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**Original Article**

# Reversed phase high performance liquid chromatographic method for simultaneous determination of phenylephrine hydrochloride and ketorolac tromethamine

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## ABSTRACT

A simple, sensitive, accurate and rapid reversed phase high performance liquid chromatographic method was developed for simultaneous determination of phenylephrine hydrochloride (PHE) and ketorolac tromethamine (KTC). The method was performed on an Eclipse plus C18 reversed phase column (100 mm × 4.6 mm, particle size 3.5 µm) using a mobile phase consisting of methanol: potassium dihydrogen ortho phosphate buffer; pH 6.5 in a ratio of 50:50 (v/v). The flow rate was 1 ml/min and detection was carried out at 277 nm for phenylephrine and 320 nm for ketorolac. The retention times of phenylephrine hydrochloride and ketorolac tromethamine were 1.03 and 2.68 min, respectively. The method was linear over the concentration range of 3-60 µg/ml for phenylephrine and 1.5-21 µg/ml for ketorolac. The described HPLC method was successfully applied for the determination of phenylephrine and ketorolac in laboratory prepared mixture containing all possible excipients present in eye drop dosage form. The mean percentage recovery was found to be 100.23% for phenylephrine and 99.74% for ketorolac. Validation of the method was carried out according to the guidelines of the International Conference of Harmonization (ICH).

**Key Words:** Pharmaceutical analysis, Optimization, Validation, Laboratory prepared mixtures, Eye drops

## INTRODUCTION

Omidria™; is a newly FDA approved combination of phenylephrine hydrochloride and ketorolac tromethamine. Phenylephrine hydrochloride (PHE; (-)-*m*-Hydroxy-α-[(methylamino) methyl] benzyl alcohol hydrochloride) is an α1-adrenergic receptor agonist that acts as a mydriatic agent by contracting the radial muscle of the iris [1, 2]. PHE is official in both BP 2008 [3] and USP 34 [4]. Ketorolac tromethamine (KTC; (±)-5-Benzoyl -2, 3- dihydro-1H- pyrrolizine -1-carboxylic acid: 2 -amino -2-(hydroxy methyl)-1, 3-propanediol) is a nonsteroidal anti –inflammatory drug that inhibits both cyclooxygenase enzymes (COX -1 and COX -2), resulting in a decrease in tissue concentrations of prostaglandins to reduce pain due to surgical trauma. Ketorolac prevents surgically induced miosis by inhibiting prostaglandin synthesis secondary to ocular surgical insult or direct mechanical stimulation of the iris [5-7]. KTC is official in both BP 2008 [8] and USP 34 [9]. The chemical structures of PHE and KTC are shown in Fig. 1. Omidria® is added to ophthalmic irrigation solutions used during cataract surgery or intraocular lens replacement and is indicated for maintaining pupil size by preventing intraoperative miosis and reducing postoperative ocular pain [10]. There are several publications describing analytical methods for the determination of PHE either alone or in combination with other drugs. These methods include capillary electrophoresis [11, 12]; voltammetry using a glassy carbon electrode modified with multi-wall carbon nanotubes [13] and spectrophotometry using ion pair complexation with alizarin [14] or 4-aminoantipyrine and copper (II) [15]. Different RP-HPLC methods have been developed for the determination of PHE in combination with other drugs such as: chlorpheniramine maleate [16], paracetamol/ chlorpheniramine maleate [17], chlorpheniramine maleate/methscopolamine nitrate [18], phenyl propanolamine hydrochloride/ guaifenesin [19] and paracetamol/chlorpheniramine maleate/ dextro-methorphan hydrobromide [20]. Different methods were developed for determination of KTC including: spectrophotometric methods [21, 22], fluorimetric method using stopped-flow sequential injection analysis [23] and chromatographic methods involving HPTLC [24]and HPLC in

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human eye sample [25], in human plasma [26] and in tablet dosage forms [27].

Literature survey revealed a few methods for the simultaneous determination of PHE and KTC in pharmaceutical dosage forms [28- 31]. The present work aims to develop a new, rapid, sensitive and fully validated RP-HPLC method for the direct and simultaneous determination of KTC, and PHE in raw materials and in laboratory prepared mixture containing both drugs at the ratio present in Omidria eye drops with the excipients. This method can be used for quality control of these drugs in pharmaceutical products.

