Research Article

A novel stability indicating RP-HPLC method development and validation for estimation of Phenylephrine hydrochloride and Bromhexine hydrochloride in their tablet dosage form.

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ABSTRACT

A novel, specific, sensitive, accurate and economical stability indicating RP-HPLC method was developed for the estimation of Phenylephrine hydrochloride and Bromhexine hydrochloride in their tablet dosage form. Separation was achieved on C\textsubscript{18} column (250 × 4.6 mm, 5\textmu m) using Optimized Mobile Phase Water: Acetonitrile (60:40 % v/v) (pH adjusted to 4.5 with 1% OPA) and flow rate maintained at 1.0 ml/min was used. Wavelength was monitored at 215 nm. Both the drugs were subjected to acid, base, oxidation, thermal and photolytic degradation conditions. The retention time of PHN and BHX were found to be 3.24 min and 7.11 min respectively. The linearity response was observed in range of 5-25 µg/ml and 4-20 µg/ml of PHN and BHX respectively. Force degradation study revealed that maximum degradation of PHN and BHX was occur in Photolytic degradation. (Standard 19.88%, Sample 18.07% and standard 19.81%, Sample 18.26%) respectively. % RSD was found to be less than 2 in precision, Robustness, LOD and LOQ. % recovery was found in range 98-102%.The proposed method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness.

KEYWORDS

Phenylephrine hydrochloride, Bromhexine hydrochloride, RP-HPLC, Force Degradation Study.
1. INTRODUCTION
Phenylephrine hydrochloride is chemically Benzenemethanol-3-hydroxy α ((methyl amino) methyl)-1-hydrochloride (alpha R)\(^1\). It is a sympathomimetic agents or (\(\alpha_1\)–adrenoceptor agonist). They cause local vasoconstriction, thereby reducing congestion and edema of the nasal mucosa. They are used to advance nasal obstruction in the common cold, sinusitis, fever, acute or chronic rhinitis, allergies of the upper respiratory tract.\(^2\)-\(^3\) The chemical structure of Phenylephrine hydrochloride is shown in figure 1. Chemically Bromhexine hydrochloride is 2, 4-Dibromo [[cyclohexyl (methyl) amino] methyl] aniline hydrochloride\(^4\). It is a Mucolytic agent or expectorant are drugs believed to increase bronchial secretion or reduce its viscosity, facilitating its removal by coughing. The chemical structure of Bromhexine hydrochloride is shown in figure 2. Both drugs are official in Indian pharmacopeia\(^5\), British Pharmacopeia\(^6\) and United States Pharmacopeia\(^7\). The combination of PHN and BHX is used in treatment of Congestion with Excessive Mucus as compared to single drug. Literature review reveals that different Spectrophotometric\(^8\)-\(^11\) and Chromatographic methods\(^12\)-\(^25\) have been reported for estimation of Phenylephrine hydrochloride alone and in combination with other drugs and for Bromhexine hydrochloride and its combination with other drugs. Published UV method for both drug PHN and BHX combination\(^26\). So, there is need to develop and validated RP-HPLC method and degradation study for these drugs. So, Aim of present work is Development and Validation of Stability indicating assay method for estimation of Phenylephrine hydrochloride and Bromhexine hydrochloride in their combined dosage form. For this purpose marketed tablets Solvin Decongestant Tablet containing 10 mg of PHN, 8mg of BHX was used.

2. MATERIALS AND METHODS

2.1. Instrumentation
The chromatography was performed on a RP-HPLC instrument equipped with UV detector and RP C\(_{18}\) column (250 mm × 4.6 mm, 5μm) was used as stationary phase. Swisser analytical balance, pH meter, an ultrasonic cleaner (Toshcon), Hot air oven.

2.2. Reagent and chemicals
Pharmaceutically pure sample of PHN and BHX were obtained as a gift samples from Manish Pharma Lab, Viramgam and Espee Formulation Pvt. Ltd, Rajkot. All solvents were of HPLC and AR grade obtained from Merck, Rankem and Chemdyes, Rajkot respectively.

