VALIDATED RP-HPLC/UV METHOD FOR THE QUANTITATION OF DIPHENHYDRAMINE IN FORMULATION VEHICLE AND ITS APPLICATION TO HOMOGENECITY AND STABILITY IN DOSE FORMULATION VEHICLE

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ABSTRACT

A novel, simple, specific, sensitive and reproducible high-performance liquid chromatography (HPLC) assay method has been developed and validated for the estimation of diphenhydramine (DPH). The HPLC method for the analysis of diphenhydramine hydrochloride in dose formulation samples using Tolbutamide as internal standard (IS). The chromatographic analysis was performed by Waters Alliance High Performance Liquid Chromatographic system equipped with UV and PC based data system with Empower Software. Chromatographic separation was achieved using Symmetry sheild, RP 18, 5 μM, (Waters) column maintained at 35°C temperature and an gradient mobile phase (0.01 M Ammonium Acetate in Milli- Q Water, pH adjusted to 5 using Acetic Acid and acetonirile) at a flow rate of 1 mL/min with a total run time of 10 min. The elute was monitored using an UV detector set at 267 nm. Method of validation was performed as per ICH guidelines and the results met with the acceptance criteria. The calibration curve was linear over a concentration range of 1001 to 14987 μg/mL (r² = 0.999). Precision & accuracy batches were analysed at 3 different occasions revealed the accuracy of DPH in formulation was in the range of 99.85 to 105.83%, 100.19 to 101.81% and 94.94 to 95.02%, respectively. (with in batches ): Inter day accuracy was 99.8, 98.3 and 98.1 respectively. The validated HPLC method was successfully applied for formulation analysis.
KEYWORDS: Diphenhydramine, HPLC method validation, Homogeneity, Formulation.

INTRODUCTION

Histamines are organic nitrogenous compound involved in localized immune response as well as regulating physiological function in the gut and acting as a neurotransmitter. Histamine is also involved in inflammatory responses. As part of an immune response to foreign pathogens, histamine is produced by basophils and mast cells which are found in the nearby connective tissues. Histamine increases the permeability of the capillaries for white blood cells and some proteins and allow them to engage in the process of pathogenesis at the site of infection. Histamine was first discovered in 1910 by Sir Henry Hallett Dale as a contaminant of ergot generated by bacterial action. It was first synthesized before its significance was known, and due to its wide range of biological activity, it has become one of the most important biologically produced amines in medicine and biology. e H1 receptor antagonists are used in the treatment of emesis as an antitussive, dermatitis, pruritus and for hypersensitivity reactions. As hypnotic in parkinsons diseases and as an ingredient in anticolc preparations.

Diphenhydramine hydrochloride (DPH) is chemically 2-(diphenyl methoxy)-N,N-dimethylamine hydrochloride) is an effective antihistaminic and has been used for the treatment of motion sickness and extra pyramidal symptoms, as an antitussive and to induce sleep at night in case of sleeplessness. Recently, it is reported that it is used in combination with other drugs as an antiemetic for the prevention of cisplatin-induced emesis an antineoplastic drug. It is also used as sedative in dentistry for children and in combination with local anaesthetics (Goodman-Hillman et al., 1996).

