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STABILITY INDICATING RP-HPLC METHOD DEVELOMENT & VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCES IN PILOCARPINE HYDROCHLORIDE OPHTHALMIC SOLUTION

Divya Dyapa*1

*1Eugia Pharma Specialities Ltd, Quthubullapur, Ameenpur, Bachupally, Hyderabad, Telangana, India.

Correspondence Author E-Mail: Divyadyapa7@gmail.com

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ABSTRACT

A Simple, Specific, Accurate and Stability indicating analytical method has been developed and validated for the determination of Related substances of Pilocarpine Hydrochloride ophthalmic solution by using RP- HPLC.

This method developed by using an HPLC equipped with gradient pumps, variable wavelength UV detector attached with data recorder and integrator software was used. YMC-trait C18 (250 X 4.6 mm, 5µm) column at 35°C was used with a flow rate of 1.0 ml/min and the compounds were detected at 215nm. Mobile phase A consisted of buffer (8.8g Dipotassium hydrogen phosphate added to 2 litre water pH adjusted to 7.6 with diluted ortho phosphoric acid and the solution was filtered through 0.45µm filter paper) and mobile phase B used buffer, methanol and acetonitrile with a ratio of 40:40:20. This method was validated according to ICH guidelines with respect to Accuracy, Precision, Specificity, Linearity, LOD, & LOQ prediction and the Stability of the method has been studied by Forced degradation studies of the sample under various stress conditions like acid, base, peroxide, thermal degradation studies. Based on the above information this method can be used for the daily routine analysis.

Keywords: RP- HPLC, Related Substances, Pilocarpine Hydrochloride, Forced Degradation Studies.

I. INTRODUCTION

Pilocarpine hydrochloride is chemically known for (3S,4R)-3-Ethyl-4-((1-methyl-1H-imidazol-5-yl) dihydrofuran-2(3H)-one having molecular formula $C_{11}H_{16}N_2O_2$. Hcl Molecular weight 208.261g.mol^{-1}. pKa value of the drug is 7.21. The solubility of the pilocarpine is very **soluble** in **water** and **insoluble** in **ether**.

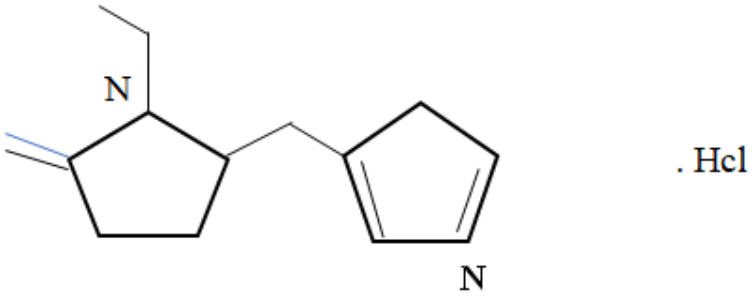

Fig 1: Structure of pilocarpine hydrochloride

It is a direct acting cholinergic parasympathomimetic agent of muscarinic receptors (M_1, M_2) present in smooth muscles. It is used to treat glaucoma, presbyopia [1,2].

Pilocarpine hydrochloride is not official in any pharmacopeia. A literature survey revealed that only few analytical techniques are available, high-performance liquid chromatographic determination of pilocarpine hcl and its degradation using β cyclodextrin column, **Mobile phase with too low pH resulted in overlap of Pilocarpic acid and Pilocarpine main peak in this method** (Kent Sternitzke et al., PubMed,DOI:10.1016/00219673(92)8007,Jan 1992), Evaluation of monolithic C_{18} HPLC columns for the fast analysis of pilocarpine Hcl in the presence of its degradation products, this method **Used both conventional & monolithic columns different columns showed different results due to that this method is not considered as specific method** (EI Deeb at al., Pharmazie, sept 2005), High performance liquid chromatography analysis of pilocarpine Hcl, Isopilocarpine, pilocarpic acid and isopilocarpic acid in eye drop preparations,**Possible disadvantage of this method is the high salting through rinsing of the HPLC system**

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after use to avoid pump seals (Jhon Kennedy et al., J Chromatgor 1981), Quantitative determination of pilocarpine, Isopilocarpine, Pilocarpic acid and Isopilocarpic acid in clinical ophthalmic pilocarpine formulation by RP_HPLC **this method only determines the quantity of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid in commercial product of pilocarpine eye drops it does not determines the related substances and its interference,** (Noordam et al., J Pharm Sci, Jan,1981) have been employed for the determination of Pilocarpine hydrochloride Hence, an attempt has been made to develop simple, precise, accurate method to determine related substances in pilocarpine hydrochloride using HPLC with uv detector, which is readily available in most of the analytical laboratories [3].

