Acta Chimica & Pharmaceutica Indica

Acta Chim. Pharm. Indica: 4(3), 2014, 170-179
ISSN 2277-288X

A PRE-COLUMN DERIVATIZATION TECHNIQUE FOR THE DEVELOPMENT AND VALIDATION OF A HPLC-UV METHOD FOR THE DETERMINATION OF AMANTADINE HYDROCHLORIDE IN BULK AND FORMULATIONS BY USING (2-NAPTHOXY) ACETYL CHLORIDE

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(*Received : 11.08.2014; Accepted :* 23.08.2014)

ABSTRACT

A simple, precise, and accurate HPLC method has been developed and validated for the quantitative analysis of amantadine hydrochloride in tablet form by using (2-Napthoxy) Acetyl chloride as derivatization agent and memantine as an internal standard. An isocratic separation was achieved using inertsil ODS-3V, 250 x 4.6, 5 µm column with a flow rate of 1.5 mL/min and UV detector at 226 nm. The mobile phase consisted of 0.02 M ammonium acetate buffer and methanol in the ratio (12:88). The retention time for Amantadine and Memantine was around 6.23 and 8.62. The method was validated for specificity, linearity, precision, accuracy, robustness, and solution stability. The specificity of the method was determined by assessing interference from the placebo. The method was linear over the concentration range 28-171 µg/mL (r^2 = 0.999) with a Limit of Detection (LOD) and Limit of Quantitation (LOQ) 0.23 and 0.69 µg/mL, respectively. The accuracy of the method was between 98.9-99.6%. The method was found to be robust and suitable for the quantitative analysis of amantadine hydrochloride in a tablet formulation.

Key words: Amantadine HCL, HPLC, Precolumn derivatization, (2-Napthoxy) Acetyl chloride.

INTRODUCTION

Amantadine hydrochloride is designated chemically as 1-adamantanamine hydrochloride. It is a stable white or nearly white crystalline powder, freely soluble in water and soluble in alcohol and chloroform. Amantadine is an antiviral drug, which also acts as an antiparkinson agent, for which it is usually combined with L-DOPA when L-DOPA responses decline (probably due to tolerance). It is a derivate of adamantane, like a similar drug rimantadine. The mechanism of action of Amantadine in the treatment of Parkinson's disease and drug-induced extrapyramidal reactions is not known. It has been shown to cause an increase in dopamine release in the animal brain, and does not possess anticholinergic activity. The mechanism of its antiparkinsonic effect is not fully understood, but it appears to be releasing dopamine from the nerve endings of the brain cells, together with stimulation of norepinephrine response. It also has NMDA receptor antagonistic effects. The antiviral mechanism seems to be unrelated. The drug interferes

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with a viral protein, M2 (an ion channel), which is needed for the viral particle to become "uncoated" once it is taken inside the cell by endocytosis. Its molecular weight and molecular formula is 187.71 and $C_{10}H_{17}N$ N.HCl. Due to lack of required chromophores, Amantadine cannot be readily assayed by HPLC-UV techniques. It's highly basic (pKa 10.71) and lipophilic (log P 2.53) nature suggests that it may show binding with (2-Napthoxy) Acetyl chloride, 9-fluorenylmethyl chloroformate, dansyl chloride etc due to interaction of its primary amine group.

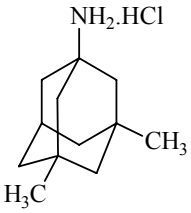


Fig. 1: Structure of memantine HCl

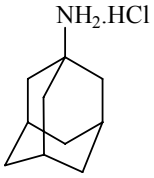


Fig. 2: Structure of amantadine HCl

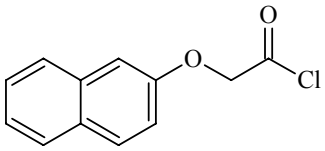
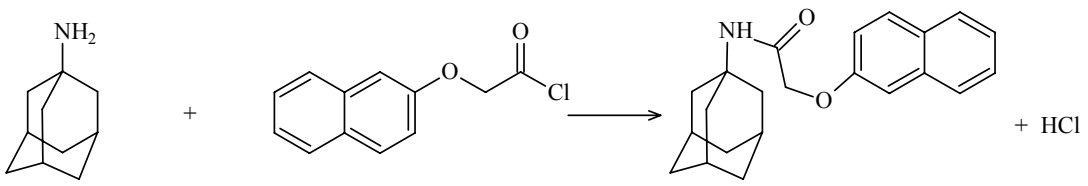


