Simultaneous Analysis of Phenylephrine HCl and Ketorolac Tromethamine in Bulk and Injectable Formulations by RP-HPLC-PDA Method

Nagarjuna PJ1, Tharun Kumar B1, Buchi N. Nalluri1,2*

1Department of Pharmaceutics and Biotechnology, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada-520010, AP, INDIA
2Siddhartha Pharma Innovation and Incubation Center at KVSR Siddhartha college of Pharmaceutical Sciences, Vijayawada-520010, AP, INDIA

ABSTRACT

Rapid, isocratic and economical RP-HPLC-PDA method was developed for the simultaneous estimation of Phenylephrine HCl (PEH) and Ketorolac Tromethamine (KTR) in bulk and injectable dosage forms. Chromatographic separation was achieved with the Agilent Eclipse C18 column (150×4.6mm;5µ) and the mobile phase composed of methanol and 2mM Ammonium acetate in the ratio 43:57v/v at a flow rate of 1mL/min. The injection volume was 5µL and eluents were monitored at 220nm. Response was a linear function of concentration in the range 30–105 μg/mL for Phenylephrine HCl (PEH) and 10–35 μg/mL Ketorolac Tromethamine (KTR); the correlation coefficients were 0.999 and 0.999, respectively. The method was validated and is suitable for the simultaneous estimation of PEH and KTR in bulk and injectable dosage forms.

Keywords: Ketorolac tromethamine, Phenylephrine HCl, Reverse phase HPLC, PDA detector.

INTRODUCTION

Phenylephrine (PEH) is a sympathomimetic amine and an α1-adrenergic receptor agonist used primarily as a decongestant, to increase blood pressure and as an agent to dilate the pupil (mydriatic). Chemically it is 1-(3-Hydroxyphenyl)-N-methylethanolamine and exhibits its pharmacological activity by acting on α1 adrenergic receptors present on peripheral smooth muscles. Ketorolac (KTR) is a non-steroidal anti-inflammatory drug which belongs to heterocyclic acetic acid derivatives class and is used as an analgesic, anti-inflammatory and antipyretic drug. KTR is used to reduce pain and inflammation. Chemically, it is 5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid with a molecular weight of 255.26. Pharmacological activity of KTR is due to inhibition of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes which are involved in the formation of pain and anti-inflammatory mediators. Chemical structures are subjected as Fig 1.
The analytical methods reported/published so far for estimation of PEH and KTR individually and in combination with other drugs like Ofloxacin, Sparfloxacin, Febuxostat, Omeprazole, Paracetamol, Cetirizine Hydrochloride, Chlorpheniramine Maleate, Tramadol include HPLC\(^3\)-\(^9\), HPTLC\(^2\)-\(^6\), UV-Visible Spectrophotometry\(^3\)-\(^7\), LC-MS\(^8\) etc. However, no methods were reported for the simultaneous estimation of PEH and KTR in bulk and injectable dosage forms.

Hence, the present investigation was aimed at developing an economical and rapid LC-MS compatible RP-HPLC-PDA method for the simultaneous estimation of PEH and KTR in bulk and injectable formulations.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Reference standards of PEH and KTR were obtained from Dr. Reddy's Laboratories, Hyderabad, India. Marketed injectable formulation reported to contain 49.6mg PEH and 16.96mg KTR/4mL. Methanol -HPLC grade (E. Merck, Mumbai), Ammonium acetate- HPLC grade (E. Merck, Mumbai), citric acid monohydrate and sodium citrate dihydrate (analytical reagent grade) were purchased from Central Drug House (P) Ltd, Mumbai and sodium hydroxide (analytical reagent grade) was purchased from S D Fine Chem limited, Mumbai. Deionized water was used throughout the experiment.

**Apparatus and Chromatographic Conditions**

Quantitative RP-HPLC was performed on a high pressure gradient High Performance Liquid Chromatography (Shimadzu HPLC, class VP series) with two LC-20AD pumps, SIL-20A auto sampler was used with 200µL loop volume, programmable variable wavelength PDA detector SPD-M20A VP, and Agilent Eclipse C\(_{18}\) column (150x4.6mm, 5 µ). The HPLC system was equipped with “LC-Solution” software to acquire and process the data. The mobile phase of methanol: 2mM ammonium acetate (43:57 %v/v) at a flow rate was 1 mL/min was used. The mobile phase was degassed by sonication before use. UV detection was performed at 220 nm and the peak identity was confirmed by comparison of spectra and retention times with those of standards.

