

# **INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES**

**Pharmaceutical Sciences**

**Research Article.....!!!**

Received: 06-08-2014; Revised; Accepted: 05-09-2014

## **RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF TROPICAMIDE AND PHENYLEPHRINE HYDROCHLORIDE IN OPHTHALMIC FORMULATION**

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### **Keywords:**

Tropicamide, Phenylephrine  
Hydrochloride, RP-HPLC,  
Ophthalmic formulation

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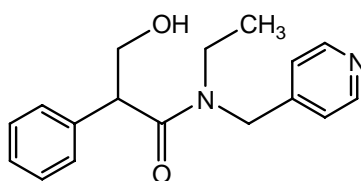
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### **ABSTRACT**

An accurate, highly sensitive, precise and reproducible isocratic RP-HPLC method was developed and subsequent validated for the simultaneous analysis of Tropicamide and Phenylephrine Hydrochloride in ophthalmic formulation. Method development was carried out on ODS Hypersil C<sub>18</sub>, 250mm × 4.6mm, 5μ(Particle Size) column. The mobile phase was a mixture of buffer (0.05M KH<sub>2</sub>PO<sub>4</sub>, pH-4) and methanol in the ratio of 60:40 v/v. The flow rate was set at 1.0 ml/min and UV detection at 216nm. The retention time of Tropicamide and Phenylephrine Hydrochloride were found to be 3.290 min and 5.063 min respectively. In the linearity study, the regression equations Tropicamide and Phenylephrine Hydrochloride were found to be  $y=200x + 18.06$  and  $y=28.11x-27.59$ . Correlation coefficient was 0.999 and 0.998 for Tropicamide and Phenylephrine Hydrochloride respectively. The proposed method was successfully applied for the quantification of ophthalmic formulation.

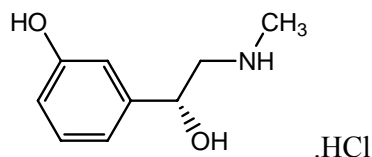
## INTRODUCTION

Chemically, Tropicamide (TRO) is N-Ethyl-3-hydroxy-2-phenyl-N-(pyridin-4-ylmethyl) propanamide. It binds to and blocks the muscarinic receptor in the muscles of the eye. Tropicamide acts by blocking the responses of the iris sphincter muscle to the iris and ciliary muscles to cholinergic stimulation, producing dilation of the pupil and paralysis of the ciliary muscle. Tropicamide is an antimuscarinic drug that produces short acting mydriasis and cycloplegia when applied as eye drops. Due to its relatively short duration of effect (4–8 hours), it is typically used during eye examinations such as the dilated fundus examination, but it may also be used before or after eye surgery.<sup>1-4</sup> In common,  $\alpha_1$ -adrenergic receptors mediate contraction and hypertrophic growth of smooth muscle cells.



**Figure 1:** Chemical structure of Tropicamide

Chemically, Phenylephrine (PHE) is (R)-3-(1-Hydroxy-2-(methylamino) ethyl) phenol appears to act likewise on all three receptor subtypes. All three receptor subtypes appear to be involved in maintaining vascular tone. Phenylephrine decreases nasal congestion by acting on  $\alpha_1$ -adrenergic receptors in the arterioles of the nasal mucosa to produce constriction; this leads to decreased edema and increased drainage of the sinus cavities.<sup>5</sup> The aim of this research work is RP-HPLC method development and validation for simultaneous estimation of Tropicamide and Phenylephrine Hydrochloride in ophthalmic formulation.



**Figure 2:** Chemical structure of Phenylephrine Hydrochloride

## MATERIALS AND METHOD

Tropicamide and Phenylephrine hydrochloride were kindly supplied as gift samples from Altra Lab Ahmedabad, HPLC grade methanol (Merck, Rankem), AR grade Potassium dihydrogen phosphate (Merck, Rankem), HPLC grade water (Merck, Rankem), Orthophosphoric acid (Merck, Rankem)

**Table: 1 HPLC apparatus and conditions**

Sr no.	Instrument	Specification and Manufacturer
1	HPLC	Analytical Tectnologies
2	Analytical Balance	Swisser
3	pH meter	Systonic-Model no-335
4	Ultra Sonic Cleaner	Tashcon, Toshiniwal process instrument pvt Ltd, Ajmer

**Preparation of solutions & reagents****Preparation of mobile phase**

Mobile phase were prepared by mixing of 500 ml of methanol with 500 ml of 1%  $\text{KH}_2\text{PO}_4$ , whose pH was previously adjusted to pH 4 by addition of orthophosphoric acid. The mobile phase prepared was degassed by ultrasonication for 20 min, so as to avoid the disturbances caused by dissolved gases. The degassed mobile phase was filtered through  $0.45\mu$  filters to avoid the column clogging due to smaller particles.

