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Analysis of Amantadine Hydrochloride-Phenyl Isothiocyanate Complex in Bulk and Pharmaceutical Dosage Forms by RP HPLC-PDA Method

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Authors' contributions

Author SKV performed the experimental work and wrote the first draft of the manuscript. Authors NRK and MJM managed the literature search. Author BNN designed and managed the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: The aim of the present investigation was to perform the precolumn derivatization of Amantadine Hydrochloride (AMT) with phenylisothiocyanate and to develop a RP-HPLC-PDA method for the quantification of Amantadine Hydrochloride-phenylisothiocyanate (AMT-PITC) complex in bulk and dosage forms which is rapid, sensitive and economical.
Study Design: Method development and Validation study.
Methodology: A Phenomenex C_{18} RP column of 250 x 4.6mm dimensions and 5µm particle size with mobile phase containing water and acetonitrile (40:60% v/v) was used at isocratic mode binary pump and eluent was monitored at 273nm.
Results & Discussion: The retention time of AMT-PITC complex was 6.3 min. The developed method showed a good linearity in the concentration range of 10-50µg/mL with a correlation coefficient >0.998. The recoveries ranged between 95-105% with a Relative Standard Deviation of (RSD) < 2%.
Conclusion: The developed method was validated as per ICH guidelines and successfully used for quantification of AMT by derivatization with PITC. The method was found to be rapid, specific and accurate.

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Keywords: Amantadine Hydrochloride, Phenyl isothiocyanate, C18 column, PDA Detection, Method Validation, Derivatization.

1. INTRODUCTION

AMT (Fig. 1) is a synthetic adamantine derivative and is chemically 1-amino tricyclodecane. AMT belongs to the category of anti viral and anti parkinsonian drug. AMT as an anti viral agent specifically inhibits influenza A virus replication and as an anti parkinsonian agent, is used to treat extra-pyramidal reactions and post therapeutic neuralgia [1]. AMT is a weak antagonist of the N-methyl D-aspartate (NMDA) type glutamate receptor, increases dopamine release and blocks its reuptake [2].

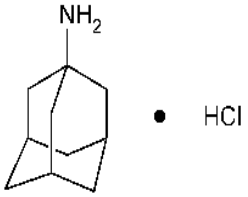

Fig. 1. Structure of Amantadine Hydrochloride

Since, AMT lacks useful chromophores in its structure and cannot be readily quantified by either UV or fluorescence detection techniques. Consequently, AMT has to be derivatised before analysis. Derivatizing agents such as Dansyl chloride (Dns-Cl), 9-fluorenyl methyl chloro formate (FMOC), 4- fluoro 7-nitro -2, 1, 3-benzoxadiazole (NBD), Ortho-Pthaldehyde (OPA), DMEQ-cocl, reacts readily with both the primary and secondary amines in basic conditions and are regarded as the derivatizing agents of choice for fluorescence detection and anthraquinone-2-sulfonyl chloride is used as a derivatizing agent for UV detection [3].

Literature survey reveals that only few HPLC methods were reported on the pre column derivatization of AMT with agents such as Dns-Cl [4], FMOC [5,6,7], NBD [8], OPA [9], DMEQ-cocl [10], 6-carboxyfluoresin-n- hydroxyfluorescein-N-hydroxysuccinimideester [11] for fluorescence detection and with anthraquinone-2-sulfonyl chloride [12] for UV detection and these derivatizing agents react with the primary amine in AMT under basic conditions and a spectrophotometric method was reported using methyl orange as derivatising agent [13]. Most of the HPLC methods reported are for analyzing AMT in biological fluids. However, no methods were reported on the derivatization of AMT with PITC for UV-detection in bulk and dosage forms. Hence, the main objective of the present investigation was precolumn derivatization of AMT with PITC and to evaluate the AMT-PITC complex for developing a new rapid and sensitive RP-HPLC-PDA method for the analysis of AMT in bulk and dosage forms.