2.3. Chromatographic Condition
Separation was achieved by RP C18 column (250mm × 4.6 mm, 5μm) as stationary phase with water: acetonitrile (60:40 %v/v) as a mobile phase and pH of 4.5 adjusted with 1% orthophosphoric acid at a flow rate of 1 ml/min. Wavelength was monitored of PHN and BHX at 215 nm with UV detector and 20μL injection volume.

2.4. Preparation of Stock Solution
Accurately weighed 10 mg of PHN and 8 mg of BHX taken into two different 100 ml volumetric flask and make up volume with methanol (100µg/ml of PHN and 80µg/ml BHX).

2.4.2. Preparation of Working Solution
PHN from stock solution pipetting out 1.5 ml and diluted up to 10 ml with methanol (15µg/ml). BHX From stock solution pipetting out 1.5 ml and diluted up to 10 ml with methanol (12µg/ml).

2.4.3. Preparation of Calibration Curve
The calibration curves were plotted over a concentration range of 5-25 µg/ml for PHN and 4-12 µg/ml for BHX. Pipetting out 0.5, 1, 1.5, 2, 2.5 ml from stock solution (100 µg/ml of PHN and 0.5, 1, 1.5, 2, 2.5 ml from stock solution 80 µg/ml of BHX) into 10 ml volumetric flask and make up the volume up to the mark with methanol.

2.5. Forced Degradation Study
2.5.1. Sample Preparation for Acid Degradation
Acid decomposition study was performed by refluxing the working solution of both drugs (1.5 ml) in 2 ml of 0.1N HCl for 3 hr at 70 ºC. After 3 hr solutions neutralized with 2 ml of same strength of base (0.1 N NaOH) and finally make up to 10 ml volume with methanol, sonicated and filtered through 0.45µm membrane filter paper and injected in to HPLC system.

2.5.2. Sample Preparation for Base Degradation
Alkali decomposition study was performed by refluxing the working solution of both drugs (1.5 ml) in 2 ml of 0.1 N NaOH for 4 hr at 70 ºC. After 4 hr solution neutralized with 2 ml of same strength of 0.1 N HCl and finally make up to 10 ml volume with methanol, sonicated and filtered through 0.45µm membrane filter paper and injected in to HPLC system.

2.5.3. Sample Preparation for Oxidative Degradation
Oxidative decomposition study was performed by refluxing the working solution of both drugs (1.5 ml) in 2 ml 3% H₂O₂ for 3 hr at 70 ºC. After 3 hr volume make up to 10 ml with methanol, sonicated and filtered through 0.45µm membrane filter paper and injected into HPLC system.

2.5.4. Sample Preparation for Thermal Degradation
Thermal decomposition study was performed by refluxing the working solution of both drugs (1.5 ml) for 30 min at 105 ºC. After 30 min volume make up to 10 ml volume with methanol, sonicated and filtered through 0.45µm membrane filter paper and injected into HPLC system.

2.5.5. Sample Preparation for Photolytic Degradation
Photolytic degradation was performed by exposing the working solution of both drugs (1.5 ml) to Sunlight for 5 hr. After time period, volume make up to 10 ml volume with Methanol, sonicated and filtered through 0.45µm membrane filter paper and injected into HPLC system.
2.6. Analysis of Marketed Formulation

The method was used for simultaneous estimation of PHN and BHX in tablet dosage forms. For the sample preparation Mobile phase was used as a solvent. Ten tablets were powdered, accurately weighed (equivalent to 10 mg) and transferred in to 100 ml volumetric flask, added about 5 ml of Mobile phase in to it, sonicated for 30 minutes with intermittent shaking, cooled to attain room temperature and added up to 1200ml of Mobile phase and mixed well. It was filtered through 0.45 µ syringe filter. Further 1.5 ml of the above filtrate was diluted to 10 ml with Mobile phase to get 15 µg/ml concentration of PHN and 12 µg/ml concentration of BHX in mixture sample respectively. Absorbance of sample solution was measured at all selected wavelength.

The content of PHN and BHX in sample solution of tablet was calculated.

