantine concentration; Di: differences; SDi: standard deviation of the differences

† 1: 25 days; 2: just prepared. ‡ 1: branch 1; 2: branch 2. § 1: habitual operator; 2 non-habitual operator.

Table IV. Mean ± SD Concentrations (µg/mL) of Memantine after Periocular Administration (Subtenon Administration) in Rabbits of 1.8 mg/kg of Body Weight*

Time (min)	Vitreous humour ± SD (µg/mL)	Plasma ± SD (µg/mL)
10	26.80 ± 0.21	0.208 ± 0.017
60	1.01 ± 0.02	0.059 ± 0.013
120	0.21 ± 0.12	0.030 ± 0.012
240	0.04 ± 0.001	0.020 ± 0.004
1440	N.D.	N.D.

* Each value represents the mean of three independent determinations. ND = not detected.

Application

Measurements of plasma and vitreous concentrations of memantine in rabbits after subtenon administration confirmed the utility of the proposed analytical methodology. The mean memantine concentrations in plasma and vitreous humour after the administration of 0.3 mL memantine (15 mg/mL) are shown in Table IV.

Conclusions

This analytical procedure was validated in terms of selectivity, recovery, linearity, precision, accuracy, ruggedness, and stability. The LOQ is adequate for use in ocular bioavailability studies. In conclusion, the results indicate that this HPLC procedure represents a highly specific and reproducible method that provides consistent quantification of memantine in the plasma and vitreous humour.

Acknowledgments

This study was supported by the project 23154/2008 of the University of Zaragoza, Spain. Belen Puente received a grant from the OTRI (Investigation Research Transference Office) from the University of Zaragoza, Spain.

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Manuscript received May 28, 2010;
revision received September 10, 2010.