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ORIGINAL ARTICLE

Development and Validation of HPLC Method for Diphenhydramine Hydrochloride

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Authors' Contributions
1 Conception & Study Design, Data Collection & Processing, Data Analysis and/or Interpretation.
2 Conception & Study Design, Critical Review.
3 Data Collection & Processing, Data Analysis and/or Interpretation.

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ABSTRACT

Aim: The aim of present method development for Diphenhydramine HCl performed on HPLC is to obtain specific, more accurate and precise results as compare to spectrophotometric method.

Methods: HPLC analysis was performed according to USP method with wavelength detection at 220nm and 1.0ml/min flow rate. Wufeng thermo HPLC system UV -100detector was used having column C18(4.6mm*250mm) 5. Methanol and water (4:1) mixture was used as mobile phase and pH was adjusted at 7.4 with the help of triethanolamine. Validation parameters like linearity, accuracy, precision, solution stability, robustness, LOD, LOQ and system suitability were successfully evaluated.

Results: The regression co-efficient for calibration curve was 0.991 and % recovery was in range (80-110%), whereas no robustness was observed in this reported method.

Conclusion: In summary, the expected linearity, accuracy and % recovery indicating that HPLC is more precise method than spectrophotometry and suggested that present method qualifies the validation criteria.

Keywords: Diphenhydramine HCl, HPLC, Method Validation, LOD, LOQ, Robustness.

INTRODUCTION

High performance liquid chromatography (HPLC) is one of the analytical method used for validation of drugs and chemical substances [1, 2]. Diphenhydramine HCl (Figure **1**) is antihistaminic drug substance, widely used in the treatment of allergic rhinitis, common cold and skin allergies. DPH is H1 receptor antagonist, white crystalline powder having melting point 168-172^{o}C. Diphenhydramine HCl is lipophilic in nature and well absorbed from nasal cavity. Its molecular weight is 291.82 g/mol (less than 1KDa), rapidly absorbed transcellularly across the nasal membrane. It can cross Blood Brain Barrier (BBB) having 40-60% bioavailability and metabolize in liver [3-5].

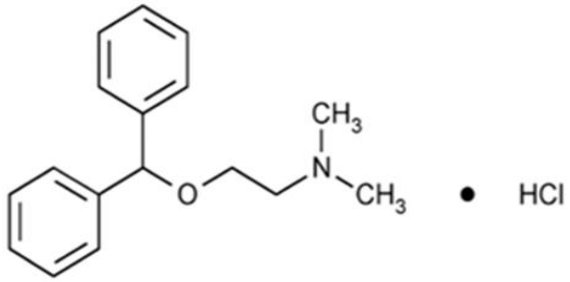

Figure 1. The chemical structure of Diphenhydramine HCl.

In present study, HPLC system using column C18 was used for the method validation of DPH HCl. Linearity, precision, reproducibility, limit of detection (LOD), limit of quantitation (LOQ), robustness and system suitability were determined. All of these

parameters are the important indicators of system functionality, sample preparation and column [6, 7].

EXPERIMENTAL

Chemicals

Diphenhydramine hydrochloride was gifted from Pfizer pharmaceuticals, Karachi (Pakistan). Methanol of HPLC analytical grade was purchased from Merck, Germany. Double distilled water was used gifted from Zakfas Pharmaceuticals Multan (Pakistan).

Instrumentation

HPLC system (Wufeng thermo UV 100 detector, China), Lamp, Ultrasonic bath (shenzhan co., China), Plungers and Plunger seals (7725i, USA), Syringe and Syringe filters, Needle assembly, vials, pH meter (PHS-3E, China), Magnetic stirrer (stuart,UC 152)

Chromatographic Condition

Column: C18 (4.6 mm * 250 mm) 5

Mobile phase: methanol: water: Triethanolamine (40:10:0.5), adjust pH at 7.4

Diluent: methanol: water (40:10)

Flow rate: 1.2mL/min

Column temperature: 25℃

UV detection: 220 nm

Injection volume: 10µL

Membrane disc filters (0.45µm pore size)

Preparation of Mobile Phase

Mobile phase was prepared by mixing the 40ml methanol (HPLC-analytical grade) with 10 ml double distilled water (4:1, V/V) and pH was adjusted at 7.4 with the help of Triethanolamine (0.5ml). Then filtered the mobile phase through 0.45µm pore size filter paper under vacuum and degassed through ultrasonic bath before use.