**Preparation of Standard Solutions**

A stock solution of PEH and KTR reference standards (10 mg/mL, respectively) was prepared in methanol and water, because of their active solubility in their respective solvent. Working standard solution (45µg/mL PEH and 15µg/mL KTR) was obtained by diluting the stock solution with 2mM ammonium acetate.

**Method Validation**

**Linearity**

The linearity of the method was checked by analyzing six solutions in the range 30-105 μg/mL for PEH (30, 45, 60, 75, 90 and 105 μg/mL) and 10-35µg/mL for KTR (10, 15, 20, 25, 30 and 35 µg/mL). Each solution was prepared in triplicate.

**Precision**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision was measured in terms of repeatability of application (System precision) and measurement (Method precision). The precision of the method was ascertained from the peak areas of six replicate injections of a fixed concentration.

**Limits of Detection and Quantification**

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can be readily detected but not necessarily quantified. It is usually regarded as the amount for which the signal-to-noise ratio (SNR) is 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte that can be quantified with acceptable precision and accuracy. It is usually regarded as the amount for which the SNR is 10:1. Two types of solution, i.e. blank and spiked with known progressively decreasing concentrations of each analyte, were prepared and analysed. The limits of detection (LOD) and quantification (LOQ) were then established by evaluating the minimum levels at which the analyte could be readily detected or accurately quantified, respectively.

**Accuracy**

Accuracy (Recovery) of the method was determined by spiking 80, 100 and 120% of known quantities of standards within the range of linearity to the synthetic solution of drug product (45 µg/mL of PEH and 15 µg/mL of KTR) and these mixture solutions were analyzed by developed method in triplicate.

**Specificity**

Specificity studies were carried for both pure drug and drug product by comparing the 3D plots with dihydrochloride. Peak purity tests were also carried out to show that the analyte chromatographic peak is not attributable to more than one component as the impurities are not available by purity index data.

**Assay**

A 1mL of the ophthalmic formulation equivalent to 30 mg of PEH and 10mg of KTR was transferred to a 10mL volumetric flask containing about 6mL of methanol, sonicated and made up to the mark with the same. The resulting solution was filtered through 0.45µm nylon membrane filter to obtain a stock solution of 3mg/mL PEH and 1mg/mL of KTR. It was further diluted with dihydrochloride, 2mM ammonium acetate to get the required concentration. The solution was injected three times into the column. The amount present in the formulation was calculated by comparing the area of standard and formulation sample of standard PEH and KTR with that of the sample.

**RESULTS AND DISCUSSION**

**Method Optimization**

The RP HPLC method was optimized to develop a simultaneous estimation of PEH and KTR. Initially 2mM ammonium acetate and methanol in the ratio 50:50 were used where PEH was eluted at around 2.821min, but KTR was also eluted at 3.056min where both the peaks appear almost closer to each other. In the next trial the diuron was changed to water, here the drugs were eluted as single peak before solvent front at 1.526 min. In the next trial diuron was changed to 2mM ammonium acetate and Agilent Eclipse C\(_{18}\) Column was used for estimation where PEH was eluted at 3.74min and KTR at 6.617 min but KTR peak tailing. The composition was changed and even flow rate was altered but there was no improvement in peak shape. In the next trial
column was switched to Inertsil C₁₈ column, with the same mobile phase and wavelength was changed to 230nm but no peak was observed.

Finally good peak shape and resolution were obtained with a mobile phase composition of 2mM ammonium acetate and methanol (57:43 v/v) on Agilent Eclipse C₁₈ column at a flow rate of 1mL/min with buffer as diluent. PEH was eluted at 1.9 min and KTR at 3.4 min. All the parameters were within the limits and the peaks were obtained within a runtime of 6 min. Wavelength was selected to be 220nm where the peak parameters were good with reproducibility and resolution with minimum interference. Method was validated according to ICH guideline (Q2B). The peak purity index was more than 0.9999 for both PEH and KTR indicating that the samples used for the analysis were pure enough. Optimized chromatogram was shown in Fig 2.

![Chromatogram of standard solution containing PEH (45µg/mL), KTR (15µg/mL), (B₁, B₂) UV spectra of PEH and KTR; (C₁, C₂) Peak purity indexes of PEH and KTR](image-url)


Method Validation

The method was validated for linearity, accuracy, and precision, limits of detection and quantification, and specificity.