2. MATERIALS AND METHODS

2.1 Reagents

AMT was gift sample from Sandoz Pharmaceuticals, India. Phenylisothiocyanate (PITC-99%) was purchased from Sigma Aldrich chemicals (HPLC grade), India. Water and acetonitrile were purchased from E. Merck (Mumbai, India), Sodium carbonate (analytical grade) purchased from Qualigens (Mumbai, India), and Sodium bicarbonate (analytical

grade) was purchased from SD Fine Chem. Industries (Chennai, India). Amantrel® (manufactured by Cipla Ltd, Malpur, Solan, B No:D21784, MFD:AUG.12, EXP: July.15) a capsule containing AMT 100mg was commercially purchased.

2.2 Equipment

A Shimadzu Prominence HPLC system provided with DGU-20A3 degasser, LC-20AD binary pumps, SIL-20AHT auto sampler, and SPD-M20A PDA detector was used. Data acquisition was carried out using LC solutions software.

2.3 Chromatographic Conditions

Mobile phase consisting of water: acetonitrile (40:60% v/v) was used in isocratic mode and the mobile phase was filtered through nylon disc filter of 0.45µm (Millipore) and sonicated for 3 min before use. The chromatographic analysis was performed on Phenomenex C_{18}- RP column (250 × 4.6mm; 5µ), at a flow rate of 1mL/min with an injection volume of 10µL. PDA detection was performed at 273nm and the separation was achieved at ambient temperature.

2.4 Preparation of Stock and Standard Solutions

Stock solution of AMT (1mg/mL) was prepared by transferring 10mg of accurately weighed AMT into a 10mL volumetric flask and dissolved in 5 mL of methanol. Finally the volume was made upto mark with remaining methanol. The working standard solutions of 10-50µg/mL were prepared by appropriately diluting the stock solution of AMT using water and methanol (70:30%v/v) as the diluent.

2.5 Preparation of Phenylisothiocyanate Solution (4% v/v)

0.4mL of PITC was transferred into a 10mL clean and dry volumetric flask. 5 mL of acetonitrile was added and vortexed for 1min and the volume was made up to the mark with acetonitrile.

2.6 Preparation of Sodium Bicarbonate solution (5% w/v):

0.5gm of sodium bicarbonate was weighed and transferred into a clean and dry 10mL volumetric flask. 5mL of HPLC grade water was added, vortexed for a period of 1 min. The volume was made up to the mark with HPLC grade water.

2.7 Preparation of Sodium Carbonate Solution (1% w/v)

0.1gm of sodium carbonate was weighed and transferred into a clean and dry 10mL volumetric flask. 5mL of HPLC grade water was added, vortexed for a period of 1 min. The volume was made up to the mark with HPLC grade water.

2.8 Derivatization Procedure for Standard and Sample Solutions

Derivatization of AMT with PITC was carried out as per literature [14]. Aliquots of AMT ranging from 10-50µg/mL were taken into a 2mL centrifuge tube. To each of the solutions

100µL of 4% v/v of PITC and 100µL of 5% w/v of sodium bicarbonate were added. The centrifuge tube was capped, shaken well and heated at 40º C in a water bath for 10 min. Later 100µL of 1% w/v sodium carbonate was added and heated again at 40ºC for 5 min and the solution was cooled down to room temperature and finally made to 1 mL with water and methanol (70:30% v/v) as diluent and filtered with 0.45µm nylon syringe filter. 10µL of the derivatised solution was injected and the detection was carried out at 273nm. All the solutions were stored at 4ºC until analysis. The derivatization scheme was presented in Fig. 2.