Preparation of Standard Solution

Stock solution (100 µg/mL) of DPH was prepared in diluent (4:1; methanol; water) in volumetric flask. Standard solutions were prepared by making further dilutions by adding mobile phase over concentration range 10, 20, 30, 40 and 50 µg/mL, to 20,40,60,80 and 100% in mobile phase respectively.

Chromatographic Method for Validation

According to the ICH-Guidelines, parameters for chromatographic validation were determined such as linearity, precision, LOD, LOQ, solution stability and system suitability [8].

Linearity

It describes the relationship between response and analyte concentration over the range. For the evaluation, linearity range depends on purpose of analytical method [9]. ICH guideline specify the minimum, five concentration levels with minimum specified range. The linearity data is accepted by observing the values of Regression coefficient ($r^2 \leq 1$), y-intercept (less than percent response obtained for analyte), slope and % relative standard deviation (% RS) [10]. In present study, linearity for DPH was determined over the concentration range of 10-50µg/mL (n=3).

Specificity of Assay

In order to detect any interference between reference standard solution of DPH, placebo and mobile phase, specificity was evaluated by comparing chromatograms of 3 replicate injections of reference standard solution and placebo [11].

Accuracy

Accuracy is the closeness of test values to the true values of method [11]. In this study, it was obtained by preparing the sample solution over range of 10-50µg/mL (n=3) and % recovery was calculated. According to USP for acceptance of accuracy criteria, the %recovery must be in range 80-110%.

Precision

Intra-day assay measures the degree of repeatability of analytical method under normal specific conditions. The intra-batch precision is determined under same condition over short interval of time. In present study, it was determined by assessing the replicating 10 injections of DPH at same concentration (10 µg/mL, n=3) during same day and under same experimental conditions.

Inter-day assay is performed on different days at different experimental conditions [12]. In present study, it was determined by selecting the three different concentrations (20,30 and 40 µg/mL, n=3) at three different consecutive days.

The mean, standard deviation and % RSD were calculated for both intra-day and inter-day precision.

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

Limit of Detection (LOD) is the lowest amount of analyte, detected at three times the noise level (S/N=3) [6] and calculated by;

LOD= 3.3 SD/m

Whereas SD is the standard deviation and m is the slope of calibration curve.

While LOQ is the lowest amount of analyte that reproducible quantified above baseline noise (S/N=10) [6] and calculated by;

LOQ= 10 SD/m

Whereas SD is the standard deviation and m is the slope of calibration curve.

In present study, 20µL injection of DPH (10 µg/mL, n=3) standard used to calculating the LOD and LOQ with % RSD.

Analytical Solutions Stability

It was measured by keeping the mobile phase and standard solutions in capped volumetric flask in laboratory for 48 hours under normal conditions and was assessed at 12 hours intervals and determined the %RSD of DPH standard solutions [13].

Robustness

It was determined by observing the changes in various experimental conditions. Three standard solutions were prepared and then analyzed by using established conditions and by make variation in some chromatographic conditions. The changes in mobile phase pH (±0.3) and composition (±3), wavelength (±1 nm) and experimental temperature (±2°C) were made and obtained the data that was then subjected for statistical analysis by using analysis of variance (ANOVA) test.

System Suitability Test

It is the most important step in HPLC analysis, used for the verification of accuracy and precision of system [14]. In present study, system suitability was performed on HPLC system by injecting 10 injections of same concentration of DPH (10 µg/mL), assessed with mobile phase. The different parameters such as theoretical plates per column, tailing factor, %RSD of peak area and %RSD of retention time were calculated.

RESULTS AND DISCUSSION

Linearity

The calibration curve of Diphenhydramine HCl was linear over the concentration range of 10-50 µg/mL Three injections of each concentration were applied and regression equation (Y=180063X+222402) was obtained by plotting the injecting concentration (µg/mL) against obtained peak area (Y). The value of correlation coefficient (r^2=0.991) has shown the significantly good relationship between injecting concentration (µg/mL) and obtained peak area (Y) as shown in Figure **2**. The data for linearity was obtained for this experiment has been tabulated in Table **1** that showed %RSD was less than 1 for each concentration of standard solutions. This linearity data is in agreement with spectrophotometrically analytical method as previously reported in literature [15].