Linearity

Linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship was evaluated across the range (30-105 µg/mL for PEH and 10-35 µg/mL for KTR) of the analytical procedure in triplicate. The range of concentrations was selected based on 80-120% of the test concentration. Peak area and concentrations were subjected to least square regression analysis to calculate regression equation. These were $Y = 7999X - 66502$ and $Y = 5639X + 1608$, with correlation coefficients of 0.999 and 0.999 for PEH and KTR, respectively. The high values of the correlation coefficients were indicative of linear relationships between analyte concentration and peak area.

Precision

Precision studies were carried out in terms of repeatability. Repeatability of the peak areas of standard sample (system precision) and assay sample (method precision) was assessed by using six replicates of concentration (45 µg/mL PEH; 15 µg/mL KTR). The data is given in Table 1 and 2 and the % RSD for both the drugs was found to be less than 2% which is acceptable according to Q2B guidelines.

| Injection | Peak area | Retention Time (min) |
|-----------|-----------|----------------------|
|           | PEH       | KTR                  |
| 1         | 290732    | 83691               |
| 2         | 290444    | 82803               |
| 3         | 290648    | 82816               |
| 4         | 290756    | 82699               |
| 5         | 290698    | 82798               |
| 6         | 290765    | 82750               |
| Average   | 290673.83 | 82926.16            |
| SD        | 120.433   | 377.18              |
| % RSD     | 0.41      | 0.455               |

| Injection | Peak area | Retention Time (min) |
|-----------|-----------|----------------------|
|           | PEH       | KTR                  |
| 1         | 328475    | 96149               |
| 2         | 328533    | 96290               |
| 3         | 328741    | 96971               |
| 4         | 328769    | 96843               |
| 5         | 328649    | 96869               |
| 6         | 328687    | 96943               |
| Average   | 328642.3  | 96677.5             |
| SD        | 116.42    | 360.60              |
| % RSD     | 0.035     | 0.373               |

Limits of Detection and Quantification

The limits of detection (LOD) and quantification (LOQ) were established by evaluating the minimum level at which the analyte could be readily detected and quantified with accuracy, respectively. LOD and LOQ of PEH were found to be 0.14 µg/mL and 0.4 µg/mL respectively while that of KTR were found to be 0.8 µg/mL and 2.57 µg/mL, indicating good sensitivity of the method.

Accuracy

Method accuracy was checked by preparing synthetic mixtures containing different amounts of PEH and KTR and analyzing the mixtures by using the developed method. Percentage recovery and %RSD were then calculated. The results obtained indicate that recoveries were excellent which are not less than 99% and %RSD were less than 2%.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants and matrix, etc. The effect of wide range of excipients and other additives usually present in the formulations of PEH and KTR in the determinations under optimum conditions were investigated.

Blank, placebo solution and sample solution were analyzed individually as per the method to examine interference. From the base shifted overlay of the chromatograms it can be inferred that there were no co-eluting or interfering peaks where PEH and KTR eluted. This shows that the peak of analyte was pure and excipients in the formulation did not interfere with the analyte. The peak purity indices values of the standard and sample peaks were found to be greater than 0.999 and these results were in good agreement with the above results. The peak purity index profile of both the drugs also confirms the absence of the impurities as subjected in Fig.3.
Figure 3(a): Overlay of the chromatograms of A-blank; B-placebo; C-standard chromatogram; D-sample chromatogram

Figure 4: 3D plots of the diluent (A), standard (B) and sample (C)
Stability of solution

The stability of the stock solution was assessed by analyzing it at different time intervals up to 7 days stored at 4°C. The percentage variation was found to be less than 2% to the initial concentration at different time points and it was observed that the solution was stable for a period of 7 days when stored at 4°C.

Assay

From prepared formulation, 0.8mL of the ophthalmic formulation equivalent to 10 mg of PEH and 3.39mg of KTR was transferred to a 10mL volumetric flask containing about 6mL of methanol, sonicated and made up to the mark with the same. The resulting solution was filtered through 0.45μm nylon membrane filter to obtain a stock solution of 1mg/mL of PEH and 0.3mg/mL of KTR. It was further diluted with diluent, 2mM ammonium acetate to get the required concentration. The solution was injected three times into the column. From the peak areas obtained, the content of PEH and KTR in the formulation was calculated. Results are subjected in Table 3.

