Research Article

High-Performance Liquid Chromatographic Ultraviolet Determination of Memantine Hydrochloride after *In Vitro* Transdermal Diffusion Studies

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The purpose of the present work was to validate an accurate and precise high-performance liquid chromatography (HPLC) method involving ultraviolet detection for the quantitative analysis of memantine hydrochloride. In order to analyze a molecule with no chromophoric groups that could be detected by a UV/visible detector, it was necessary to extract the drug and to perform a dansylation reaction that enabled the UV/visible detection of the derivatized molecule. Separation was carried out with a 150 mm Kromasil C18 column at room temperature. The detection response, at 218 nm, was found to be linear in the concentration range from 0.5 to 50 $\mu\text{g/mL}$. The method was validated for specificity, linearity, precision, accuracy, limit of detection, limit of quantification, and robustness. The limit of detection (LOD) was 0.144 $\mu\text{g/mL}$, and the limit of quantification (LOQ) was 0.437 $\mu\text{g/mL}$. The dansylated memantine complex was stable for at least five days in all the conditions evaluated. The potential use of this method has been demonstrated by the quantification of memantine hydrochloride contained in samples from the study of its *in vitro* transdermal permeation.

1. Introduction

In recent years, the population has aged considerably. Advanced age is associated with damage of brain function, and the prevalence of degenerative diseases such as Alzheimer's disease has, therefore, risen. Alzheimer's disease is a progressive and fatal brain disease that manifests itself through many symptoms. The earliest observable symptom is memory loss, which becomes more intense as the disease advances, eventually constituting a severe state of amnesia [1, 2].

Memantine (a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist) was developed in order to

achieve better results in the treatment of this pathology. An alteration of the glutamatergic system provokes neurotoxicity and cognitive failure [3]. Memantine acts on the glutamatergic system by blocking NMDA receptors and inhibiting their overstimulation by glutamate [4]. Due to its mechanism of action, memantine can delay an Alzheimer's patient's cognitive and functional failure [5].

Randomized, placebo-controlled trial clinical studies have demonstrated the efficacy and safety of memantine in patients with mild to moderate vascular dementia [6]. As a result, this antagonist, which has a different mechanism of action to that of acetylcholinesterase inhibitors, has been

approved for the treatment of the symptoms that characterize the moderate-serious state of Alzheimer's disease [7].

The tolerability and safety data for memantine obtained in clinical trials show that adverse events due to this antagonist are infrequent and mild and include confusion, dizziness, headache, and fatigue [8].

The purpose of our research was to study the transdermal administration of memantine. The samples obtained in these experiments included endogenous substances released from the skin that could have interfered with subsequent analyses.

A review of the literature reveals that different analytical methods have been described for the determination of memantine hydrochloride. They include HPLC with UV/Visible detection, alone [9, 10] or in tandem with mass spectrometry (MS) [11, 12], and fluorescence detection [10, 13–15]. But they rely on an infrastructure that is not available to small laboratories, present too many interferences from endogenous compounds present in the skin, or provide an elevated range of concentration, thus not suitable for the purpose of our research.

In the present work, we have developed and validated a specific, accurate, precise, and robust HPLC method for the quantitative determination of memantine hydrochloride after *in vitro* transdermal diffusion studies by means of ultraviolet detection after dansyl derivatization procedure.

2. Material and Methods

2.1. Reagents and Chemicals. Memantine hydrochloride (1-amino-3,5-dimethyladamantane hydrochloride) was purchased from Sigma-Aldrich Química, S. A. and had a stated purity of $\geq 98\%$. Potassium bicarbonate (KHCO_3) and potassium hydroxide (KOH) were also provided by Sigma-Aldrich Química, S. A. Dansyl chloride (5-(Dimethylamino)naphthalene-1-sulfonyl chloride) was provided by Iberlabo, S. A. Acetonitrile (ACN), methanol and ammonium di-hydrogen phosphate (96–102%, w/w) were obtained from AnálisisVínculos, S. L., and hydrochloric acid was purchased from J. T. Baker. All compounds were of analytical grade. Ultrapure water used to prepare all solutions was obtained from a Barnstead NANOpure system.