It is indicated for its powerful hypnotic effect and for this reason it is often used as a non-prescription sleep aid and as mild anxiolytic (Marti A et.al.). The DPH also acts as an antiemetic (Helms RA et.al. 2006). Several methods have been proposed for determining diphenhydramine hydrochloride in pharmaceutical preparations including capillary electrophoresis (Gomez et.al. 2002), fluorometry (Alatayud, et.al., 1992), flow injection analysis spectrophotometry. Chromato-graphic methods such as gas chromatography, liquid chromatography and high performance liquid chromatography (HPLC) (Yuan, H.P Yuan, H.P). Diphenhydramine is used commonly to treat drug-induced extra pyramidal symptoms and to treat mild cases of Parkinson's disease. Cromolyn and nedocromil, prevents the release of histamine, Where as diphenhydramine competes with free histamine for binding at HA-
receptor sites (Estelle *et al.* 2004). This drug is having multiple uses and used alone as well as in combination therapy with many adverse effects and for this reason it is required to develop a sensitive extraction and confirmatory method for the identification and quantitation of Diphenhydramine (Imma Ferrer, *et al.* 2004). Many analytical Chemists focus on a single type of instrument as UV Spectrophotometer by which analytical method may be developed and helpful for routine analysis in quality control laboratories and to establish quality assurance parameters. The aim of the present study is to develop an accurate, reproducible and adequately sensitive HPLC method to determine diphenhydramine. The proposed method can be applied for determining diphenhydramine in formulation analysis studies.

**EXPERIMENTAL**

**Chemicals and reagents.** Diphenhydramine Hydrochloride and Tolbutamide was procured from Sigma Aldrich, USA. HPLC grade acetonitrile from, JT Baker, Ammonium acetate from Sigma Aldrich, USA, Acetic acid, AR Grade from Rankem Ranbaxy Fine Chemicals, New Delhi. All other chemicals/reagents were of research grade and used without further purification.

**HPLC operating conditions.** The HPLC system used was *Waters 2695 Alliance system* (Waters, Milford, USA) equipped with performance PLUS inline degasser along with an auto-sampler used to inject 25µL aliquots of the processed samples to HPLC Column: Symmetry shield, RP 18, 5 µM, (Waters) from LGC Promochem India Limited, Bangalore, which was maintained at ambient room temperature (25 ± 1°C). GRADIENT phase comprising acetonitrile, Ammonium acetate buffer (pH 5.0) (v/v) delivered at a flow-rate of 1 mL/min was used for attaining chromatographic resolution of Diphenhydramine and IS Internal standard).

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Solvent Flow Rate (mL/min)</th>
<th>Mobile Phase A (%) Ammonium acetate</th>
<th>Mobile Phase B (%) Acetonitrile</th>
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<tr>
<td>0.01</td>
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**Preparation of stock and CC QC solutions.**
Standard blank samples were spiked in 25 µl of water into 225 µl diluents and vortexed thoroughly. Standard zero samples by spiking 25 µl of water into 225 µl IS working solution
and vortexed thoroughly. Calibration curve standards and Quality control samples by spiking 25 µl of working solution into 225 µl IS working solution and vortex to mix thoroughly. Transfer about 200 µl into inserts kept in pre-labeled 1 ml glass vials and capped with polyethylene plugs/ caps.

Sample preparation
The test item for each dose were weighed on separate butter paper, and carefully transferred into a 10mL measuring cylinder. Small aliquot of the vehicle was added to dissolve the test substance and final volume was made up to the mark (10 mL) with the vehicle to get the desired concentration (2mg/mL & 8mg/mL). These dose formulations were placed at room temperature (19°C - 24°C).

Validation procedures
A full validation according to the OECD Principles of Good Laboratory Practice for the testing of chemicals as specified by International [C (97) 186/Final] Legislation. ICH Guidelines: Validation of analytical procedures: Text and methodology Q2 (R1) and Australian pesticides and veterinary medicines authority guidelines for the validation of analytical methods for active constituents, agricultural and veterinary chemical products, October 2004.

System suitability
System suitability test was performed on six different days prior to each day of method validation at the medium QC level. Analytical batch of system suitability consisting of six injections of medium QC level in diluent solution. Samples were prepared by spiking 25 µL of MQC working solution into 225 µL IS working solution and vortexed thoroughly and analysed by using HPLC.