## MATERIAL AND METHOD:

***Apparatus and Software:*** HPLC measurements were performed on Agilent Technologies 1260 Infinity instrument equipped with a G1311C quaternary pump, G1329B automatic injector and G1314F variable wavelength detector (VWD) detector. Data were processed using Agilent Open LAB CDS Chemstation Edition software for LC system.
The pH measurements were made with HANNA pH 211 Microprocessor pH-meter with double junction glass electrode.

***Chromatographic conditions:*** The Separation was carried out on an Eclipse plus C18 column (100mm ×4.6mm ×3.5 µm particle size) purchased from Agilent Inc., USA. A mobile phase consisting of methanol: phosphate buffer (50 mM) at pH 6.5 (50:50v/v) and was prepared daily, filtered, sonicated and delivered isocratically at a flow rate of 1 ml/min. The UV detector was programmed at a wavelength of 277 nm for the first 2 minutes for determination of PHE then; the signal was changed and balanced at 320 nm for the last minute for determination of KTC. The injection volume was 15µl.

***Chemicals and Reagents:*** Phenylephrine hydrochloride was purchased from Sigma-Aldrich Co., Germany with a purity of 99.8%. Ketorolac tromethamine was kindly supplied by Sigma Company for Pharmaceutical Industries, Quesna, Menofia, Egypt. The purity of KTC was found to be 99.18% according to the official method [2]. Methanol HPLC-grade was used. Potassium dihydrogen orthophosphate, ortho phosphoric acid and all other chemicals were of analytical grade.

***Stock and Working Standard Solution:***
***Stock standard solutions:*** Stock standard solutions of PHE and KTC were prepared in methanol to obtain solutions with concentration 1.0 mg/ml of PHE and KTC.

***Preparation of working standard solutions***: Accurately measured volumes of stock standard solutions of PHE and KTC were transferred into a series of 10ml volumetric flasks and diluted appropriately with mobile phase to obtain working standard solutions with concentration range 3-60 µg/ml for PHE and 1.5-21 µg/ml for KTC.

***Construction of Calibration curves:*** A volume of 15 µl of each working standard solution was injected at the optimum chromatographic conditions. Calibration curves were obtained by plotting the peak area of each working solution versus its corresponding concentration. Regression equations were calculated.

***Preparation of laboratory prepared mixture:*** Omidra® is a 4 ml clear, colorless, sterile solution containing phenylephrine hydrochloride 12.4 mg/ml equivalent to 10.16 mg/ml (1% w/v) of phenylephrine and ketorolac tromethamine 4.24 mg/ml equivalent to 2.88 mg/ml (0.3% w/v) of ketorolac in a single-patient-use vial, with a pH of approximately 6.3.

Omidra® is not available in local market, so a laboratory prepared mixture simulated to this dosage form was prepared by mixing 124 mg phenylephrine hydrochloride and 42.4 mg ketorolac tromethamine. This mixture was transferred to a 10 ml volumetric flask and dissolved in citrate buffer pH 6.3 (consists of citric acid monohydrate, sodium citrate dehydrate, sodium hydroxide for pH adjustment). The solution was then sonicated for 15 min for complete dissolution of both drugs and made up to volume with buffer solution. The solution was filtered and the first 1.0 ml of the filtrate was discarded.

An aliquot of 250 µl of the filtrate was transferred to a 100 ml volumetric flask and made up to final volume with mobile phase to obtain a solution containing 31 µg/ml PHE and 10.6 µg/ml KTC. A volume of 15 µl of these solutions was injected under the optimized chromatographic conditions. Each drug concentration was calculated from its own calibration curve.

## RESULTS AND DISCUSSION

The present work describes a simple, precise and accurate RP-HPLC method for simultaneous determination of PHE and KTC. The method was developed by studying the effect of different factors on the retention times of both drugs. The method was then optimized, validated and applied to laboratory prepared mixtures.