2.7. Method Validation

2.7.1. Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solutions of PHN and BHX were compared.

2.7.2. Linearity and Range

The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 5 – 25 µg/ml and 4 – 20 µg/ml for PHN and BHX, respectively. The solutions of each concentration were injected under the operating chromatographic condition as described earlier. Chromatograms were recorded. Plot the calibration curve of Peak area verses concentration and determine Correlation co-efficient and Regression equations for PHN and BHX. These operations were done five times and mean responses were calculated. % RSD was calculated. It should not be more than 2%.

2.7.3. Precision

PHN 15 µg/ml solution and BHX 12 µg/ml solution were analyzed and the absorbance of the each solution was measured six times, absorbance was measured and % RSD was calculated.

2.7.3.1. Intraday precision

Three replicates of three concentrations (5, 15 and 25µg/ml of PHN and 4, 12 and 20µg/ml of BHX), were analyzed at short interval of time, absorbance was measured and % RSD was calculated.

2.7.3.2. Interday precision

Three replicates of three concentrations (5, 15 and 25µg/ml of PHN and 4, 12, 20µg/ml of BHX), were analyzed at three consecutive days and absorbance was measured and % RSD was calculated.
2.7.4. Accuracy
Accuracy of the method was determined in terms of % recovery of standard. Recovery studies were carried out by spiking standard drug solution at the level of 80%, 100% and 120% to the pre-analyzed sample solution of PHN and BHX (10 and 8 μg/ml respectively). In this method the known concentration of standard drug was spiked to the assay sample. Each sample was prepared in triplicate at each level and injected. The amount of PHN and BHX were estimated by applying obtained values to the regression equation of the calibration curve. Acceptance criteria: 98 – 102 %.

2.7.5. Limit of Detection and Limit of Quantification
The limit of detection (LOD) and limit of quantitation (LOQ) of the method were determined by following equations.
LOD = 3.3 × σ/S
LOQ = 10 × σ/S
Where, σ = the standard deviation of Y- intercept of 5 calibration curves.
S = the mean slope of the 5 calibration curves

2.7.6. Robustness
Combined standard solutions of PHN (15μg/ml) and BHX (12μg/ml) were prepared and analyzed changing mobile phase, flow rate and pH by measuring the corresponding responses 3 times.

3. RESULTS AND DISCUSSION
3.1. Method Development
3.1.1. Optimized Chromatogram
Mobile phase Water: Acetonitrile: (60:40%v/v), pH adjusted to 4.5 with 1% Orthophosphoric acid (figure 3).

3.2. Force degradation study
Acid degradation base degradation, oxidative degradation, thermal degradation and photolytic degradation are depicted as chromatograms and given in figure 4, 5, 6, 7 and 8 respectively.

3.3. Analysis of Marketed Formulation
Applicability of the proposed method was tested by analyzing the commercially available tablet formulation named Solvin Decongestant Tablet. The accuracy of the method was determined by calculating the recoveries of PHN and BHX by the standard addition method at three concentration levels (80, 100 and 120%). The percentage recoveries of PHN and BHX were found to be in the range of 99.38 – 99.43% and 99.21 – 101.24% respectively (Table 2). Percentage Assay of PHN and BHX were found to be in an acceptance limit so this method can be used for analysis of this combination.
3.4. Validation of Stability Indicating RP-HPLC Method

3.4.1. Specificity
In the specificity study, blank, standard and sample were injected into the system. The chromatograms of blank do not show any interference at the retention time of PHN and BHX as it can be seen from respective chromatograms. Chromatographic condition of diluent was shown that there is no interference from the diluent (figure 9, 10).

3.4.2. Linearity
The linearity study was carried out for both drugs at different concentration levels. The linearity of PHN and BHX was in the range of 5 - 25 µg/ml and 4 - 20 µg/ml respectively. The % RSD of all results were less than 2%. The $r^2$ value was found 0.999 for both the drug (figure 11, 12 and 13) and (Table 3 and 4).