Table 3: Assay Results of PEH and KTR (n=3)

| Formulation | Drug | Label claim (mg/mL) | Amount found (mg) (mean±SD) | % Assay | % RSD |
|-------------|------|---------------------|-----------------------------|---------|-------|
| Omidria®    | PEH  | 12.4                | 12.48±0.18                  | 100.72  | 1.44  |
|             | KTR  | 4.24                | 4.27±0.02                   | 100.93  | 0.46  |

CONCLUSION

A Rapid and accurate reversed-phase HPLC method has been established for simultaneous determination of PEH and KTR. Finally, it can be concluded that the proposed RP-HPLC-PDA method was validated fully as per the International Conference on Harmonization (ICH) Guidelines, and found to be applicable for routine quality control analysis for the estimation of PEH and KTR. The results of linearity, precision, accuracy and specificity, proved to be within the limits. The method provides selective quantification of PEH and KTR without interference from blank, placebo and degradants. The proposed method is sensitive, reproducible, reliable, rapid, and specific and also has the unique advantage of LC conditions being compatible with MS detection.

ACKNOWLEDGEMENT

The authors are thankful to Dr. Reddy’s Laboratories, Hyderabad for providing the reference standards of PEH and KTR and also to Siddhartha Academy of General and Technical Education, Vijayawada for providing the laboratory facilities to carry out the research work.

CONFLICT OF INTEREST: Nil

REFERENCES

[1] Tripathy KD, Essentials Of Pharmacology, 6th ed.; JAYPEE Brothers Medical Publishers (P) Ltd; 2006.P. 120-129, 184,194,200.
[2] The Indian Pharmacopoeia, 6th ed.; The Indian Pharmacopoeia Commission: Ghaziabad; 2010.P.1543, 1899.
[3] Rang, H. P, Dale M. M. Ritter J. M., Flower R. J, Henderson, G. Rang and Dale’s pharmacology, 7th ed.; Churchill Livingstone: London, 2012.P. 319, 11-13, 128, 429.
[4] Bertram G. Katang, Susan B. Masters, Anthony J Trevor, Basic and clinical Pharmacology, 12th ed.; Tata Mc GRAW-HILL: 2012, P. 133t, 136f, 141, 148,642.
[5] Balan P, Kannappan N, Bhimadhuni GN, Simultaneous estimation of Olopatadine Hydrochloride and Ketorolac Tromethamine in bulk and pharmaceutical dosage forms by RP-HPLC method. Int journal of research in pharmaceutical sciences, 2014; 5(1):14-19.
[6] Pratap B, Dey A, Rao G, Analytical method development and validation for simultaneous estimation of Fevuboxast and Ketorolac in bulk and pharmaceutical dosage form in rat plasma by RP-HPLC. IAJPR, 2014; 4(4):1729-77.
[7] Dubey, S. K, Duddelly, S, Jangala, H., Saha R. N. Rapid and sensitive reverse phase high performance liquid chromatography methods for estimation of Ketorolac in pharmaceutical dosage forms, Indian J. Pharm Sci, 2013; 75(1):9-93.
[8] Rahul S, Ashish P, Pooja C. Method development and validation for simultaneous estimation of Ketorolac and Sparfloxacin by RP- HPLC. Indian Journal of Pharmaceutical and Biological Research, 2013; 1(4):95-101.
[9] Syed NR, Ashfaq M, Ullah Khan I, Ifrana M. Stability indicating HPLC method for simultaneous determination of ofloxacin and ketorolac tromethamine in pharmaceutical formulation. Analytical methods, 2012; 4(7):2112-26.
[10] Boyka GT, Ivanka PP, P lamen TP, HPLC determination of Ketorolac tromethamine in tablet dosage forms. Der Pharmacia Sinica. 2012; 3(4): 400.
[11] Dharri P, Mehul P, Ketan P. Simultaneous RP-HPLC estimation of Moxifloxacin hydrochloride and Ketorolac tromethamine in ophthalmic dosage forms. Asian journal of research chemistry, 2012; 5(5):698.
[12] Fegade JD, Bhole RP, Chaudhary RY, Patil VR. Simultaneous estimation of Ofloxacin and Ketorolac tromethamine in ophthalmic dosageform by Reverse phase high performance Liquid chromatography. Asian journal of chemistry, 2009; 21(5):3360-66.
[13] Khushbu BP, Krupa CT, Dilip GM. Stability indicating HPLC method for simultaneous estimation of Ciprofloxacin and Phenylephrine in pharmaceutical dosage forms. Pharmacophore, 2014; 5(2):262-72.
[14] Mukesh M, Richa R, Yertika G, Dharmendra K, Anamendra K C, Anand G, Ranjith S. Development and validation of RP-HPLC method for the determination of Chlorphenamine Maleate and Phenylephrine in pharmaceutical dosage forms. Int journal of comprehensive pharmacy. 2014; 5(05):1-4.
[15] Ananad J, Selpannap M. Development and validation of RP- HPLC method for simultaneous estimation of Citrizine HCl and Phenylproporphine HCl in combined dosage form. IAJPR, 2013; 3(5):3863-70.
[16] Mahesh P, Karajane S, Mane A, Bhat A, Shinde P. Analytical method development and validation of Acetaminophen, Caffeine, Phenylephrine hydrochloride and Dextromethorphone hydrochloride in tablet dosage form by RP-HPLC. Int journal of pharmaceutical science invention. 2013; 2(2):09-15.
[17] Kumar A, Sharma R, Nair A, Saini G. Development and validation of RP-HPLC method for simultaneous estimation of
Nimesulide, Phenylephrine Hydrochloride, Chlorphenermine maleate and Caffeine anhydrous in pharmaceutical dosage forms. Acta Pol Pharma. 2012, 69(6):1017-22.