***Method Development:*** Many factors affect the separation and resolution of both drugs in their mixtures. These factors include organic to aqueous ratio, pH and concentration of potassium dihydrogen orthophosphate in the mobile phase. These factors were studied separately to optimize the chromatographic separation considering the resolution and the system suitability parameters.

By considering the pKa values of both drugs which are 8.9 for PHE and 3.45 for KTC, the first chosen pH was 7.2 which is 1.5 pH units far from pKa of each drug. So, one form of each drug will predominate at the chosen pH value. Then at fixed pH 7.2, the ratio of organic phase was changed in the range from 80% to 40% while keeping other factors constant. At a ratio 80:20 of organic to aqueous phase, the drugs were unresolved as shown in Fig. 2. As the ratio of organic phase decreased, the resolution increased but the run time also increased. So, the ratio 50:50 was chosen as it gives better resolution with short run time and high efficiency as shown in Fig. 3. Then the pH value was changed in the range from 3.5 to 7.2 while keeping other factors constant. The optimum pH selected is 6.5 which gave the highest efficiency as shown in Fig.4.

Finally, concentration of potassium dihydrogen orthophosphate changed from 50 to 200 mM. The optimum concentration selected is 50 mM which gave better miscibility with the methanol and acceptable buffering capacity.

The wavelength was initially adjusted at 277 nm which is λmax of PHE but measuring at this wavelength for both drugs show broad peaks with low sensitivity for KTC, so the wavelength was adjusted to be at 277 nm for the first 2 minutes to determine PHE at its λmax, then at 320 nm for the last minute to determine KTC at its λmax. Different trials were carried out and optimum conditions were selected to give higher resolution and higher symmetry. All system suitability parameters are shown in Table 1.

The proposed HPLC method was applied successfully for the determination of both drugs in their combination with good resolution as shown in Fig.5. where their retention times were 1.03 and 2.68 minutes for PHE and KTC, respectively.

***Validation of the developed method:*** The validity of the method was studied regarding linearity, specificity, accuracy, and precision according to ICH guidelines (ICH, 2005) [32].

***Linearity:*** Linearity was studied to determine the range over which analyte response is linear as a function of concentration. This study was performed by preparing standard solutions at seven different concentrations and analyses were performed in triplicates. The method was found to be linear over a concentration range of 3-60 μg/ml for PHE and 1.5-21 μg/ml for KTC. Regression equations were calculated; the results of slope, intercept, standard deviation about the slope and intercept are summarized in Table 2.

***Accuracy & Precision:*** Precision was considered at two levels: repeatability and intermediate precision. Repeatability, or intra-day precision, was determined by performing nine analyses at three different concentrations covering the linearity range on the same day. The inter-day precision studies were determined by estimating the corresponding responses for the same samples on different days.

Three replicate standard solutions at three different concentrations of PHE (15, 35, 55 μg/ml) and KTC (4.5, 13.5, 16.5 μg/ml) were assayed on the same day and on three different days. Mixtures of three concentrations of both drugs (PHE: KTC) at a ratio 10:3 which is similar to their ratio in the marketed dosage forms were prepared. The found concentrations of each drug in mixtures and the mean % recoveries were calculated from its corresponding regression equations. The results of % recoveries were used as a measure for accuracy of the method and the results of standard deviation (S.D.) and percentage relative standard deviation (% R.S.D.) were used to assess determination of repeatability and intermediate precision of the method.

The experiment was performed in triplicate. The mean % recovery and % R.S.D. were calculated for each concentration. The results obtained are summarized in Tables 3 and 4 indicating good accuracy and precision of the method.

***Specificity:*** As there is no marketed formulation available in local market, laboratory prepared mixtures with possible excipients were prepared at the dosage form ratio and analyzed under the optimum chromatographic conditions. The method specificity was determined by % recovery obtained from analyses of laboratory prepared mixtures. The mean % recovery obtained by the proposed method was found to be 101.3% ± 0.27% for PHE and 100.97% ± 0.04% for KTC. This indicates that there are no interferences from the excipients.

***Limit of Detection:*** The limit of detection of an individual analytical procedure is "the lowest amount of analyte in a sample which can be detected but not necessarily to be quantitated as an

exact value". The limit of detection (LOD) was calculated as follow:

LOD = 3.3 σ/ S

where

σ = Relative standard deviation of y-intercepts of regression line.