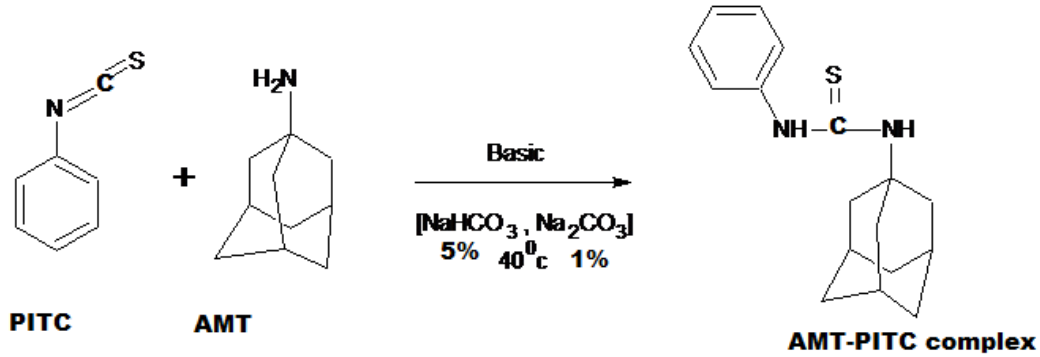

Fig. 2. Reaction between AMT and PITC

2.9 Validation of the Hplc Method

The proposed method was validated as per ICH guidelines [15].

2.9.1 Linearity

A linear relationship was evaluated across the range of the analytical procedure with a minimum of five concentrations. A series of standard solutions of AMT-PITC complex were prepared over a concentration range of 10-50µg/mL (10, 20, 30, 40, 50µg/mL) from stock solution. Linearity was evaluated by a plot of peak areas as a function of analyte concentration, and the test results were evaluated by appropriate statistical methods where by slope, intercept, regression (R^2) and correlation coefficients (R) were calculated.

2.9.2 Precision

Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Repeatability was assessed by using a minimum of six determinations at 100% of the test concentration. The standard deviation and the relative standard deviation were reported for precision. Less than 2% RSD for peak areas indicate the precision of the developed method and the data was presented in Table 1.

Table 1. Linearity, Precision and Accuracy data

Validation data	
Linearity (n=3)	Range 10-50 µg/mL y =430768x+200000 R=0.999 R^2=0.998
Precision (n=6)	Average peak area of the standard sample (%RSD) 4543657.3(0.646%)
Accuracy (n=3)	Mean Percent Recovery (%RSD)
Level of addition	
80%	96.33(0.599%)
100%	96.41(0.875%)
120%	103.18(0.946%)

2.9.3 Specificity

Specificity is a measure of the degree of interference in the analysis of the complex sample mixtures such as analyte mixed with the formulation excipients or the known impurities. Specificity of the method was carried out by comparing chromatogram of the placebo (in house made) with that of the sample for checking any interference peaks.

2.9.4 Accuracy

Accuracy was established across the specified range of the analytical procedure. Accuracy of the method was tested by spiking 80, 100, and 120% standards to 20µg/mL of test AMT-PITC solution. These solutions were analyzed by developed method in triplicate. The % recovery and the %RSD were calculated at each level of addition.

2.9.5 Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on calibration curves and were expressed as LOD = (3.3 ×σ)/m; LOQ= (10.0×σ)/m (Where, σ is the standard deviation of the y-intercepts of the three regression lines and m is mean of the slopes of the three calibration curves).

2.9.6 Robustness

Robustness of the method was determined by altering the experimental conditions such as flow rate and wavelength intentionally. The chromatographic parameters viz., capacity factor, tailing factor, theoretical plate number and % assay were recorded. To study the effect of flow rate, the flow rate was changed by 20% and the effect of wavelength was studied by changing wavelength by ± 2nm.

2.9.7 System suitability

System suitability was carried out by injecting a standard concentration of 10µg/mL at different injection volumes in the range of 10µL to 50µL. The system suitability test parameters were noted and % RSD was calculated.

2.9.8 Assay

Twenty Capsules were weighed and finely powdered and the powder sample equivalent to 10mg of AMT was accurately weighed and transferred into a 10mL volumetric flask. 5 mL of methanol was added to solubilize and the solution was vortexed for 5min and made upto the mark with methanol. The above solution was centrifuged for 3min at 3000rpm and filtered using Nylon disposable syringe filter (13 mm, 0.45µm). Aliquots of the filtrate (20µg/mL) were subjected to derivatization process as mentioned in the section 2.8 to attain the AMT-PITC complex and samples were analyzed in triplicate. The amount present in the each capsule was quantified by comparing the peakarea of standard AMT-PITC complex with that of the sample.