Table 1. Linearity of HPLC Method for Assay of Diphenhydramine HCl (n=3).

Injected concentration	Retention time (min)	DPH height (µv)	DPH Peak area (µvs)	DPH mean Peak area SD	DPH mean Peak area %RSD
10 µg/mL	14.17	22740	1710462	0.816497	4.77×10^{-5}
20 µg/mL	14.19	53553	4049980	0.816497	2.02×10^{-5}
30 µg/mL	14.29	84725	5830335.5	1.247219	2.14×10^{-5}
40 µg/mL	14.28	108995	7584088.5	0.816497	1.08×10^{-5}
50 µg/mL	14.40	132298	8946539.1	0.77603	8.67×10^{-6}

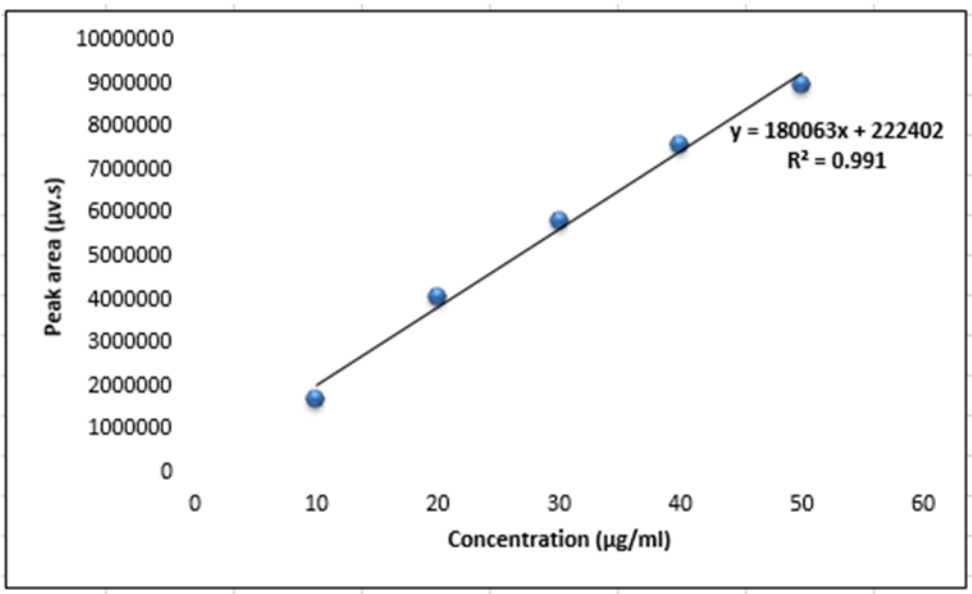

Figure 2. Linearity curve of Diphenhydramine HCl of reported analytical method.