S = the slope of the calibration curve (of the analyte).

***Limit of Quantitation:*** The limit of quantitation of an analytical procedure is "the lowest amount of analyte in a sample, which can be quantitatively determined with suitable accuracy and precision". Limit of quantitation (LOQ) was calculated from the equations:

**LOQ = 10 σ / S**

where

σ = Relative standard deviation of y-intercepts of regression line.

S = the slope of the calibration curve (of the analyte).

The limit of detection and limit of quantitation were found to be 0.49 and 1.49 µg/ml for PHE and 0.15 and 0.46 µg/ml for KTC. The values indicate that the method is sensitive enough for routine analysis of both drugs in commercially available eye drops.

## CONCLUSION

The developed method is simple, accurate, robust, sensitive and precise for the simultaneous estimation of ketorolac tromethamine and phenylephrine hydrochloride in their pharmaceutical dosage forms. The excipients present in eye drop dosage form did not interfere in the analysis, which proved the specificity of the method for these drugs. The sample preparation is simple; the analysis time is short which about 3 minutes is. Hence, the proposed method can be used for the routine quality control analysis in the combined formulation either in authentic samples or in dosage forms.

**Table 1: Results of System Suitability Tests for the Determination of Phenylephrine and Ketorolac by the Proposed RP-HPLC Method**

| Parameter | Phenylephrine | Ketorolac |
|---|---|---|
| **Theoretical plates (N)** | 1859 | 5859 |
| **HETP*** | 0.0538 | 0.0171 |
| **Tailing factor (T)** | 1.253 | 1.199 |
| **Resolution (Rs)** | 13.87 | |

*HETP: height equivalent to a theoretical plate (mm).

**Table 2: Quantitative Parameters for Determination of PHE and KTC with the Proposed RP-HPLC Method.**

| Parameter | Phenylephrine | Ketorolac |
|---|---|---|
| **Linearity*** | 3-60 | 1.5-21 |
| **Intercept** | - 1.964 | - 15.62 |
| **Slope** | 8.792 | 47.61 |
| **Correlation Coefficient** | 0.9999 | 0.9999 |
| **Retention Times*** | 1.03 | 2.68 |
| **LOD*** | 0.4921 | 0.1525 |
| **LOQ*** | 1.4913 | 0.468 |
| **$S_{y/x}$ *** | 2.4433 | 3.6787 |

* Linearity (□g/ml), Retention Time (min), LOD: Limit of detection (□g/ml), LOQ: Limit of quantitation (□g/ml), $S_{y/x}$: residual standard deviation of the regression line.

**Table 3: Evaluation of Accuracy for the Determination of PHE and KTC.**

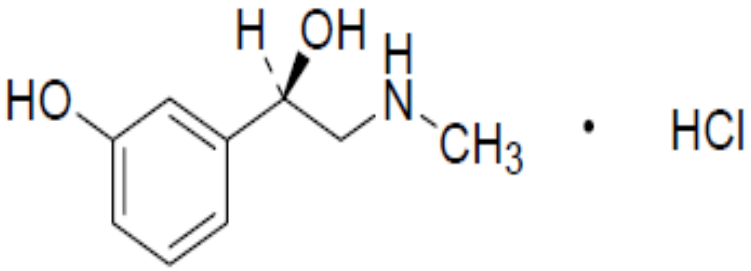
| Drug | Concentration taken* | Mean Concentration Found* | % Recovery | S.D. | Mean % Recovery±S.D. |
|---|---|---|---|---|---|
| **PHE** | 15<br>35<br>55 | 14.952<br>35.358<br>54.990 | 99.683<br>101.023<br>99.981 | 0.147<br>0.077<br>0.229 | 100.23<br>±<br>0.703 |
| **KTC** | 4.5<br>13.5<br>16.5 | 4.469<br>13.411<br>16.593 | 99.312<br>99.338<br>100.565 | 0.013<br>0.020<br>0.006 | 99.74<br>±<br>0.716 |