Fig. 3: Structure of 2-naphthoxy acetyl chloride

Amantadine is official in USP, I.P. and J.P. monograph. Literature survey states that various analytical methods have been reported such as Spectrophotometric[1], HPLC-fluorescence detection[2-9] and HPLC- UV[10,11] by using various derivatization agents. Also few LCMS and GC-FID/MS methods were reported[12-20] for the determination of Amantadine hydrochloride in bulk and biological samples. However all these methods are time consuming, Non-economical and not routine for commercial scale. So, we felt to develop a simple, precise and accurate RP-HPLC method for the determination of Amantadine hydrochloride in bulk and formulation by using Memantine as an internal standard and (2-Napthoxy) acetyl chloride[21] as a derivatization agent[22]. The developed method was validated as per ICH Guidelines[23,24].



Adamantan-1-amine hydrochloride
Mol. formula: $C_{10}H_{17}N$
Mol. wt.: 151.25

2-(Naphthalen-2-yloxy)acetyl chloride
Chemical formula: $C_{12}H_9ClO_2$
Mol. wt.: 220.65

(Adamantan-1-yl)-2-(naphthalen-2-yloxy)acetamide
Chemical formula: $C_{22}H_{25}NO_2$
Mol. wt.: 335.45

Fig. 4: Reaction mechanism

EXPERIMENTAL

Materials and methods

Chemicals and solvents

Pure standard of amantadine hydrochloride and memantine hydrochloride were provided as a gift sample by Torrent Pharmaceuticals, Ahmadabad. Amantadine tablets were procured from local market Hyderabad, India. Ammonium Acetate (AR grade), Sodium Hydroxide (GR grade) were procured from Merck India. (2-Naphthoxy) Acetyl chloride (GR grade) was procured from Aldrich, USA. Toluene (HPLC grade), Methanol (HPLC grade), Triethyl amine (GR grade) were procured from SDFCL Mumbai, India and High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

Instrumentation and apparatus

The chromatography analysis was performed using Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/visible detector or 2998 PDA detector, degasser, quaternary pump, and auto sampler system. The output signals were monitored and processed using Empower 2 software. Cintex digital water bath was used for hydrolysis studies. Photo-stability studies were carried out in the photo-stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India). The pH of the solutions was measured by a pH meter (Mettler-Toledo, Switzerland). Class A Volumetric flasks, pipettes, beakers, measuring cylinders and centrifuge tubes of Borosil glass were used.

Chromatographic conditions

Separation of Amantadine was achieved on Inertsil ODS-3V, 250 × 4.6, 5 µ column and mixture of 0.02 M ammonium acetate buffer and methanol in the ratio of 22:88 (v/v) as mobile phase at flow rate of 1.5 mL/minute with isocratic mode. Detection of Amantadine was performed at 226 nm. Column temperature was maintained at 40°C. Sample injection volume was 20 µL and total run time of the method was 12 minutes. Mixture of Toluene and Triethylamine was used as diluent.

Preparation of solutions

Preparation of derivatization reagent

Dissolved 300 mg of (2-naptoxy) acetyl chloride in 200 mL of toluene.

Preparation of diluent

2 mL of Triethylamine was added to 1000 mL of Toluene and mixed well.

Preparation of mobile phase

120 mL of 0.02 M ammonium acetate buffer (1.5 g in 1000 mL of water) was added to 880 mL of methanol. Degassed by sonication in an ultrasonic bath for 10 minutes.

Preparation of standard stock solution

1000 mg of Amantadine hydrochloride pure standard was added to 500 mL of volumetric flask and added about 250 mL of water and sonicated to dissolve and finally made up the volume with water. 4 mL of the above solution is transferred to 50 mL volumetric flask and made up the volume with water (160 ppm).

Preparation of internal standard stock solution

1000 mg of Memantine hydrochloride pure standard was added to 500 mL of volumetric flask and added about 30 mL of water and sonicated to dissolve and finally made up the volume with water. 4 mL of the above solution is transferred to 50 mL volumetric flask and made up the volume with water.

Preparation of sample solution

Twenty commercial tablets were weighed and powdered. A quantity of the powder equivalent to 100 mg of Amantadine hydrochloride was accurately weighed, transferred to 500 mL volumetric flask and is dissolved in 300 mL of the water. Sonicated the solution for few minutes to dissolve the drug completely. Further diluted 4 mL of the above solution to 50 mL with water and proceeded for derivatization process.