3.4.3. Precision
For, Repeatability, % RSD of PHN was found to be 1.06 %, while for BHX, it was found to be 0.81 % (Table 5)

3.4.3.1. Intraday Precision
For, Intraday precision, % RSD of Phenylephrine hydrochloride was found to be 0.60 – 1.06 %, while for Bromhexine hydrochloride, it was found to be 0.69 – 0.93 % (Table 6).

3.4.3.2. Interday Precision
For, Interday precision, % RSD of Phenylephrine hydrochloride was found to be 0.79– 1.80 %, while for Bromhexine hydrochloride, it was found to be 0.94 – 1.53% (Table 7). For Repeatability, Intraday precision and Interday precision, % RSD for both drugs were found to be less than 2. So, it can be concluded that proposed method for estimation of PHN and BHX is precise in nature.

3.4.3.3. Accuracy (Standard Addition Method)
Accuracy of the method was confirmed by recovery study from marketed formulation at three level 80%, 100% and 120 % of standard addition. For, Accuracy, % Recovery for PHN was found to be 99.77–101.54 %, while for BHX, it was found to be in range of 99.80–100.55% (Table 8 and 9). Result obtained reveals that % recovery of PHN and BHX were within acceptance criteria given in ICH guideline i.e. 98-102%.

3.4.3.4. Limit of Detection and Limit of Quantification
Calibration curve was repeated for 5 times and the standard deviation (S.D) of the intercepts was calculated. Then LOD and LOQ were measured as follows (Table 10). The proposed method can detect and quantify small amount of drugs with precision. So, it can be concluded that the proposed method is very sensitive in nature.
3.4.3.5. Robustness
Varying conditions of temperature, pH and mobile phase composition were carried out and % RSD was found less than 2% (Table 12).

4. CONCLUSION
A novel, specific, sensitive, accurate and economical stability indicating reversed-phase high-performance liquid chromatographic method was developed for the estimation of Phenylephrine hydrochloride and Bromhexine hydrochloride in their tablet dosage form.
Both the drugs were subjected to acid and base hydrolysis, oxidation, thermal and photolytic degradation conditions.
Maximum degradation of Phenylephrine hydrochloride was occur in Photolytic degradation. Standard (19.88%) and Sample (18.07%).
Maximum degradation of Bromhexine hydrochloride was occur in photolytic degradation. Standard (19.81%) and Sample (18.26%).
Validation parameters prove that method is repeatable, sensitive and selective for the analysis of Phenylephrine hydrochloride and Bromhexine hydrochloride in tablet dosage form.

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6. REFERENCES
1. Phenylephrine hydrochloride “Drug profile”, Accessed: September 2015.
   http://www.sebt.com/datasheet-203677-r-phenylephrine-hydrochloride.html
2. Tripathi KD. (2008-10). Essentials of Medical Pharmacology. (6th ed.). New Delhi, (chapter 9 and 16).
3. Phenylephrine “Drug profile”, Accessed: September 2015.
   http://www.drugbank.ca/drugs/DB00388
4. Bromhexine hydrochloride “Drug profile”, Accessed: October 2015.
   http://www.abcam.com/bromhexine-hydrochloride-ab143585.html
5. Indian Pharmacopoeia (2010). Government of India Ministry of Health & Family Welfare, The Indian Pharmacopoeia Commission, Ghaziabad, India. 277, 398, 1900.
6. The United States Pharmacopoeia USP 32-NF (2009). 27, Rockville MD USA, 3284-3285.
7. (2009), “British Pharmacopoeia”, Published by the stationary office on behalf of Medicines and Healthcare products Regulatory agency, 468-89.

8. Savic G, Nikolic, Bankovic V (2008). Development and Validation of Spectrophotometric method for phenylephrine hydrochloride Estimation in Nasal Drop Formulation. Macedonian Journal of Chemistry and Chemical Engineering. 27(2):149-156.

9. Chien DS, Schoenwald RD (1985). Fluorometric determination of phenylephrine hydrochloride by liquid chromatography in human plasma. J. Pharm. Sci., 74(5): 562-64.