*****: mean of three determinations (concentration in units of µg/ml).

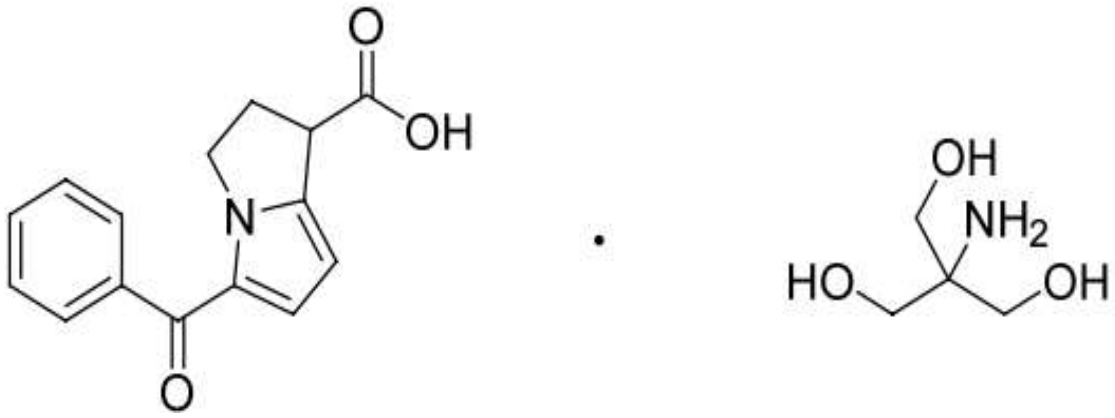
**Table 4: Evaluation of the Intra-day and Inter-day Precision for the Determination of PHE and KTC.**

| Drug | Concentration taken | Intraday | | | Interday | | |
|---|---|---|---|---|---|---|---|
| | | Mean concentration Found* | % Recovery | % R.S.D | Mean concentration Found* | % Recovery | % R.S.D |
| **PHE** | 15<br>35<br>55 | 14.777<br>35.144<br>54.827 | 98.513<br>100.413<br>99.686 | 0.066<br>0.112<br>0.382 | 14.906<br>35.343<br>54.899 | 99.373<br>100.981<br>99.816 | 0.114<br>0.191<br>0.083 |
| **KTC** | 4.5<br>13.5<br>16.5 | 4.468<br>13.405<br>16.596 | 99.283<br>99.295<br>100.580 | 0.003<br>0.016<br>0.015 | 4.467<br>13.403<br>16.577 | 99.259<br>99.281<br>100.469 | 0.003<br>0.009<br>0.029 |