3. RESULTS AND DISCUSSION

The present investigation was carried out with a view to develop a RP-HPLC-PDA method for the quantification of AMT in the form of derivatised AMT-PITC complex. Mobile phase optimization initially carried out with Phenomenex C_{18} column (250 x 4.6 mm; 5µm) using 5mM ammonium acetate and acetonitrile combination (40:60% v/v) at a flow rate of 1mL/min and water and acetonitrile (70:30% v/v) as diluent. Under these conditions the peak of AMT-PITC complex was elutes with fronting at 6.4 min. In other trial, ammonium acetate was replaced with water and keeping acetonitrile (40:60% v/v) at a flow rate of 1mL/min and under these conditions a unsymmetry AMT-PITC complex peak was eluted at 6.4 min. Finally, the mobile phase of water and acetonitrile (40:60% v/v) at flow rate of 1mL/min using water and methanol (70:30%v/v) as diluent was selected and under these conditions a sharp AMT-PITC peak was elued at 6.3 min with a total runtime of 10 min. For quantitative analytical purpose the wavelength was set at 273 nm, which provided better reproducibility without interference. The method was validated as per ICH guidelines. The peak purity index was found to be greater than 0.999 and indicating peak purity of the AMT-PITC complex. The resultant chromatogram was shown in Fig. 3 along with UV spectra.

3.1 Method Validation

The method has been validated as per ICH-Guidelines for following parameters.

3.1.1 Linearity

The range of reliable quantification was set at the concentrations of 10-50µg/mL of AMT-PITC complex. This range was selected based on 80-120% of the standard concentration used for accuracy and were analyzed in triplicate. Peak areas and concentrations were subjected to least square regression analysis to calculate regression equation. The correlation coefficient (R) was found to be 0.998 and indicates a linear response over the range used. The calibration curve data was given in Table 1.

3.1.2 Precision

Precision studies were carried out in terms of repeatability. Repeatability of standard application was assessed by using six replicates of concentration (20µg/mL) and the data was given in Table 1. The % RSD was found to be below 2 for peak areas and these results confirms the closeness of the data values to each other.