Derivatization procedure of standard and sample

Accurately transferred 5 mL of standard stock solution and 5 mL of internal standard stock solution into 20 mL test tube separately for both sample and standard solutions. Added 2 mL of 5 N NaOH to each test tube and cyclomixed on a vortex mixer for 2 minutes and added 3 mL of diluent and cyclomixed for 2 minutes. Finally left the solutions on bench top for 5 minutes. 2 mL of upper toluene layer from the above solutions is transferred into a 20 mL test tube and added 2 mL of derivatization reagent and cyclomixed for 2 minutes. Kept the solutions on bench top for 5 minutes. Added 3 mL of methanol to the solutions and cyclomixed for 1 minute. Injected each solution into the HPLC.

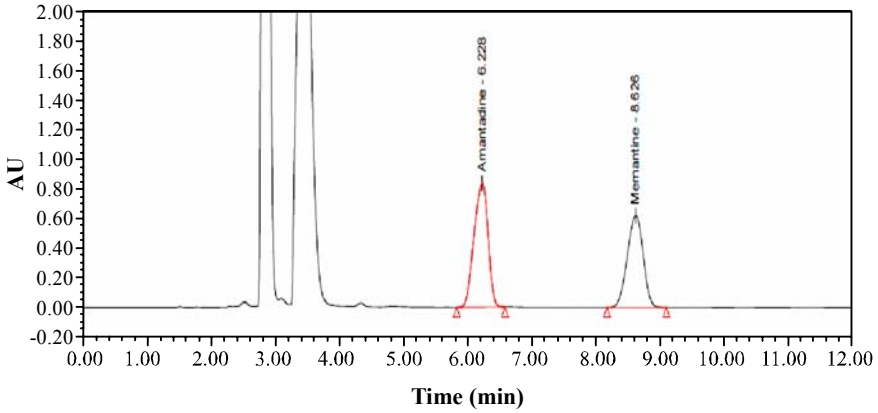


Fig. 5: Standard chromatograms of amantadine hydrochloride

Method development & optimization

The present investigation was carried out with a view to develop a RP-HPLC-PDA method for the quantification of Amantadine in the form of derivatised complex. Mobile phase optimization initially carried out with Inertsil ODS-3V, 250 x 4.6, 5 µm using 0.02 M ammonium acetate buffer and Acetonitrile combination (12:88% v/v) at a flow rate of 1.5 mL/min and Toluene as diluent. Under these conditions the peak of Amantadine –NAC complex was elutes with fronting at 6.6 min.

In other trial, Acetonitrile was replaced with methanol and keeping 0.02 M ammonium acetate buffer (88:12% v/v) at a flow rate of 1.5 mL/min and under these conditions a unsymmetry Memantine-NAC complex peak was eluted at 6.5 min. Finally, the mobile phase of 0.02 M ammonium acetate buffer and Methanol combination (12:88% v/v) at flow rate of 1.5 mL/min using Triethylamine and Toluene (0.02:99.98) as diluent was selected and under these conditions a sharp Memantine-NAC peak was eluted at 6.2 min with a total run time of 10 min.

Few chromatographic parameters such as derivatization process time optimization, NAC volume optimization, NAC concentration optimization were evaluated to obtain a specific, linear and accurate method.

NAC volume optimization

Different volumes of NAC solution i.e. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mL was added to the above mentioned solutions (sample and standard) under derivatization procedure to confirm complete reaction of Amantadine. Peak areas ratios were monitored to obtain a value of 1.0, which indicates 2.0 mL is the optimized volume of NAC Reagent.

Derivatization process time optimization

Sample and standard solutions after addition of derivatization reagent were stored at room temperature for different time intervals i.e. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 minutes to fix the reaction time. Peak areas ratios were monitored to obtain a value of 1.0, which indicates 2.0 min is optimized time to complete the reaction.

Table 1: Optimized chromatographic conditions

Parameters	Condition
Mobile phase	0.02 M ammonium acetate buffer and methanol in the ratio (12:88)
Diluent	Triethylamine and toluene
Column	Inertsil ODS-3V, 250 x 4.6, 5 µm
Flow rate	1.5 mL/min
Wavelength	226 nm
Injection volume	20 µL

Method validation

The method was validated for specificity, linearity and range, precision, accuracy, LOD and LOQ, robustness and system suitability as per International Conference on Harmonization (ICH) guidelines.

Specificity

The specificity of the developed HPLC method for the determination of Amantadine in bulk drug and pharmaceutical preparation (COMANTREL) was evaluated by non-interference of placebo.

Non-interference of placebo

To check the non-interference of placebo, the placebo solution was prepared in the same way as that of the sample solution in the presence of all inactive ingredients of the tablet formulations, but without Amantadine.