10. Feng S, Zhao Q, Jiang J, et al (2013) Determination of phenylephrine in human plasma using ultra performance liquid chromatography tandem mass spectrometry. J. Chrom. B. Ana. Tech. Bio. Life. Sci., 28-32.

11. Ptacek P, Klima J, Macek J (2007). Development and validation of a liquid chromatography-tandem mass spectrometry method for the determination of phenylephrine in human plasma and its application to a pharmacokinetic study. J. Chrom. B. Ana. Tech. Bio. Life. Sci., 263-268.

12. Wagh RS, Hajare RA, Tated A et al (2011). Absorption Correction Method and Simultaneous Equation Method for the Simultaneous Estimation of Ebastine and Phenylephrine hydrochloride in Bulk and in Combined Tablet Dosage Form. International Journal of Research in Pharmacy and Chemistry. 1(4): 812-819.

13. Wankhede SB, Lad KA, Chitlange SS (2012). Development and Validation of UV-Spectrophotometric Methods for Simultaneous Estimation of Cetirizine hydrochloride and Phenylephrine hydrochloride in Tablets. International Journal of Pharmaceutical Sciences and Drug Research. 4(3): 222-226.

14. Ghodasara RB, Prajapati AM (2013). Spectrophotometric Method for Simultaneous Estimation of Ambroxol hydrochloride, Levocetirizine Dihydrochloride and phenylephrine hydrochloride in Tablet Dosage Form. International Research Journal of Pharmacy. 4(1): 197-200.

15. Tuljapure DS, Gowekar NM, Yadav SS, Mogale A (2012). Development and Validation of RP-HPLC Method for Simultaneous Estimation of Levocetirizine Dihydrochloride and Phenylephrine in Bulk and In Tablet Dosage Form. American Journal of Pharmatech Research. 2(4): 669-670.

16. Patel PU, Patel HB (2014). RP-HPLC Method for Simultaneous Estimation of Ciprofloxacin and Phenylephrine in Combined Dosage Forms. International Journal of Pharmaceutical Sciences and Nanotechnology. (7): 2631-2637.

17. Rekulapally VK, Rao VU (2015). A novel stability indicating RP-HPLC method development and validation for simultaneous estimation of phenylephrine, acetaminophen, guaifenesin and dextromethorphan in tablet dosage form. Scholar Research Library. 7(7): 329-339.

18. Patel KB, Thula KC, Maheshwari DG (2014). Stability Indicating HPLC Method for Simultaneous Estimation of Ciprofloxacin and Phenylephrine in Pharmaceutical Dosage Form. Pharmacophore. 5(2): 262-272.
19. Kishk SM, Salama I, Mostafa S, Mohamed El-Sadek (2014). Stability–Indicating Chromatographic Method for the Determination of Benzonatate, Diphenhydramine, Guifenesin, and Phenylephrine. Journal of Liquid Chromatography & Related Technologies. 37(5): 726-747.

20. Su-ying LIU, Su-xiang FENG, Guo-chou HUANG, Xiao-qing LI (2007). Content analysis of bromhexine hydrochloride in tablets by HPLC. Journal of Guangdong College of Pharmacy.

21. Dhoka M, Gawande V, Joshi P (2010). High Performance Liquid Chromatographic Method for Determination of Amoxicillin Trihydrate and Bromhexine hydrochloride in Oral Dosage Forms. International Journal of Pharmacy and Pharmaceutical Sciences. (2):129.

22. Kumar A, Nanda S (2011). A validated high performance liquid chromatographic method for estimation of bromhexine and terbutaline in bulk and tablet dosage forms. Pharmaceutical Methods. 2(4): 218–222.

23. Sonawane LV, Bari SB (2010). Development and Validation of RP-HPLC Method for The Simultaneous Estimation of Amoxicillin Trihydrate and Bromhexine hydrochloride from Oily Suspension. Pharmaceutica Analytica Acta. 1(2): 1-6.

24. Pekamwar SS, Kalyankar TM, Kokate SS (2014). RP-HPLC method development and validation for simultaneous estimation of bromhexine and ciprofloxacin in tablet dosage form, Scholar Research Library. 6(4): 97.