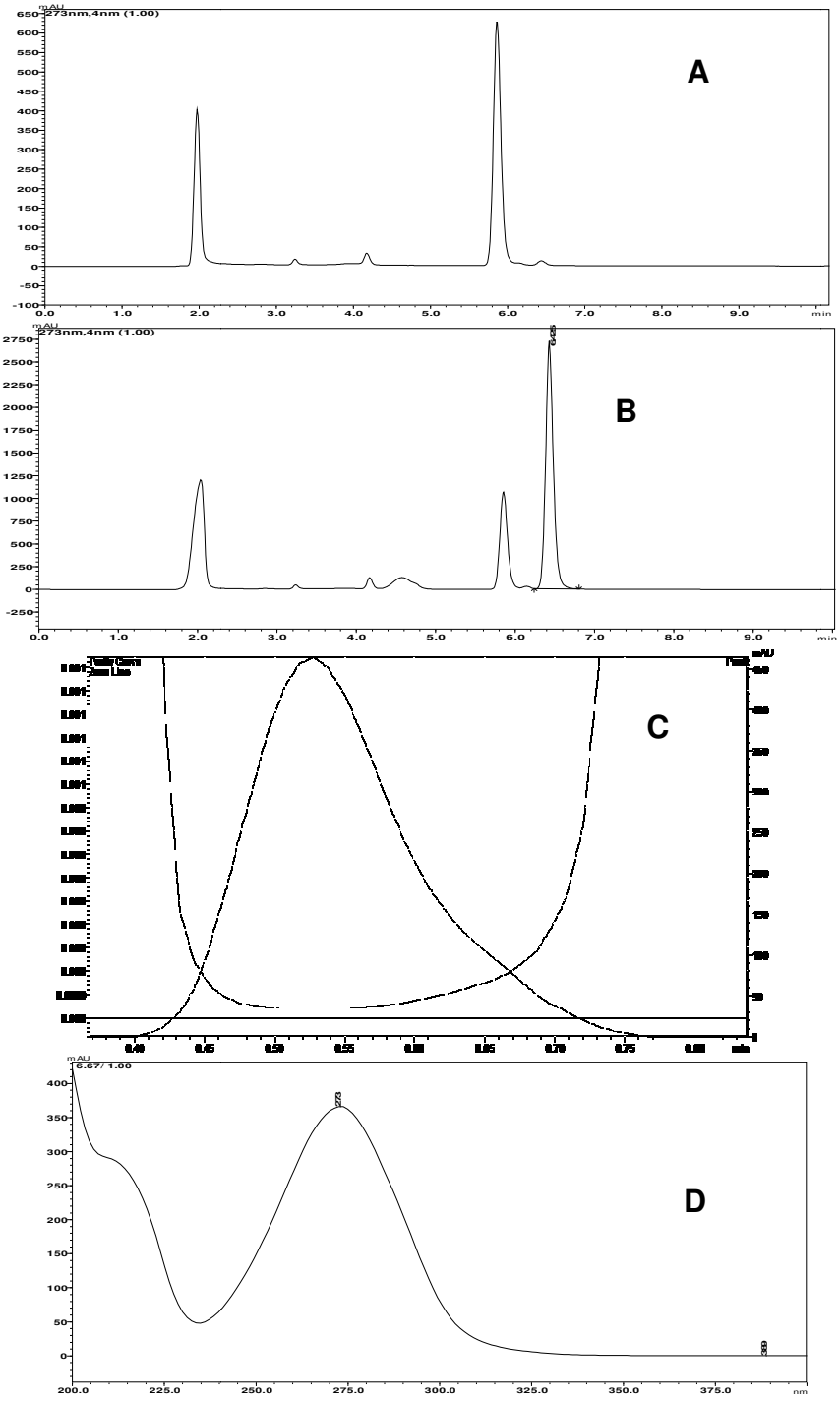

Fig. 3. A- Chromtogram of Diluent; B - Standard Chromatogram of AMT-PITC complex (10 µg/mL); C - Peak purity index and D - UV spectrum

3.1.3 Specificity

The specificity of the method was established by injecting the solutions of diluent, placebo, standard and test sample (formulation) individually to examine any interference. From the overlay of chromatograms as shown in (Fig. 4) and the 3D plots of placebo and test samples, it can be inferred that there were no co-eluting peaks at the retention time of AMT-PITC complex. These results show that peak of analyte was pure and the excipients in the formulation did not interfere with the analysis. The peak purity indices for sample and standard were found to be greater than 0.999 and this confirms specificity of the method.

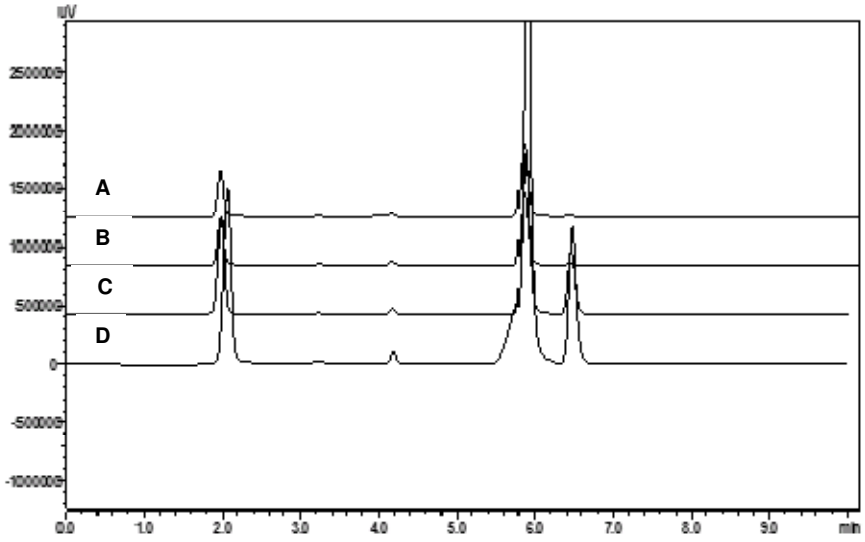

Fig. 4. Overlaid chromatograms of the Diluent (A), Placebo (B), Standard (C) and Sample (D) preparations