*****: mean of three determinations (concentration in units of µg/ml).




**(a) Phenylephrine Hydrochloride**




**(b) Ketorolac Tromethamine**

**Fig. 1. Chemical structure of (a) PHE and (b) KTC.**

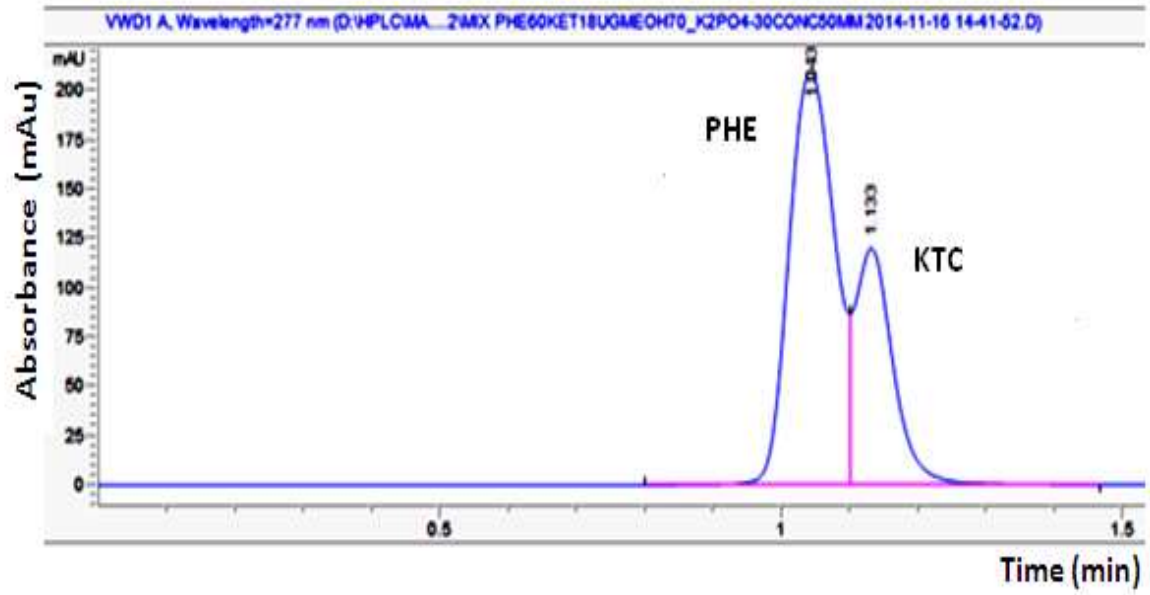



**Fig. 2. Chromatogram of unresolved peaks of PHE and KTC at initial chromatographic conditions: using mobile phase consisting of methanol: $KH_2PO_4$ buffer in ratio of 80:20 (v/v), pH= 7.2, at flow rate= 1ml/ min), temp.= 25°C and inj. vol.= 15 µl using variable wavelength detector on an Eclipse plus C 18 column at λ= 277 nm.**

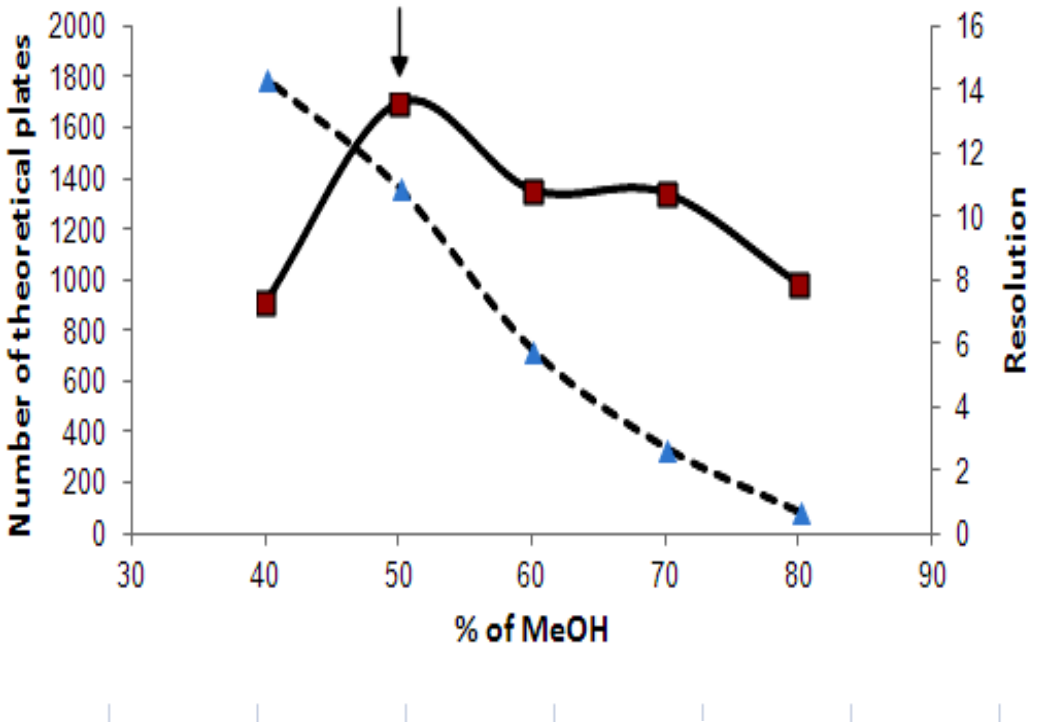



**Fig. 3. Effect of % of methanol on the resolution ( ) and number of theoretical plates ( ).Other chromatographic conditions: $KH_2PO_4$ buffer (50m M), temp.= 25°C, inj. vol.= 15 µl, pH= 7.2 and at flow rate= 1ml /min.**