2.2. Preparation of Standard Solutions. A 5 mg/mL stock solution of memantine hydrochloride was prepared in isotonic buffer [NaCl (150 mM)-HEPES (20 mM) (pH 7.4)].

Six standard solutions (50, 25, 10, 5, 1, and 0.5 $\mu\text{g/mL}$) were prepared by further dilution of the memantine stock solution with an isotonic buffer. These standard fresh solutions were used for the calibration curves.

2.3. Extraction and Derivatization. Different protocols were carried out to optimize the extraction procedure, all of which consisted of mixing an aliquot of the memantine solutions (which varied between 180 and 220 μL) with acetonitrile (in a volume of 160 to 200 μL) in an Eppendorf tube (1.5 mL). In order to separate the organic and aqueous layers, the mixture was rendered alkaline by adding a KOH solution (3 M)

FIGURE 1: Diagram of the dansylation reaction with memantine.

(180–220 μL) or an alkaline buffer solution [KOH (1.5 M)- KHCO_3 (1 M) (pH 10.6)] (200 μL). It was then vortex-extracted for 1 minute and centrifuged at 13000 rpm for 1.5 minutes. Following centrifugation, two phases were clearly separated; the lower layer contained the aqueous phase and the upper layer contained the organic phase, which consisted of ACN containing the extracted memantine.

Different protocols were also assayed in order to optimize our derivatization procedure. An aliquot of 70 μL of the acetonitrile containing the extracted memantine was removed from the upper layer and placed in a PCR tube (0.2 mL) or an Eppendorf tube (1.5 mL) and 40–60 μL of an alkaline-buffered solution [KOH (1.5 M)- KHCO_3 (1 M) (pH 10.6)] or 50 μL of a KOH solution (3 M) were added. The solution was then mixed with the solution of the derivatizing agent [40–60 μL of a dansyl chloride solution (1.7 mg/mL in ACN), were created immediately prior to being used]. The mixture was shaken vigorously for 5 seconds and maintained in the dark at 50°C during the derivatization reaction. After derivatization (35–55 minutes), the mixture was cooled in ice water for 10 minutes. Once again, two phases were obtained. An aliquot of 70 μL of the acetonitrile was removed from the upper layer, and 10 μL of HCl (1 N) was added to halt the reaction.

The derivatization reaction is shown in Figure 1, which shows how memantine was derivatized into a dansyl-memantine complex that provided the chromophoric groups that enabled the UV/visible detection [16].

Once the chemical procedure had terminated, the derivatized memantine solutions were stored at -20°C until HPLC analysis.

2.4. Instrumentation and Chromatographic Conditions. A Waters system equipped with a Waters 600 quaternary pump (including a Waters 996 UV/VIS diode-array detector), and a Waters 717 Plus automatic injector was employed for HPLC analysis. Acquisition and treatment of computerized data were performed with the Millennium Chromatography Software. The instrumentation components were purchased from Waters Cromatografía, S. A.

Chromatographic separation of the analytes was achieved at room temperature ($25^\circ\text{C} \pm 2^\circ\text{C}$) using a Kromasil C18

(150 × 4.6 mm) reverse-phase column packed with 5 μm C18 silica particles. A mixture of hydrochloric acid water solution (0.01 M; pH 2.4)-methanol (15 : 85, v/v) pumped at a flow rate of 1.2 mL·min⁻¹ was used as a mobile phase. The injection volume was 50 μL . Absorbances were measured at 218 nm.

2.5. Validation. The analytical method was validated with six different concentrations of memantine hydrochloride (0.5, 1, 5, 10, 25, and 50 $\mu\text{g/mL}$). Each concentration was assayed five times to determine within-day reproducibility. In order to detect inter-day variation, the procedure described previously was repeated on ten different days.

Calibration curves were obtained by least square linear regression analysis of the peak area obtained as a function of the concentration of the drug. An average curve was constructed using all the data from all the calibration curves obtained.