25. Porel A, Haty S, Kundu A. (2011). Stability-indicating HPLC Method for Simultaneous Determination of Terbutaline Sulphate, Bromhexine hydrochloride and Guaiifenesin. Indian Journal of Pharmaceutical Sciences. 73(1): 46-56.

26. Mevada DR, Bhalodiya K, Maniar B, Dadhania K, Faldu S (2014). Development and Validation of First Order Derivative Spectrophotometric Method for Simultaneous Estimation of Bromhexine hydrochloride and Phenylephrine hydrochloride in their Combined Pharmaceutical Dosage Form. Pharma Tutor Magazines. 2(6): 132-138.

Table 1: Summary of Force Degradation Condition

| Stress Type | Stress Condition          | PHN       | BHX       |
|-------------|--------------------------|-----------|-----------|
|             |                          | % Degradation of standard | % Degradation of sample | % Degradation of standard | % Degradation of sample |
| Acid        | 0.1 N HCl, 2ml for 3 hr, 70 ºC | 14.98% | 15.49% | 15.14% | 16.11% |
| Base        | 0.1 N NaOH, 2ml for 4 hr, 70 ºC | 16.84% | 17.29% | 15.80% | 17.02% |
| Oxidation   | 2 ml 3% H₂O₂ for 3 hr     | 14.56% | 15.05% | 19.46% | 17.65% |
At 105 °C for 30 min

At Sun-light for 5 hr

Table 2: Analysis of Marketed Formulation

| Batch no. of Tablet | Label Claim (mg) | % Amount of Drug Found (mg) | % Assay of PHN ± S.D (n=3) | % Assay of BHX ± S.D (n=3) | % RSD PHN | % RSD BHX |
|---------------------|------------------|----------------------------|-----------------------------|-----------------------------|-----------|-----------|
|                     | PHN              | BHX                        | PHN                         | BHX                         |           |           |
| EBD034003AS         | 10               | 8                          | 10.36                       | 7.88                        | 99.43±0.63| 101.64±1.6 |
| EBD015003AK         | 10               | 8                          | 9.98                        | 8.13                        | 99.38±0.56| 99.21±1.50|

Table 3: Linearity (Calibration Curve)

| Conc.( µg/ml) | Mean Area ± SD | % RSD |
|---------------|----------------|-------|
| PHN BHX      | PHN BHX        | PHN BHX |
| 5 4           | 650.798 ± 12.09| 767.027 ± 2.01 | 1.85 | 0.26 |
| 10 8          | 960.726 ± 11.40| 1105.321 ± 4.48| 1.18 | 0.40 |
| 15 12         | 1313.438 ± 8.65| 1549.923 ± 11.43| 0.65 | 0.73 |
| 20 16         | 1617.137± 5.50  | 1909.031± 5.81   | 0.34 | 0.30 |
| 25 20         | 1968.044± 15.35 | 2322.883± 7.05   | 0.77 | 0.30 |

Table 4: Optical parameters for PHN and BHX

| PARAMETER               | RESULT          |
|------------------------|-----------------|
| PHN                    | BHX             |
| Range                  | 5- 25µg/ml      | 4-20 µg/ml      |
| Regression Equation    | y = 65.81x + 314.7 | y = 97.88x + 356.2 |
| Correlation co-efficient ($r^2$) | 0.999 | 0.998 |
Table 5: Repeatability of PHN and BHX (n=6)

| CONC. (µg/ml) | PHN | BHX |
|---------------|-----|-----|
|               | AREA         |      | AREA         |
| 15            | 1308.159     | 12   | 1543.702     |
|               | 1279.002     |      | 1546.818     |
|               | 1313.429     |      | 1549.959     |
| MEAN          | 1306.809     | MEAN | 1542.389     |
| SD            | 13.885       |      | 12.589       |
| % RSD         | 1.06         | % RSD | 0.81         |