Selectivity
Selectivity was performed with six different batches of Milli-Q water and the Milli-Q water spiked at LLOQ (lower limit of quantification) concentration in diluent solution. Samples were prepared by spiking 25 µL each of Milli Q water and LLOQ working solution into 225 µL IS working solution respectively and vortex to mix thoroughly and analysed by using HPLC.
Autosampler carryover test
Mobile phase was directly injected and samples of LLOQ and ULOQ (upper limit of quantification) were prepared by spiking 25 µL each of LLOQ and ULOQ working solution into 225 µL IS working solution, respectively and vortex to mix thoroughly and analysed by using HPLC.

Linearity of calibration standards
Linearity of calibration standards were carried out on six different days (three times along with P&A batches on three different days and remaining three times during homogenicity and stability analysis on three different days). A standard curve comprising of 8 non-zero standards including lowest and highest concentration in duplicate (excluding Standard Blank and Standard Zero). Calibration standards for analysis were prepared by spiking 25 µL of respective working solution into 225 µL IS working solution and vortex to mix thoroughly and analysed by using HPLC.

Precision and accuracy (P&A)
Precision and accuracy was determined on three different days following injection of standard blank, zero standard blank and 8 non zero calibration standard with duplication of LLOQ and ULOQ injection. The precision and accuracy samples was comprising six sets each of LQC, MQC and HQC representing the entire standard curve range.

Fortification Levels of Test substance
Homogenicity, Bench-top and long term stability tests for dose formulations were carried out at the following concentrations of diphenhydramine in the vehicle (Milli Q water):
- 2.0 mg/mL
- 8.0 mg/mL

Preparation of Dose Formulations
The test item for each dose were weighed on separate butter paper, and carefully transferred into a 10mL measuring cylinder. Small aliquot of the vehicle was added to dissolve the test substance and final volume was made up to the mark (10 mL) with the vehicle to get the desired concentration (2mg/mL & 8mg/mL).
Homogenicity
A volume of 0.5mL of dose formulation was withdrawn from top, middle and bottom level in duplicates from measuring cylinder with the help of micropipette into 1.5mL eppendorf tube and used for the analysis of homogenicity. On each occasion of sampling, each dose formulation was mixed well before sampling. One sample from vehicle and six samples from each dose level (low and high) was drawn and analysed for the test item concentration. Samples were prepared by spiking 25 µL of vehicle as blank, 2 aliquot from top layer, 2 aliquot from the middle layer and 2 aliquot from the bottom layer into 225 µL IS working solution, respectively and vortex to mix thoroughly and analysed by using HPLC.

STABILITY
Stability of test substance in vehicle was determined at different time intervals (short term/bench top stability at 0, 1, 2, 4 and 8 hr and long term stability at day-3 and day-7). Stability of test substance was evaluated in Milli-Q water at 2 and 8 mg/mL concentrations. The stability samples were analyzed using a freshly spiked calibration curve and quality control samples (2 replicates of LQC, MQC and HQC). Six replicates of stability samples was analyzed per concentration and mean back-calculated concentrations of stability samples was compared against mean of first day (initially observed) concentrations for stability evaluation. On each occasion of sampling, each dose formulation was mixed well before sampling.

Processing procedure: 25 µL each of six replicates of stability samples were spiked into 225 µL IS working solution respectively and vortex to mix thoroughly and analysed by using HPLC.

Bench-top stability and Long-term stability: Stability samples was kept at ambient temperature and an aliquot was subjected for analysis after completion of stability duration i.e. 0, 1, 2, 4 and 8 hrs. Samples were processed and stored in refrigerated condition till analysis and for long term stability samples was kept at ambient temperature and an aliquot was subjected for analysis after completion of stability duration i.e. 3 and 7 days.