II. MATERIALS

- Pilocarpine hydrochloride was obtained as gratis reference standard (Batch no.-).
- Impurity A, B, & C was also obtained as gratis reference standard.
- AR grade methanol, Gradient grade acetonitrile, & WFI grade water were used.
- All the chemicals i.e. Dipotassium hydrogen orthophosphate anhydrous, Orthophosphoric acid (88%w/w), Hydrogen peroxide, Hydrochloric acid, & Sodium hydroxide were of AR grade.

III. METHODS

Instrumentation & analytical conditions:

HPLC system of waters make with empower software, fitted with YMC trait C_{18} (250x4.6mm),5µ column. The autosampler injection volume of 20µl loop. A water HPLC system equipped with the UV detector [4]. Waters system was used to operate at gradient programme through the column with pH 7.6 buffer, methanol and acetonitrile as mobile phase at a flow rate 1.0 mL/min and the run time 60 min. The injection volume of 20µl and the detection was at 215nm. The HPLC was operated at 35°c of column temperature and 25°c of sample cooler temperature. Data collected through the software connected system.

Preparation of standard solution:

25mg of pilocarpine standard was weighed and transferred into the 50ml clean, dry volumetric flask, 20ml of diluent(water) was added and sonicated to dissolve, then made up the solution upto the mark with diluent and mixed well. Further, transfer 2ml into 100ml flask made up the solution upto the mark with diluent(10µg/ml) and mixed well.

Preparation of sample solution:

1% v/v pilocarpine hydrochloride solution was weighed 1gm into the 20 ml clean, dried, volumetric flask and made upto the mark with diluent and mixed well(500µg/ml).

Preparation of impurities solution:

1.07mg of Isopilocarpic acid impurity, 1.13mg of Pilocarpic acid impurity and 0.96mg of Isopilocarpine impurity were weighed and transferred into the 10ml clean, dried volumetric flask and 5ml of diluent was added and sonicated for 30min to dissolve and diluted upto the mark with the same diluent(10µg/ml).

Validation procedure:

It is important to demonstrate that the developed method is suitable for its intended use or not. This method is validated according to its ICH guidelines.

Pilocarpine hydrochloride ophthalmic solution related substances determination method validated for Linearity, Accuracy, Precision, System suitability and Robustness.

Precision and linearity were performed by using impurity spiked solution from 5%,10%,25%,50%,100%,150% and 200%.

The above preparations were injected into the HPLC system on the same day, %RSD was calculated for precision and linear regression analysis was evaluated for linearity [5]. The accuracy was determined by adding known amount of standard to the sample and the percentage recovery was calculated [6]. The robustness was determined by changing the method conditions like flow rate, mobile phase composition and column temperature to evaluate the variation impact on the method.

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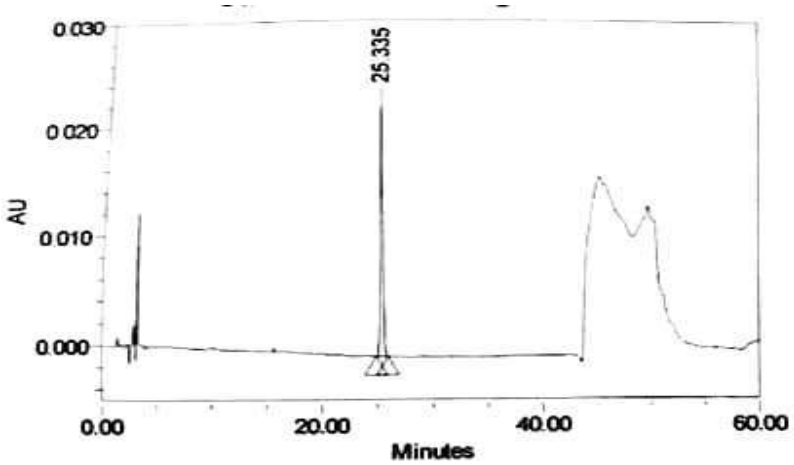

Fig 2: RT of Pilocarpine HCL

IV. RESULTS AND DISCUSSION

Development and optimization of HPLC method:

To get the better separation and satisfactory results optimized chromatographic conditions [7], (as shown in the table no 1), YMC trait C_{18}(250x4.6mm,5µm) column was used for separation.