Absence of interference was investigated by analyzing 10 blank isotonic solutions in order to determine the specificity of the methods. The isotonic buffer used to prepare the standard solutions for calibration [NaCl (150 mM)-HEPES (20 mM) (pH 7.4)] was considered a blank isotonic solution.

The linearity of the calibration curves was tested by statistical comparison (Student's *t*-test) of the slopes and intercepts of the calibration curves with zero and the correlation coefficient with 1, respectively.

Accuracy expresses the closeness of agreement between the calculated value and the accepted reference value, namely the conventionally true value. It was defined as the relative error of known concentration solutions. Measures had to be within $\pm 10\%$ for all concentrations to be considered acceptable [17, 18]. The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous drug solution under prescribed conditions. It provides information regarding random error. The variance of repeatability and intermediate precision, as well as the corresponding relative standard deviation (R.S.D.), were calculated from the estimated concentrations. To be considered acceptable, the R.S.D. had to be lower than 10% at all the concentrations analyzed [17, 18].

The detection limit (LOD) is the lowest amount of an analyte that can be detected but not necessarily quantified as an exact value. The quantification limit (LOQ) is the lowest amount of the analyte that can be quantitatively determined with defined precision under the experimental conditions. Assuming a normal distribution of measured concentration values, LOD and LOQ were calculated from the residual standard deviation of the regression data according to the criteria $\text{LOD} = 3.3 \times (\text{S.D.}/b)$ and $\text{LOQ} = 10 \times (\text{S.D.}/b)$ [19], where S.D. was the residual standard deviation and *b* was the slope of the linear regression equation.

The robustness of a method is its ability to remain unaffected by small changes in operating conditions. To determine robustness, the experimental conditions were deliberately altered at three different levels and evaluated for chromatographic response and retention time [20–22].

Factors were changed one at a time in order to study the effect. These alterations were carried out by varying the detector wavelength (± 2 nm (216 nm–220 nm)), the buffer and methanol ratio of the composition of the mobile phase (82 : 18–88 : 12, v/v), the pH of the mobile phase (2.2–2.6), and the flow rate of the mobile phase (1.0–1.4 mL·min⁻¹).

2.6. Stability Test. Six groups of 100 $\mu\text{g/mL}$ memantine hydrochloride saline-buffered solution were prepared to determine the stability of the dansylated memantine complex.

The first group was prepared by dilution of a 5 mg/mL memantine stock solution in order to obtain solutions at the appropriate concentrations. Once extraction and derivatization had finalized, solutions were quantified by HPLC in the dark. The rest of the groups were prepared in an identical manner and following extraction and derivatization, the solutions were stored in darkness under different conditions: freezer (-80°C and -20°C), refrigerator (4°C), laboratory stove (37°C), and at ambient temperature ($25^\circ\text{C} \pm 2^\circ\text{C}$). All these groups were assayed to determine the residual concentration of memantine at 8, 24, 48, and 120 hours after preparation.

2.7. Application of the Method to Determine In Vitro Transdermal Diffusion of Memantine Hydrochloride. The validated HPLC method was used to quantify memantine hydrochloride in the samples obtained from *in vitro* transdermal diffusion studies. These experiments were performed employing vertical standard diffusion cells (Franz type) purchased from DISA (Milan, Italy) with a diffusion area of 0.567 cm² and skin from pig ear as the membrane.

Pig ears were generously provided by the Faculty of Medicine, University of Valencia (Valencia, Spain) immediately following the death of the animal.

One mL of a solution of memantine hydrochloride (2.7 mg/mL) prepared in an isotonic buffer [NaCl (150 mM)-HEPES (20 mM), pH 7.4] was placed in the donor compartment. The receptor compartment (4.2 mL volume) was filled with the same isotonic buffer (pH 7.4), which was thermostated at $(36.93 \pm 0.13)^\circ\text{C}$ ($n = 36$) and stirred by a rotating Teflon-coated supermagnet placed inside the cell to prevent boundary layer effects. Two hundred μL samples were removed manually from the receptor chamber at pre-determined time intervals. The volume of sample removed was replaced with the same volume of buffer pH 7.4. The memantine hydrochloride contained in each sample was recorded in order to calculate the accumulative amount in the receptor compartment. At the end of the *in vitro* transdermal diffusion experiments the amount of drug retained in the skin was determined by the HPLC method after extraction by shaking the skin for 12 h with 1 mL of the isotonic buffer (pH 7.4). The transdermal flux (*J*) was estimated from the slope of the linear region (steady-state portion) of the plot of the accumulated amount of memantine hydrochloride ($\mu\text{g}/\text{cm}^2$) against time (hours).