Table 6: Intraday Precision (n=3)

| Conc. (µg/ml) | PHN | BHX |
|---------------|-----|-----|
|               | Mean Area ± SD | % RSD | Mean Area ± SD | % RSD |
| 5             | 642.713 ± 6.874 | 1.06 | 4             | 757.396 ± 6.914 | 0.91 |
| 15            | 1298.704 ± 11.223 | 0.86 | 12            | 1532.233 ± 10.708 | 0.69 |
| 25            | 1946.645 ± 11.832 | 0.60 | 20            | 2295.871 ± 21.479 | 0.93 |

Table 7: Interday Precision (n=3)

| Conc. (µg/ml) | PHN | BHX |
|---------------|-----|-----|
|               | Mean Area ± SD | % RSD | Mean Area ± SD | % RSD |
| 5             | 641.8673 ± 11.563 | 1.80 | 4             | 757.0116 ± 11.609 | 1.53 |
| 15            | 1300.4753 ± 14.615 | 1.12 | 12            | 1534.8236 ± 14.545 | 0.94 |
| 25            | 1952.3656 ± 15.497 | 0.79 | 20            | 2300.7943 ± 21.926 | 0.95 |

Table 8: Accuracy (% Recovery study) of PHN

| Conc. (µg/ml) | % of Std. Spiked Drug (µg/ml) | Conc. of Spiking Drug (µg/ml) | Total Conc. after spiking (µg/ml) | Amount found (µg/ml) | % Recovery | % Recovery (Mean±S.D) | % RSD |
|---------------|-------------------------------|-------------------------------|-----------------------------------|----------------------|------------|------------------------|-------|
| 17.88         | 99.33                         |                               |                                   |                      |            |                        |       |
Table 9: Accuracy (% Recovery study) of BHX

| Conc. (µg/ml) | % of Std. Spiked | Conc. of Spiking Drug (µg/ml) | Total conc. after spiking (µg/ml) | Amount Found (µg/ml) | % Recovery | % Recovery (Mean± S.D) | % RSD |
|---------------|------------------|------------------------------|-----------------------------------|----------------------|------------|------------------------|-------|
| 80            | 6.4              | 14.4                         | 14.30                             | 99.30                | 14.39      | 99.93                  | 0.52  |
| 8             | 100              | 8                            | 16                                | 15.98                | 15.45      | 100.62                 | 0.65  |
| 120           | 9.6              | 17.6                         | 17.56                             | 99.77                | 17.65      | 100.28                 | 0.45  |

Table 10: LOD and LOQ of PHN and BHX

| PARAMETER     | PHN       | BHX       |
|---------------|-----------|-----------|
| MEAN SLOPE    | 65.71     | 97.79     |
| S.D           | 0.824     | 1.267     |
| LOD (µg/ml)   | 0.027     | 0.063     |
| LOQ (µg/ml)   | 0.084     | 0.19      |

Table 11: System Suitability Parameter

| PARAMETER                  | PHN             | BHX             |
|----------------------------|-----------------|-----------------|
| Retention Time (R<sub>t</sub>) (min.) | 3.247 ± 0.0030  | 7.110 ± 0.0026  |
| Tailing Factor (T<sub>r</sub>)       | 1.680 ± 0.0015  | 1.400 ± 0.005   |
Number of theoretical plates (N) 4290 ± 2.645 7493 ± 3.605
Resolution (R_s) 14.666 ± 0.0036

Table 12: Robustness

| PARAMETER       | VARIATION | PHN       |          |          | BHX       |          |
|-----------------|-----------|-----------|----------|----------|-----------|----------|
|                 |           | Mean area ± SD | % RSD | Mean area ± SD | % RSD |
| Flow rate       | 1.2       | 1276.88 ± 13.42 | 1.05   | 1504.03 ± 26.14 | 1.73   |
| (1ml/min)       | 0.8       | 1356.09 ± 15.18 | 1.11   | 1599.40 ± 14.89 | 0.93   |
| Mobile Phase    | 58:42     | 1273.27 ± 21.10 | 1.65   | 1504.05 ± 17.0 | 1.13   |
| Ratio (60:40%v/v)| 62:38     | 1335.26 ± 21.99 | 1.64   | 1579.87 ± 20.89 | 1.32   |
| Mobile Phase    | 4.3       | 1249.77 ± 17.05 | 1.36   | 1474.24 ± 16.81 | 1.14   |
| pH              | 4.7       | 1339.94 ± 22.85 | 1.70   | 1583.63 ± 16.89 | 1.06   |