RESULTS OF VALIDATION PARAMETER
System Suitability and Selectivity: The % CV of peak area ratio of six consecutive injections on six different days was in the range of 0.147 to 3.852, which met the acceptance criteria (i.e., % CV of Peak Area Ratio should be less than or equal to 5%). The % CV of
retention time of DPH and Tolbutamide (IS) on six different days was in the range of 0.056 to 0.251 and 0.021 to 0.121 respectively which met the acceptance criteria (i.e., % CV of retention time of analyte and internal standard should be less than or equal to 2% of the mean retention time of the analyte and internal standard). Thus, it is concluded that the system was suitable for the analysis. There was no interference observed at the retention time of diphenhydramine and internal standard in all six replicates of Milli-Q water batches v/s Milli-Q water, spiked at LLOQ concentration analysed. The results met the acceptance criteria (i.e. response of interfering peak(s) at the retention time of the diphenhydramine and internal standard peak should be ≤ 20% & ≤ 5 % respectively, in the corresponding LLOQ standard). These results suggest the ability of the method to differentiate and measure Diphenhydramine and internal standard in presence of other constituents of matrix in the sample.

**Linearity of Calibration Standards**

Linearity of calibration standards analysed along with three different precision & accuracy batches reveals the accuracy of all the standard curve points (one out of duplicates were not considered for calculation) which were in the range of 97.99 to 101.16%, 99.21 to 100.77% and 98.93 to 100.75 % respectively. All the three different batches met acceptance criteria (i.e.75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). The correlation coefficient ($r^2$) of calibration plots was greater than 0.999 in all batches, which met the acceptance criteria (i.e. correlation coefficient ($r^2$) value for standard curve should be ≥ 0.98).

**Within-run (Intra-Day):** Precision & accuracy batches analysed on 3 different days revealed accuracy of DPH in vehicle in the range of 99.85 to 105.83%, 100.19 to 101.81% and 94.94 to 95.02%, respectively at LQC level, 98.25 to 101.11%, 98.27 to 99.62% and 96.17 to 96.33%, respectively at MQC level and 98.03% to 101.46%, 98.11 to 100.73% and 95.76 to 95.88%, respectively at HQC level, which met the acceptance criteria (i.e. at least 67% of the total QC samples and at least 50% of QC samples at each level should have back-calculated concentration values within ±15% of the nominal concentrations and 75% of standards must have accuracy within or equal to 85 to 115% of theoretical).

Precision & accuracy batches analysed on 3 different days have precision (%CV) of 2.07, 0.57 and 0.03%, respectively at LQC level, 1.06, 0.59 and 0.06%, respectively at MQC level and 1.34, 0.98 and 0.07%, respectively at HQC level, which met the acceptance criteria (i.e., %CV for low, medium and high QC concentrations should not exceed 15%).
CHROMATOGRAMS

Figure-1 CALLIBERATION CURVE

Figure-2. Blank formulation vehicle with without IS

Figure-3. Blank formulation vehicle with IS
Figure-4 LLOQ with IS

Figure-5 ULOQ with IS

Figure- 6 formulation samples
Between-run (Inter-Day): Inter day accuracy was 99.8, 98.3 and 98.1 at LQC, MQC & HQC levels, respectively. Inter day Precision (%CV) was 3.06, 1.89 and 1.69 at LQC, MQC & HQC levels, respectively which met the acceptance criteria (i.e., mean accuracy should be within ±15% of the nominal concentration at LQC, MQC and HQC and %CV for low, medium and high QC concentrations should not exceed 15%).