3. Results and Discussion

In this work, we describe a new, specific, accurate, precise, and robust HPLC method for the qualitative and quantitative analysis of samples of a noncompetitive NMDA receptor antagonist, namely, memantine hydrochloride.

As previously described, because of memantine hydrochloride's on-existent UV absorbance, a conjugation reaction (dansyl derivatization) is necessary in order to introduce a chromophoric group that allows UV/Visible detection (see Figure 1). For this purpose, an extraction procedure was performed before the derivatization reaction to avoid interference peaks of skin endogenous compounds and to concentrate the memantine presented in isotonic solutions. The results of the optimization of the memantine extraction procedure are shown in Table 1.

There are several factors that can have a bearing on the extraction of memantine. The volume of the aliquot determines the total amount of memantine to be extracted but also can modify the separation of the organic and aqueous layers and their appearance. Two other relevant factors are the volume of the organic dissolvent (acetonitrile (ACN)) and the volume and nature of the alkaline solution (KOH or KOH-KHCO₃) employed, as the procedure can alter the extent of the separation of the organic and aqueous layers.

The best protocol for optimizing the extraction procedure consisted of mixing an aliquot of 200 μ L of the memantine solution with 180 μ L of ACN. In these conditions, the concentration of memantine was sufficient for the analysis of the most diluted isotonic solutions, and the volume of ACN allowed the upper layer to be recovered adequately.

The addition of 200 μ L of an alkaline buffer solution [KOH (1.5 M)-KHCO₃ (1 M) (pH 10.6)] did not produce an appropriate separation of the organic and aqueous layers. For this reason the solution was rendered alkaline by adding 200 μ L of a KOH solution (3 M). This volume was selected because higher or lower volumes did not modify the separation of the layers or, indeed, the concentration recovery.

Once the memantine was extracted from the isotonic solutions, the derivatization procedure was performed. As in the previously described protocol, several factors can modify the derivatization of the memantine extracted from isotonic solutions. Many conditions can modify the output of dansyl derivatization, such as the capacity of the tube in which the reaction takes place, the volume and nature of the alkaline solution used (pH of medium), the volume of the dansyl chloride solution, and the reaction time [23]. The results of the optimization of the memantine conjugation reaction are shown in Table 2.

PCR tubes (0.2 mL) were selected because when the reaction was carried out in Eppendorf tubes (1.5 mL), the concentration recovery obtained was $19.8 \pm 4.4\%$. This low value was probably due to the temperature at which the reaction took place (50°C), which would have led to part of the solvent (especially ACN) evaporating inside the tube. When an Eppendorf tube was used, an empty space appeared high up inside the tube, which interfered with the reaction; this did not occur when a PCR tube was employed, as the recipient was practically full with the reagents.

FIGURE 2: Dansylated memantine spectra under UV/Visible detection.

Cleaner chromatograms can be obtained if a minimum amount of dansyl is used [24], but it is important that there is enough reagent to ensure the complete dansylation of all the memantine present in isotonic solutions. Other authors have selected different concentrations for derivatization in the range of 2–30 mg/mL of dansyl in ACN [23, 25–27], but the concentration used in our study (1.7 mg/mL) provided enough dansyl for an optimal derivatization of memantine and did not produce any interference with the separation and detection achieved with HPLC. The volume of the dansyl ACN solution that we employed (50 μ L) produced a concentration recovery of 100% (see Table 2).