**Preparation of TRO and PHE standard stock solutions**

Accurately weighed quantity of TRO (10 mg) and PHE (10 mg) were transferred into two different 10 ml volumetric flask separately, dissolved in Mobile Phase and diluted up to mark with it. This will give a stock solution having strength of  $1000\mu\text{g/ml}$  of both.

**Preparation of TRO and PHE standard solutions**

From the standard stock solution 1 ml of TRO and 1 ml of PHE solution is taken in two different 10 ml volumetric flask and make up to mark with mobile phase to get  $80\mu\text{g/ml}$  of TRO and  $500\mu\text{g/ml}$ . From the above solution 1ml of TRO and 1ml of PHE solution is taken in 10 ml volumetric flask and make up to mark with mobile phase and dilute up to mark with it. This will give a standard solution having strength of  $8\mu\text{g/ml}$  of TRO and  $50\mu\text{g/ml}$  of PHE.

**Simultaneous equation method**

In the present study, standard solution of TRO and PHE were scanned over the range of 200–400 nm wavelengths. TRO showed one absorbance maxima at 209nm where as PHE showed one absorbance maxima at 215nm. Simultaneous estimation of both TRO and PHE a common absorption point was selected as wavelength maxima at 216 nm.

$$C_X = \frac{A_1a_2y_2 - A_2a_1y_1}{A_{X1}a_2y_2 - a_{X2}a_1y_1} \dots\dots\dots 1$$

$$C_X = \frac{A_1 a_{X2} - A_2 a_{X1}}{A_{Y1} a_{X2} - A_{Y2} a_{X1}} \dots\dots\dots 2$$

Where,  $A_1$  and  $A_2$  represent absorbance of mixture at  $\lambda_1$  and  $\lambda_2$ , respectively,  $a_{X1}$  and  $a_{X2}$  are the absorptivities of TRO at  $\lambda_1$  and  $\lambda_2$  respectively and  $a_{Y1}$ ,  $a_{Y2}$  are the absorptivities of PHE at  $\lambda_1$  and  $\lambda_2$  respectively.  $C_X$  and  $C_Y$  are the concentrations of Tropicamide and Phenylephrine Hydrochloride in  $\mu\text{g/ml}$ , respectively.

### Preparation of sample solution

The eye drop was weighed equivalent to 8mg TRO and 50mg PHE and transfer in to 25 ml volumetric flask. Dilute with mobile phase and sonicated for of 30 min. time interval with intermittent shaking. Content was brought back to room temperature and dilute to volume with mobile phase (stock test solution). The stock solution was filtered through  $0.45\mu\text{m}$  nylon syringe filter. Pipette out 4 ml filtered stock solution in to 10 ml volumetric flask and dilute with mobile phase (test solution). From the above solutions prepare solutions having concentration TRO ( $8\mu\text{g/ml}$ ) and PHE ( $50\mu\text{g/ml}$ ) with mobile phase.

## RESULT

### Linearity

Calibration curves were constructed by plotting peak areas vs. concentrations of TRO and PHE, and the regression equations were calculated. The calibration curves were plotted over the concentration range  $4\text{--}12\mu\text{g/ml}$  for TRO and  $25\text{--}70\mu\text{g/ml}$  for PHE. Accurately measured standard solutions of TRO (0.5 ml, 0.75 ml, 1 ml, 1.25 ml, 1.5 ml) and PHE (0.5 ml, 0.75 ml, 1 ml, 1.25 ml, 1.5 ml) were transferred to a series of 10 ml of volumetric flasks and diluted to the mark with mobile phase. Aliquots ( $20\mu\text{L}$ ) of each solution were injected under the operating chromatographic conditions described above.

### Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and the LOQ of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

$$\text{LOD} = 3.3 \times \sigma/S$$

$$\text{LOQ} = 10 \times \sigma/S$$

Where  $\sigma$  = Standard deviation of the response

S = Slope of calibration curve.

## Precision

### Intermediate precision (Reproducibility)

The Intraday and Interday precision of the proposed method was determined by analyzing the corresponding responses 3 times on the same day and on 3 different days different concentrations of standard solutions of TRO and PHE. The results were reported in terms of relative standard deviation (RSD).

### Accuracy (% Recovery)

The accuracy of the method was determined by calculating recovery of TRO and PHE by the standard addition method. Known amounts of standard solutions of TRO and PHE were added at 80%, 100% and 120% levels to prequantified sample solutions of TRO and PHE. The amounts of TRO and PHE were calculated by applying obtained values (n=6) to the regression equation of the calibration curve.

### Robustness

The robustness of the method was evaluated by assaying the test solutions after slight but deliberate changes in the analytical conditions i.e. flow rate ( $\pm 0.2$  ml/min), proportion of buffer and methanol (58:42 and 62:38 v/v), and pH of the buffer ( $\pm 0.2$ ).

### Solution stability

The stability of standard solutions was determined by comparing results of area of analyte. The area values were determined within 48 hours and also assay of sample solution was determined within 48 hours.