Homogenicity- Linearity of calibration standards
Linearity of calibration standards analysed along with homogeneity, Day-1 & short term / bench top stability samples revealed accuracy of all the standard curve points which were in the range of 99.18 to 100.97% which met acceptance criteria (i.e., 75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). The correlation coefficient ($r^2$) of calibration plots was greater than 0.999, which met the acceptance criteria (i.e., correlation coefficient ($r^2$) value for standard curve should be $\geq 0.98$). Quality control samples analysed with homogeneity, Day-1 & short term / bench top stability samples were having accuracy in the range of 95.3 to 96.2% at LQC level, 96.27 to 96.99% at MQC level and 97.98% at HQC level, which met the acceptance criteria (i.e., at least 67% of the total QC samples and at least 50% of QC samples at each level should have back-calculated concentration values within ±15% of the nominal concentrations and 75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical for the LLOQ). Accuracy of dose formulation samples (2 replicates from top layer, 2 replicates from the middle layer and 2 replicates from the bottom layer of each dose formulation) were found to be in range of 92.21 to 92.32% at 2mg/mL concentration and 93.85 to 93.85% at 8mg/mL concentration which met the acceptance criteria (i.e., at least 67% of the total samples and at least 50% of samples at each level should have back-calculated concentration values within ±10% of the nominal concentrations). The % CV of 2 & 8 mg/mL of dose formulation was found to be 0.17 & 0.03, respectively, which met the acceptance criteria (i.e., %CV of accepted samples should be less than or equal to 10%). Dose formulation of test substance in Milli Q water was found to be homogenous at 2 & 8mg/mL concentration.

Stability: Linearity of calibration standards analysed along with Day-3 & 7 stability samples revealed accuracy of all the standard curve points which were in the range of 99.03 to 100.85% and 99.36 to 100.96% respectively, which met acceptance criteria (i.e., 75% of standards must have accuracy within or equal to 85 to 115% of theoretical and 80 to 120% of theoretical and 80 to 120% of theoretical for the LLOQ).
theoretical for the LLOQ). The correlation coefficient ($r^2$) of calibration plots was greater than 0.999 in all batches. **Precision & accuracy for stability** control samples were analysed with Day-3 & 7 stability samples were having accuracy in the range of 94.27 to 94.45% (day-3) & 98.75 to 100.26% (day-7) at LQC level, 96.13 to 97.48% (day-3) & 96.25 to 98.41% (day-7) at MQC level and 96.9 to 98.49% (day-3) & 98.12 to 97.96% (day-7) at HQC level, which met the acceptance criteria. **Bench-top stability** the dose formulation samples at 2 & 8 mg/mL concentration were found to be stable on bench top at ambient temperature up to 8 hr. The % change of 2 & 8 mg/mL dose formulation was found 0.51 and -1.35 at 1 hr, -0.85 and -1.04 at 2 hr, -3.46 and -2.50 at 4 hr and -0.90 and -0.91 at 8 hr, respectively. **Long-term stability** the dose formulation 2 & 8 mg/mL samples were found to be stable for 7 days at ambient temperature. The % change of 2 & 8 mg/mL dose formulation was found 0.51 and -2.57 on day-3 and -1.53 and 0.01 on day-7 respectively.

**CONCLUSION**

Dose formulation of diphenhydramine in Milli Q water was found to be stable at room temperature for 7 days at 2 & 8 mg/mL concentration. Formulation samples were stable till 8 hours on bench top and were homogeneous. The current study will provide a guidance to researchers to design and conduct formulation analysis and support toxicokinetic studies.

**REFERENCES**

1. Agilent Technologies, Inc., 2008, Published in the USA September, 2008; 17: 5989-5319EN.


17. Helms RA, Quan DJ, Textbook of therapeutics: drug and disease management, Lippincott Williams & Wilkins, Philadelphia, Ed 8th, 2006; 1301.


21. John M. Weiler, MD; John R. Bloomfield, PhD; George G. Woodworth, PhD; Angela R. Grant, BS; Teresa A. Layton, BSN; Timothy L. Brown, MS; David R. McKenzie, MS; Thomas W. Baker, MS and Ginger S. Watson, PhD, 7th March 2000, “Effects of Fexofenadine, Diphenhydramine, and Alcohol on Driving Performance: A Randomized, Placebo-Controlled Trial in the Iowa Driving Simulator”, Annals of Internal Medicine, Vol. 132, Number 5.


33. October 2004, “Australian pesticides and veterinary medicines authority guidelines for the validation of analytical methods for active constituents, agricultural and veterinary chemical products”.