As stated previously, reaction time and temperature are also important factors that determine the efficacy of a derivatization procedure. Other authors have employed different temperatures and reaction times to those used herein; Kang et al. chose a reaction time of 30 minutes at 80°C [23], Minocha et al. and Smith and Davies established a reaction time of 60 minutes at 60°C [25, 26], and the derivatization procedure of Escribano and Legaz lasted 16 hours and was carried out at room temperature [27]. As these results show, the higher the temperature applied, the less time was required for the reaction to be completed. We have determined a temperature of 50°C and a period of 45 minutes as optimal conditions (see Table 2). Although higher temperatures can reduce the reaction time, they also increase the rate at which the solvent evaporates.

The alkaline buffer solution employed and the volume employed were found to be relevant factors. The addition of 50 μ L of a KOH solution (3 M) produced a concentration recovery of $10.0 \pm 1.0\%$. When an alkaline buffer solution [KOH (1.5 M)-KHCO₃ (1 M) (pH 10.6)] was used, the optimal volume for the reaction was 50 μ L (see Table 2).

We employed a 150 mm reverse phase column (Kromasil C18) to produce the highest resolution of the dansylated memantine peaks. Dansylated memantine spectra resulted in three maximum peaks of absorbance at 218.0, 249.7, and 332.6 nm (Figure 2). A wavelength of 218 nm was selected since it permitted the quantification of lower concentrations of memantine without interferences of skin endogenous compounds.

TABLE 1: Optimization of the memantine extraction process.

Condition	Volume modification (μL)	Real concentration ($\mu\text{g/mL}$)	Concentration recovered (%)	R.S.D. (%)	
Solvent extraction	Sample	180	20.0	102	0.500
		200	25.0	100	1.30
		220	31.4	96.5	8.64
	ACN	160	30.0	101	8.32
		180	25.0	100	1.30
		220	21.4	94.7	0.84
	KOH	180	25.0	99.6	4.60
		200	25.0	100	1.30
		220	25.0	98.4	1.48

TABLE 2: Optimization of the derivatization of the memantine extracted.

Condition	Volume modification (μL)	Real concentration ($\mu\text{g/mL}$)	Concentration recovered (%)	R.S.D. (%)	
Dansylation	Dansyl ACN	40	27.3	94.8	0.43
		50	25.0	100	1.30
		60	24.0	103	1.56
	KOH-KHCO ₃	40	25.0	79.5	2.37
		50	25.0	100	1.30
		60	25.0	82.8	4.20
	Reaction time (minutes)	35	25.0	97.9	1.41
		45	25.0	100	1.30
		55	25.0	98.7	1.56

FIGURE 3: Dansylated memantine (D.M.) chromatogram (25 $\mu\text{g/mL}$).

A representative dansylated memantine chromatogram is shown in Figure 3. The retention time of dansylated memantine obtained was 6.95 ± 0.01 minutes (mean $\pm$ S.D.; $n = 10$).

Ten blank isotonic solutions were analyzed to evaluate specificity. No significant interfering peaks were observed at the retention time for the dansylated memantine obtained.

Ten calibration curves were assayed; these calibration curves were constructed by plotting the concentration of the analyte against the peak area response. The linearity between the response (y) and the corresponding concentration (x) over the 0.5–50 $\mu\text{g/mL}$ concentration range was evaluated.

The results of the least square linear regression analysis revealed correlation coefficients ≥ 0.999 . The slope of the calibration curve was statistically different from zero ($P < 0.05$), and the intercept was not statistically different from zero ($P > 0.05$).

Intraday and interday precision and accuracy were evaluated by analyzing each concentration of memantine standard

FIGURE 4: The accumulated amounts in receptor compartments ($\mu\text{g/cm}^2$) plotted against time (hours).

and its relative error values. Sets of five replicates were studied on the same day and on ten different days. Accuracy and precision were within acceptable limits: the values obtained for all concentrations were below 10% (see Table 3).

The limit of detection of dansylated memantine was found to be 0.144 $\mu\text{g/mL}$, and the limit of quantification was established as 0.437 $\mu\text{g/mL}$.