Deepali ST, Gawekar NM, Yadav SS, Mogala AS, Development and validation of RP-HPLC method for simultaneous estimation of Levo-citirizine Dihydrochloride and Phenylephrine in bulk and tablet dosage forms. American Journal of Pharm. tech research. 2012; 2(4):669-676.

Ozan J, Pirol, M, Ozden T, Simultaneous determination of Paracetamol, Phenylephrine hydrochloride, Orolamine citrate and Chlorpheniramine maleate by HPLC in Pharmaceutical dosage forms. E-Journal of chemistry. 2011; 8(3):1275.

Wicharn J, Puanjkaew L, Development of HPTLC method for determination of phenylephrine hydrochloride and brompheniramine maleate tablets. Int journal of pharmacy and pharmaceutical sciences. 2014; 6(6):106-109.

Patil V, Phalke S, Kale S, Patil R, Vithaldas S, Development and validation of HPTLC method for the simultaneous analysis of Gatifloxacin and Ketorolac tromethamine in eye drops. Journal of chemical and pharmaceutical research. 2013; 5(9):135.

Sejal PG, Mohit G.D, Tejas CB, Mrinalini C.D, Development and validation of stability indicating HPTLC method for determination of Ofloxacin ketorolac tromethamine in combination. Journal of adv scientific research. 2011; 2(3):77-82.

Swati S, Mashru R. C, Simultaneous determination of phenylephrine hydrochloride and tropicamide in ophthalmic dosage forms with three derivative spectrophotometric methods. Journal of the Chilean chemical society. 2010; 55(4).

[18] Dharal NB, Namita HT, Chilka P, Shah VH, Udhayay UM, Simultaneous estimation of Ketorolac tromethamine and Omeprazole by ultraviolet spectroscopy. Int journal of Research in Pharmacy and science. 2013; 3(2):146.

[19] Jitendra DF, Harshal P.M, Rajesh Y. C, Vijay R. P. Simultaneous spectrophotometric estimation of Ofloxacin and Ketorolac Tromethamine in ophthalmic dosage forms. International Journal of Chem Tech Research. 2009; 1(2):189-194.

[20] Sawant R, Joshi R, Lanke P, Bhangale G, Simultaneous estimation and validation of Paracetamol, Phenylephrine hydrochloride and Chlorpheniramine maleate in tablets by spectrophotometric method. Asian journal of pharmaceutical research and health care. 2011; 3(2):23-28.

[21] Wankhede SB, Lad KA, Chitilange SS, Development and validation of UV Spectrophotometric methods for simultaneous estimation of Citrizine hydrochloride and phenylephrine hydrochloride in tablets, International journal of pharmaceutical sciences and drug research. 2012; 4(3):222-26.

[22] Raju B, Ramesh M, Borkar RM, Padiya R, Benerjee SK, Srinivas R, Development and validation of liquid chromatography – mass spectrometric method for simultaneous determination of Moxifloxocin and Ketorolac in rat plasma: Application to pharmacokinetic study. Biomedical chromatography. 2012; 26(11):1341-47.

[23] Sheng F, Qian Z, Ji J, Pei Hu, Determination of phenylephrine in human plasma using ultra performance liquid chromatography-Tandem mass spectrometry, Journal of chromatography B. 2013, 28-32.