Table 13: Summary of Validation Parameter

| PARAMETER               | PHN       |          |          | BHX       |          |
|-------------------------|-----------|----------|----------|-----------|----------|
|                         |           |          |  Precision |
|                         |           | Linearity |          |          |
|                         |           | Regression Equation |   | y = 65.81x + 314.7 | y = 97.88x + 356.2 |
|                         |           | Correlation Co-efficient (r^2) |   | 0.998 | 0.999 |
|                         |           | Range (µg/ml) |   | 5- 25 µg/ml | 4-20 µg/ml |
|                         |           | Precision |   | 1.06% | 0.81 % |
|                         |           | (n=6) |   |          |          |
|                         |           | Intraday Precision |   | 0.60 - 1.06% | 0.69 - 0.93% |
|                         |           | Interday Precision |   | 0.79 - 1.80% | 0.94 - 1.53% |
| Accuracy (% Mean Recovery) |           |   | 99.77-101.54 % | 99.80-100.55% |
| (n=3)                   |           | LOD (µg/ml) |   | 0.027 | 0.063 |
|                         |           | LOQ (µg/ml) |   | 0.084 | 0.19 |
| Analysis of Marketed    |           | Analysis of Marketed Formulation |   | 99.38 – 99.43% | 99.21 – 101.24% |
Table 14: Summary of Robustness

| CONDITION                | PHN (µg/ml) | BHX (µg/ml) |
|--------------------------|-------------|-------------|
| %RSD FLOW RATE           | 1.05 – 1.11 | 0.93 – 1.73 |
| MOBILE PHASE COMPOSITION | 1.64 – 1.65 | 1.13 – 1.32 |
| MOBILE PHASE pH          | 1.36 – 1.70 | 1.06 – 1.14 |

Figure 1: Structure of Phenylephrine hydrochloride

Figure 2: Structure of Bromhexine hydrochloride

Figure 3: Chromatogram of PHN (15µg/ml) and BHX (12µg/ml) in Water: Acetonitrile (60:40 %v/v) (pH adjusted to 4.5 with 1% OPA)
| Trial | Drug | Retention Time (min) | Peak Area   | Efficiency | Resolution |
|-------|------|----------------------|-------------|------------|------------|
| Final | PHN  | 3.247                | 1339.869    | 4290       | 14.663     |
|       | BHX  | 7.110                | 1615.332    | 7493       |            |

Both peaks was very well separated and symmetrical peak shape with good resolution was observed.

Figure 4: Chromatogram of Sample (PHN 15µg/ml and BHX 12µg/ml) for Acid Degradation

Figure 5: Chromatogram of Sample (PHN 15µg/ml and BHX 12µg/ml) for Base Degradation

Figure 6: Chromatogram of Sample (PHN 15µg/ml and BHX 12µg/ml) for Oxidative Degradation
**Figure 7:** Chromatogram of Sample (PHN 15µg/ml and BHX 12µg/ml) for Thermal Degradation

**Figure 8:** Chromatogram of Sample (PHN 15µg/ml and BHX 12 µg/ml) for Photolytic Degradation

**Figure 9:** Chromatogram of Blank (Diluent)
Figure 10: Chromatogram of Standard and Sample Solution for PHN (15µg/ml) and BHX (12µg/ml)

Figure 11: Overlain Chromatogram of PHN (5-25 µg/ml) & BHX (4-20 µg/ml)

Figure 12: Calibration curve of PHN in HPLC

\[ y = 65.81x + 314.7 \]
\[ r^2 = 0.999 \]

Figure 13: Calibration Curve of BHX in HPLC

\[ y = 97.88x + 356.2 \]
\[ r^2 = 0.998 \]