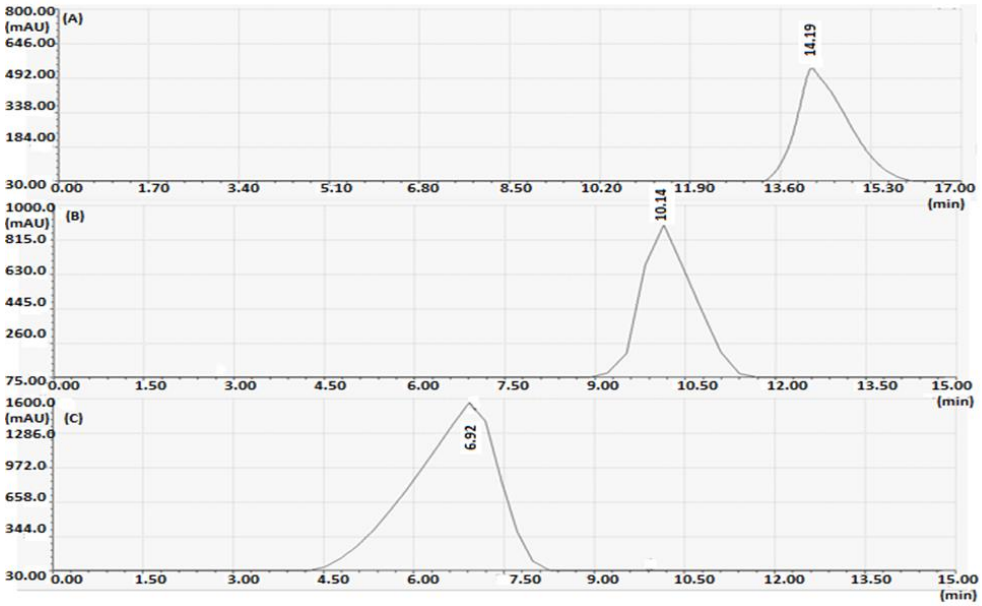

Figure 3. HPLC chromatogram of (A) reference standard solution DPH (B) Placebo (C) Mobile.

Specificity of Assay

The specificity of this method was analyzed for reference standard solution of DPH, Placebo and mobile phase by applying separate injections of each. There was no interfering peak was observed as in Figure **3**.

Accuracy

The accuracy was determined by repeating three injections of each concentration (10, 20, 30, 40 and 50 µg/mL) of DPH in mobile phase at 220nm. The mean % recovery for each concentration was calculated as described in Table **2** that was in range (80-110%) according to USP with %RSD less than 1.

Table 2. Accuracy of DPH-HCl from Standard Solutions of Known Concentrations (n=3).

Injected concentration	Obtained concentration	% Recovery	% RSD
10 µg/mL	8.264 µg/mL	82.64	0.988
20 µg/mL	21.256 µg/mL	106.28	0.768
30 µg/mL	31.144 µg/mL	103.814	0.786
40 µg/mL	40.883 µg/mL	102.21	0.798
50 µg/mL	48.45 µg/mL	96.90	0.842

Precision

Repeatability (Intra-Day Assay)

The intra-day precision was evaluated by injecting the 10 injections of 10 µg/mL concentration at same experimental conditions. Mean % RSD for Retention time, peak Height, peak area and obtained concentration was less than 1 as tabulated in Table **3**.

Intermediate Precision (Inter-Day Assay)

Inter-day precision was evaluated by injecting the three injections of selected three concentrations (20, 30 and 40 µg/mL) on different consecutive days. The retention time, peak area, obtained concentration and % recovery was observed and data showed mean % RSD for % recovery was less than 1 as tabulated in Table **4**.

Table 3. Repeatability of HPLC Assay for DPH-HCl by Replicate Injections (n=10) for Concentration (10µg/mL).

Injection number	Retention time (min)	Peak height (µv)	Peak area (µv s)	Obtained conc. (µg/mL)
1	14.17	22740	1710462	8.2641
2	14.16	22736	1710455	8.264
3	14.165	22738	171459	8.264
4	14.17	22740	1710462	8.2641
5	14.165	22738	1710459	8.264
6	14.15	22733	1710448	8.264
7	14.167	22739	1710457	8.264
8	14.157	22736	1710443	8.264
9	14.162	22737	1710459	8.264
10	14.168	22739	1710461	8.264
mean	14.1634	22737.6	1710457	8.26402
SD	0.006	2.059	5.97	$4*10^{-5}$
% RSD	0.042	0.009	0.0003	$4.84*10^{-6}$

Table 4. Demonstration of Intermediate Precision of HPLC Assay for DPH-Hcl Reported Method.

Injected concentration (µg/mL) (n=3)	RT (min)	Peak area (µv s) (n=3)	Obtained conc. (µg/mL) (n=3)	% Recovery	%RSD
20	14.19	4049980	21.256	106.28	0.768
30	14.29	5830335.5	31.144	103.814	0.786
40	14.28	7584088.5	40.883	102.21	0.798