Linearity

Linearity of detector response for Amantadine was established by analyzing serial dilutions of a stock solution of the standard. Six concentrations ranging from 25% (28.5 µg/mL) to 150% (171 µg/mL) of the test concentration of 114 µg/mL were prepared. Internal standard solution was added to each dilution as described in the derivatization procedure, such that each solution contains 114 µg/mL of Amantadine HCl

and proceeded for Derivatization. The final concentration of each solution in mg per mL was calculated and plotted against peak area ratio of Amantadineto Memantine. The slope, y-intercept and correlation coefficient (R) were calculated.

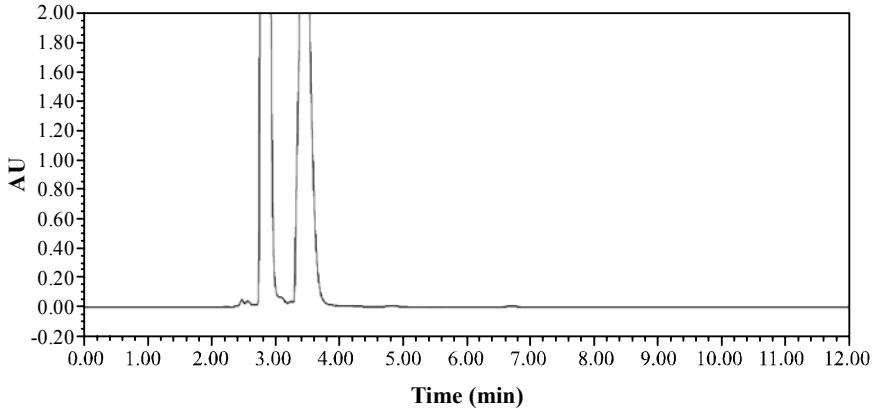


Fig. 6: Placebo chromatogram of amantadine hydrochloride

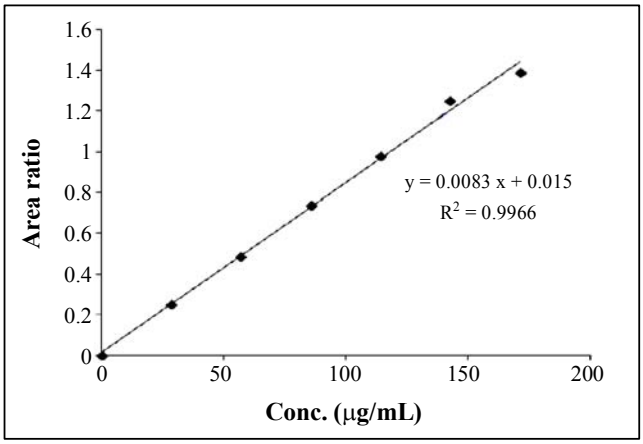


Fig. 7: Calibration curve of amantadine hydrochloride

Precission

The intra-day precision of the assay method was evaluated by carrying out 6 independent assays of a test sample of Amantadine at 100% concentration level against a standard Memantine. The % RSD of the obtained assay values was calculated. The inter-day precision study was performed on three different days i.e. day 1, day 2 and day 3 at 100% concentration level. The % RSD of the obtained assay values on 100% concentration level was calculated.

Accuracy

The accuracy of the method was performed by recovery study of Memantine in the dosage form at three concentration levels. A fixed amount of preanalyzed sample was taken and standard drug was added at 50%, 100% and 150% levels. Internal standard solution was added to each set as described in the derivatization procedure. Each level was repeated three times. The content of Amantadine per tablet was calculated. The percentage recovery ranges from 98.9-99.6% and the mean recovery of Memantine was 99.3% that shows there is no interference from excipients and the lower values of RSD of assay indicate the method is accurate.

Robustness

The robustness test was performed by deliberately making the changes in the flow rate and buffer concentrations. Retention time, tailing factor, resolution, and theoretical plates were measured to demonstrate the robustness of the method. Robustness was conducted on the sample solutions prepared from the tablet formulation.

System suitability

System suitability was carried out by injecting a standard concentration of 114.0 µg/mL at six replicates and system suitability parameters were determined. The system suitability test parameters were noted and % RSD was calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

It was performed based on the signal-to-noise ratio. A standard solution of 6.3 µg/mL of Amantadine solution was prepared to check the signal-to-noise ratios of the analytes. Then further dilutions were made for LOD and LOQ determination.

Application to analysis of pharmaceutical formulations

The proposed method was applied for the estimation of Amantadine (Commercial Tablets-COMANTREL) in their tablet formulations. About twenty tablets were taken and pulverized to a fine powder, and then tablet powder equivalent to the average weight of one tablet was taken. The drugs were extracted with mobile phase for carrying out the analysis.