The results of the robustness of the method are shown in Table 4. Variation of the detector wavelength, composition, pH, and flow rate of the mobile phase had no significant effect on the retention time and chromatographic response of the method, thus indicating that the method was robust.

TABLE 3: Intraday and interday precision and accuracy of the HPLC method.

Real concentration ($\mu\text{g/mL}$)	Between-day variability ($n = 10$)		Within-day variability ($n = 5$)	
	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
0.5	5.49	7.17	6.17	5.82
1	8.84	6.35	6.08	4.20
5	2.21	6.83	0.47	4.93
10	3.99	2.67	3.78	2.35
25	0.47	2.05	3.19	5.11
50	0.40	3.75	0.01	5.34

TABLE 4: Robustness of the HPLC method: wavelength and mobile phase composition parameters.

Condition		Modification	Area (mean $\pm$ S.D.)	R.S.D. (%)
Detector wavelength (nm)		216	13324579 $\pm$ 894588	6.71
		218	13874190 $\pm$ 427445	3.08
		220	13422096 $\pm$ 894449	6.66
pH		2.2	16507082 $\pm$ 949458	5.75
		2.4	14097231 $\pm$ 565342	4.01
		2.6	15038293 $\pm$ 945649	6.29
Mobile phase	Composition (v/v)	82 : 18	14864090 $\pm$ 1292173	8.69
		85 : 15	14097231 $\pm$ 565342	4.01
		88 : 12	15537918 $\pm$ 410088	2.64
	Flow rate ($\text{mL}\cdot\text{min}^{-1}$)	1.0	14063017 $\pm$ 1175676	8.36
		1.2	13531768 $\pm$ 1047552	7.74
		1.4	15590846 $\pm$ 1113410	7.14

TABLE 5: Stability of the dansyl-memantine complex in solution. Concentration of the molecule complex is expressed as the percentage respective to the initial concentration (mean $\pm$ S.D.).

Time (days/hours)	37°C	25°C	4°C	-20°C	-80°C
0.33/8	96.2 $\pm$ 1.9	100 $\pm$ 1	97.8 $\pm$ 1.6	99.2 $\pm$ 0.4	101 $\pm$ 1
1/24	92.8 $\pm$ 1.5	102 $\pm$ 0.01	101 $\pm$ 0.2	101 $\pm$ 3	100 $\pm$ 0.9
2/48	93.4 $\pm$ 4.2	97.7 $\pm$ 1.9	97.8 $\pm$ 0.5	101 $\pm$ 3	104 $\pm$ 0.3
5/120	98.2 $\pm$ 3.8	92.8 $\pm$ 1.6	96.5 $\pm$ 0.8	95.5 $\pm$ 4	94.5 $\pm$ 3.2

Six groups, each stored under different conditions, were employed to evaluate the stability of the dansyl-memantine complex. As can be seen in Table 5, all the assayed conditions maintained a concentration of memantine-derivate above 90% for at least five days.

The potential use of this method has been demonstrated by the study of *in vitro* transdermal permeation of memantine hydrochloride. Memantine contained in samples obtained from *in vitro* diffusion experiments has been quantified by means of the HPLC method validated and reported in this paper. The accumulated amount of memantine in receptor compartment ($\mu\text{g}/\text{cm}^2$) plotted against time (hours) is shown in Figure 4. The memantine transdermal flux value across porcine skin was $7.10 \pm 1.50 \mu\text{g}/\text{cm}^2\cdot\text{h}$ [mean $\pm$ S.D.; $n = 8$], and the amount of drug retained in skin after 10 hours of diffusion was $86.3 \pm 18.2 \mu\text{g}/\text{cm}^2$ [mean $\pm$ S.D.; $n = 8$].

4. Conclusions

We have developed and validated a new isocratic HPLC-UV method for the acute and precise determination of memantine hydrochloride. The results of the validation prove the method to be satisfactory in terms of specificity, limit of detection and quantification, accuracy, precision, and robustness. Therefore, the method is suitable for the routine determination of memantine hydrochloride presented in samples obtained from transdermal diffusion studies.

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