3.1.4 Accuracy

Accuracy of the proposed method was ascertained by performing recovery studies using the standard addition method by spiking the known quantities of standard at 80%, 100%, 120% to the test solution of 20µg/mL. The solutions were subjected to derivatization process as mentioned in the section 2.8 to form the AMT-PITC complex. These solutions were analyzed in triplicate at each level of addition. The %RSD and the % recovery were within the acceptable limit in all cases (Table 1). The proposed method enables very accurate quantitative estimation of AMT as AMT-PITC complex.

3.1.5 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection for AMT-PITC complex was found to be 1.534µg/mL and the peak was detected without any base line disturbances at this concentration. The limit of quantification for AMT-PITC complex was found to be 4.649 µg/mL.

3.1.6 Robustness

As part of the robustness, deliberate changes in the flow rate and wavelength were made to evaluate the impact on the method. Retention times were significantly changed with flow rate and no change in the retention time was observed in wavelength change. Percent assay values were also estimated under these changed conditions and the results were given in Table 2. The parameters like capacity factor, theoretical plate number and assay were not changed and were within the limits. These results indicated that the method is robust in terms of changed flow rate and wavelength.

Table 2. Robustness data

Chromatographic parameter	Retention time (min)	Theoretical plates #	Capacity factor (K')	Tailing factor (T_f)	% Assay
Flow rate (mL/min)					
0.8	7.90	17479	0.098	1.175	100.72
1.0	6.37	15891	0.097	1.208	100.54
1.2	5.33	14554	0.098	1.219	100.51
Wave length (nm)					
271	6.37	15915	0.103	1.208	101.42
273	6.37	15891	0.107	1.208	102.10
275	6.37	15870	0.104	1.207	100.94

3.1.7 System suitability

System suitability testing is an integral part of the analytical procedure. System suitability studies were carried out by injecting 10µg/mL standard concentration of AMT-PITC complex at different injection volumes ranging from 10µL to 50µL. The %RSD values for system suitability test parameters like retention time [R_t = 6.37 (0.19%)], tailing factor [T_f = 1.20 (0.10%)] and theoretical plate number [**#** = 18922.98 (1.82%)] were less than 2, indicating the present conditions were suitable for the analysis of AMT as AMT-PITC complex in bulk and dosage forms like capsules.

3.1.8 Assay

Assay of AMT in capsules was performed by the proposed method and the % assay was calculated as an average of 3 determinations (100.03 ± 0.11). These results indicate that the present HPLC method can be successfully used for the assay of AMT as derivatised AMT-PITC complex in bulk and dosage forms.

3.1.9 Stability of the stock solution

The stability of the stock solution was determined by analyzing the samples under refrigeration (8±1ºC) at different time intervals up to 48hrs. The % variation in assay values at different time intervals were found to be less than 2% of the initial zero time interval solution, thus indicating that the solutions were stable for a period of 48hrs when stored at 8º C.

4. CONCLUSION

Finally it can be concluded that a simple and economical RP-HPLC-PDA method was developed for the analysis of AMT as derivatised AMT-PITC complex in bulk and capsule dosage forms. The method was validated as per ICH Guidelines, and found to be applicable for routine quality control analysis of AMT using isocratic mode of elution. The results of linearity, precision, accuracy and specificity, proved to be within the limits. The method provides selective quantification of AMT as derivatised AMT-PITC complex without interference from diluent and placebo.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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