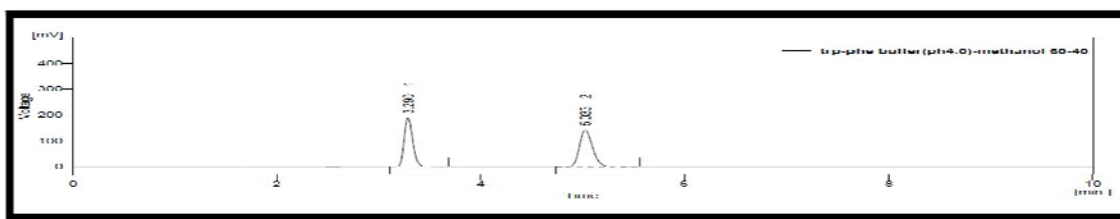


Figure 3: Typical chromatogram Tropicamide and Phenylephrine Hydrochloride

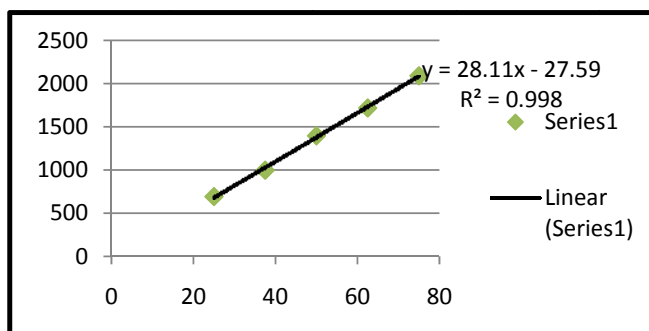


Figure 4: Linearity curve of Phenylephrine Hydrochloride

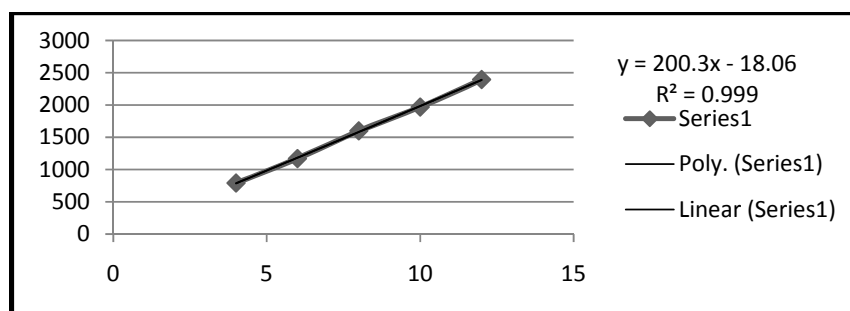


Figure 5: Linearity curve of Tropicamide

Table 2: Summary of Validation Parameters

Parameter	Result	
	Phenylephrine hydrochloride	Tropicamide
Linearity Range	25-75 µg/ml	4-12 µg/ml
Correlation Coefficient ( $r^2$ )	0.999	0.998
Regression Equation	$y = 28.11x + 27.59$ $R^2 = 0.999$	$y = 200.3x + 18.06$ $R^2 = 0.998$
Accuracy (%recovery) n=3	99.37	99.80
<b>Precision</b>		
Method Precision(%RSD)	0.9990	1.3356
<b>Intermediate Precision</b>		
Intraday(%RSD)	0.77-1.44	0.79-1.58
Interday(%RSD)	0.88-1.00	0.74-1.14
LOD(µg/ml)	0.327	0.123
LOQ(µg/ml)	0.991	0.375

## DISCUSSION

An economical, simple, sensitive, precise and accurate method has been developed for the simultaneous determination of Tropicamide and Phenylephrine Hydrochloride in Ophthalmic Formulation. The developed method involves formation and solving of simultaneous equation which is further based on absorptivity coefficients of two drugs at wavelength maxima of tropicamide and Phenylephrine. After the measurement of absorbance of sample solution at both the wavelengths the amount of drug in the sample can be found by substituting the values in the two equations (Eq. 1 and 2). The framed equation was validated as per ICH guidelines. Statistical analysis and drug recovery data showed that simultaneous estimation method was simple, rapid, economical, sensitive, precise and accurate and can thereby be easily adopted for routine quality control analysis.

Results of this analysis confirmed that the proposed method was suitable for the simultaneous determination of these drugs in pharmaceutical formulations with virtually no interference of the

additives. Hence, the proposed method can be successfully applied in simultaneous estimation of TRO and PHE in ophthalmic formulation.

## ACKNOWLEDGEMENT

The authors are kindly thanks Altra Laboratory, Ahmedabad, for providing samples of TRO and PHE respectively. We also wish to thank Miss. Payal P. Chauhan HOD department of pharmaceutical chemistry, Dr. Samir Shah Principal, Sardar Patel College of Pharmacy, Anand, Gujarat, India, for providing analytical facilities.

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