RESULTS AND DISCUSSION

A reverse phase isocratic liquid chromatographic technique was developed, optimized and validated for the determination of Amantadine in bulk and tablet dosage forms with UV detection at 226 nm by using Inertsil ODS-3V, 250 x 4.6, 5 µm with mobile phase composition of 0.02 M ammonium acetate buffer and methanol in the ratio (12:88) in optimized isocratic program. The results of optimized HPLC condition were shown in Table 1. Linearity for the proposed method was established by least squares regression analysis of the calibration curve. Calibration curves were linear over the concentration range of 28-171 µg/mL for Amantadine with a correlation coefficient (r^2) of 0.996 ± 0.002. The calibration curve was shown in Fig. 7 and the results of Linearity were given in Table 2. The precision of proposed method was good with a % RSD of below 2.0%, which indicates the method is precise. The results are presented in Table 3. The % recoveries of Amantadine were found in the range of 98.9-99.6 and the % mean recoveries found to be 99.3 for Amantadine, which indicates the method is accurate. The results of recovery studies were shown in Table 4. The Number of theoretical plates was 12136, Resolution between Memantineand Amantadine is 6.2 and the tailing factor was 1.0 for Amantadine, which assures efficient performance of the column. Results of system suitability parameters were given in Table 5. Typical chromatogram of Standard Amantadine was shown in Fig. 5. Specificity was demonstrated by the placebo studies and through forced degradation studies. The LOD and LOQ of Amantadine were found to be 0.23 and 0.69 µg/mL. In all the deliberate varied chromatographic conditions (flow rate, pH variation, buffer concentration), the system suitability parameters like tailing factor, resolution, and theoretical plates were not much affected, which shows that the method is robust. The proposed method was successfully applied to the assay of Amantadine in commercial tablets (COMANTREL). The percentage recoveries of the drug were based on the average of five replicate determinations. The results are shown in Table 6. The analysis of commercial tablets results were well within the limits which indicates that the method can be applied for Routine commercial analysis.

Table 2: Results of linearity

S. No.	Concentration (µg/mL)	Memantine area	Amantadine area	Area ratio
1	171	31431581	46204425	1.47
2	142.5	31431581	38503688	1.225
3	114	31431581	30802950	0.98
4	85.5	31431581	23102212	0.735
5	57	31431581	15401475	0.49
6	28.5	31431581	7700737	0.245

Table 3: Results of precission

S. No.	Intraday precision		Interday precision	
	Area ratio	% of Assay	Area ratio	% of Assay
1	0.972	97.2	0.954	95.4
2	0.981	98.1	0.931	93.1
3	0.950	95.0	0.961	95.1
4	0.980	98.0	0.950	95.0
5	0.991	99.1	0.971	97.1
6	0.961	96.1	0.960	96.0
Average		97.3	97.2	95.3
Standard deviation		1.5	Standard deviation	1.3
% RSD		1.4	% RSD	1.3

Table 4: Results of accuracy

Level	Concentration added (µg/mL)	Concentration found (µg/mL)	% Recovery	Mean recovery
50%	57	56.8	99.6	
100%	114	113.2	99.3	99.3
150%	171	169.1	98.9	

Table 5: Results of system suitability

Parameters		USP limits	Results
Theoretical plates		NLT 2000	12136
Tailing factor	Memantine	NMT 2.0	1.2
	Amantadine	NMT 2.0	1.2
Resolution		NLT 2.0	6.2
Retention time	Memantine		8.6
	Amantadine		6.2
% RSD of Peak Response Ratio		NMT 2.0%	1.3

Table 6: Assay results of market formulation

Market formulation	Label claim (mg)	Quantity of API found (mg)	Assay %
Comantrel-100 mg consern pharma	Amantadine	Amantadine	Amantadine
NA	100	99.6	99.6

CONCLUSION

A simple, precise and accurate stability indicating RP-HPLC method was developed and validated as per ICH Guidelines by using (2-Napthoxy) Acetyl chloride as Derivatization agent due to lack of chromophores groups in Amantadine molecule. Memantine is used as an internal standard, which increases the sensitivity of the method. Developed method can be used for the routine commercial analysis of Amantadine formulations.

ACKNOWLEDGMENT

The authors are very thankful to management of Pullareddy Institute of Pharmacy and Sipra Labs, Hyderabad, India for providing facilities for this work. They are also very thankful to Torrent Pharmaceuticals, Ahmadabad, India for providing samples for this research work.

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