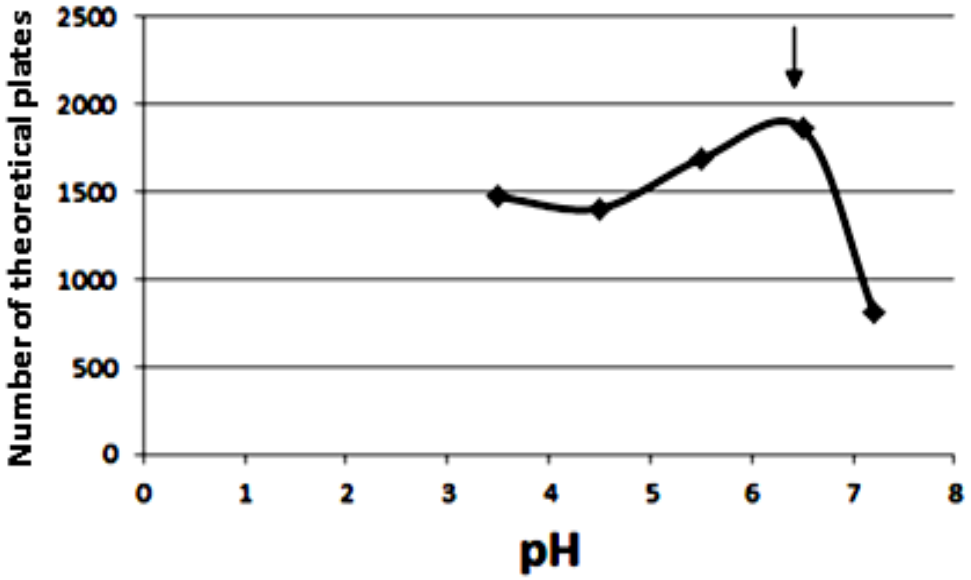



**Fig. 4. Effect of pH on number of theoretical plates. Other chromatographic conditions: using mobile phase consisting of methanol: potassium dihydrogen ortho phosphate buffer in ratio of 50:50 (v/v) and flow rate= 1ml/ min, temp. 25°C and inj. vol.= 15 µl.**

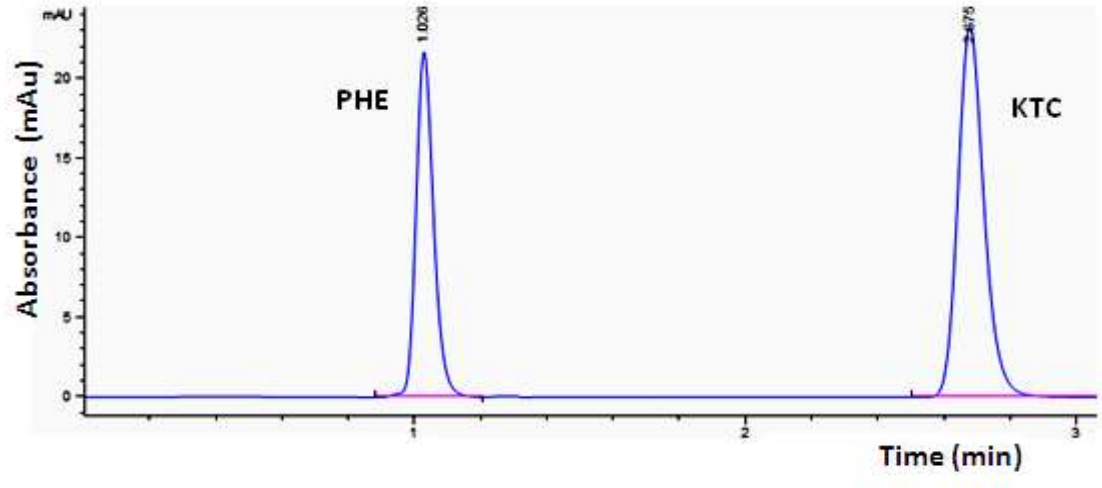



**Fig. 5. Chromatogram of PHE and KTC at a ratio 10:3 as in dosage form using mobile phase consisting of methanol: potassium dihydrogen ortho phosphate buffer in ratio of 50:50 (v/v) ; pH= 6.5 at flow rate = 1 ml/min, temp.= 25°C and inj. vol.= 15 µl.**

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