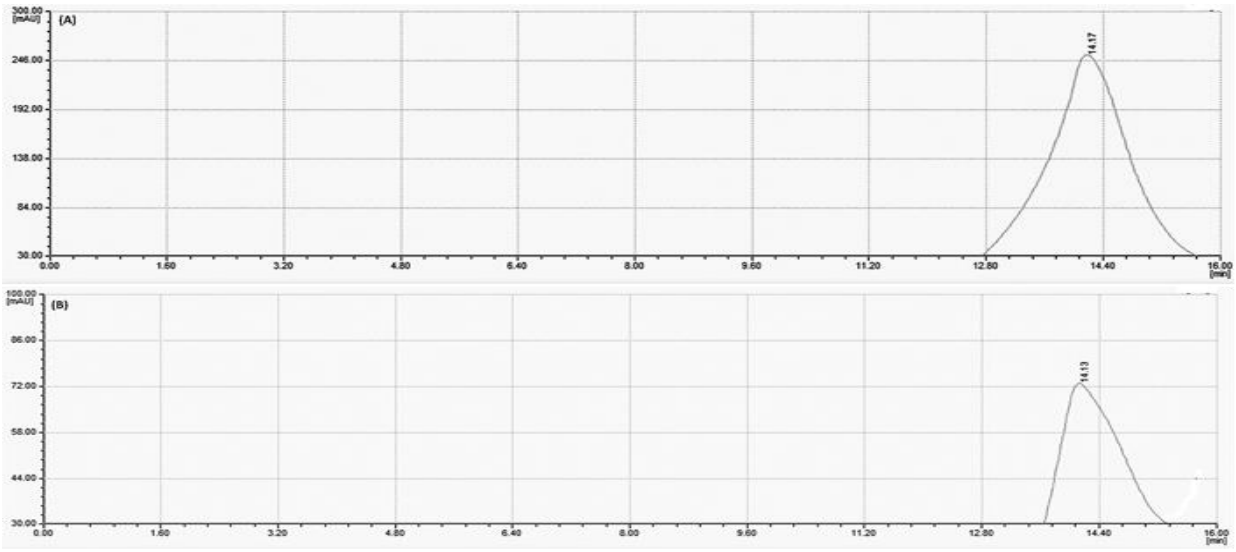

Figure 4. HPLC Chromatogram of DPH solution for (A) LOD & (B) LOQ.

LOD and LOQ

Limit of Detection and Limit of Quantitation by injecting the known lowest concentration (10µg/mL) at three and ten times S/N response for DPH was 1.49×10^{-5} µg/mL and 4.5×10^{-5} µg/mL respectively. The graph of LOD and LOQ has shown in Figure **4** (A&B).

Analytical Solution Stability

The stability of Diphenhydramine hydrochloride and mobile phase was calculated by comparing area percent and area response two standards at 10µg/ml over specific time for 48 hours. The standard solution has shown no significant change in DPH concentration throughout this time period as described in Table **5**. This was indicated by RSD less than 1% changes in peak area, obtained concentration and recovery between T=0 hours and T=48 hours. This data also showed no significant quantitative change in % recovery and as well retention time within 48 hours.

Robustness

Changes observed for pH (±0.3) and composition of mobile phase (±3 %), wavelength determination (±1 nm) and experimental temperature (±2°C), produced no affect in present developed method. This indicates developed analytical method has high level of robustness as no significant differences were observed by changing the chromatographic conditions.

System Suitability Test

In present study, this test was performed to determine the accuracy sand precision of HPLC system by injecting 10 injections of 10 µg/mL DPH. The results showed that mean % RSD for peak area and retention time was less than 1(0.0003 and 0.042 respectively). While the tailing factor was less than 2 (1.06) and theoretical plates were greater than 2000 (7076.7) as shown in Table **6**.

Table 5. Stability of DPH-HCl in Solution (n=3).

Time (hours)	Retention time (min)	Peak Area (µv) RSD (%)	Obtained conc. (µg/mL) RSD (%)	Recovery (%) RSD (%)
0	14.17	$4.77 *10^{-5}$	0.009	0.988
24	14.21	$4.79 *10^{-5}$	0.012	0.991
48	14.24	$4.83 *10^{-5}$	0.015	0.995

Table 6. System Suitability of HPLC Assay for DPH-HCl (n=10).

System Suitability Parameters	Acceptance Criteria	Results
Injection precision for Peak area	RSD ≤ 1%	0.0003
Injection precision for RT(min)	RSD ≤ 1%	0.042
Tailing factor (T) for DPH-HCl peak	T ≤ 2	1.06
Theoretical plates (N) for DPH-HCl peak	N = > 2000	7076.7

CONCLUSION

HPLC analysis of drug substances and validation is complexed and time consuming method. But in spite of all these, it is more precise and accurate analytical technique. This article is intended in providing the guidance to perform validation method for HPLC that generates useful data in order to meet all requirements of USP, ICH and FDA for the validation of DPH analysis.

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