The mobile phase A consists of pH 7.6 buffer, methanol (90:10%v/v) and mobile phase B consists of buffer, methanol and acetonitrile (40:40:20%v/v). The flow rate of 1.0mL/min was delivered with the detection wavelength 215nm.

Table 1: Gradient programme of optimized method

Time	Mobile phase(A)	Mobile phase(B)
0.01	88	12
3.00	88	12
25.00	65	35
40.00	55	45
40.10	20	80
47.00	20	80
47.10	88	12
60.00	88	12

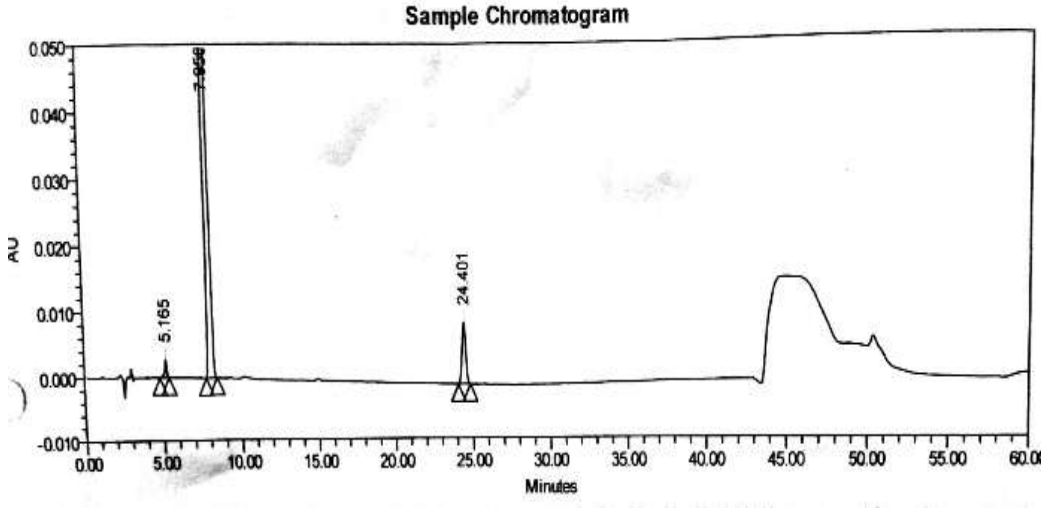

Fig 3: Chromatogram of pilocarpic acid

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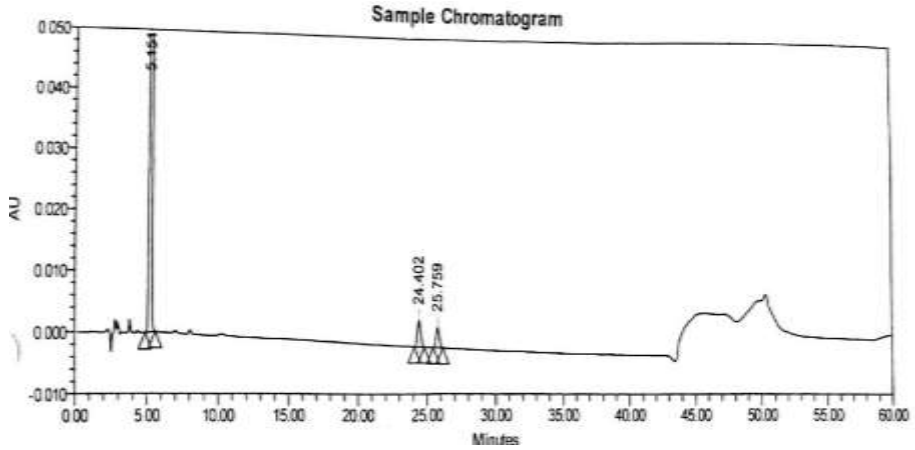

Fig 4: Chromatogram of isopilocarpic acid

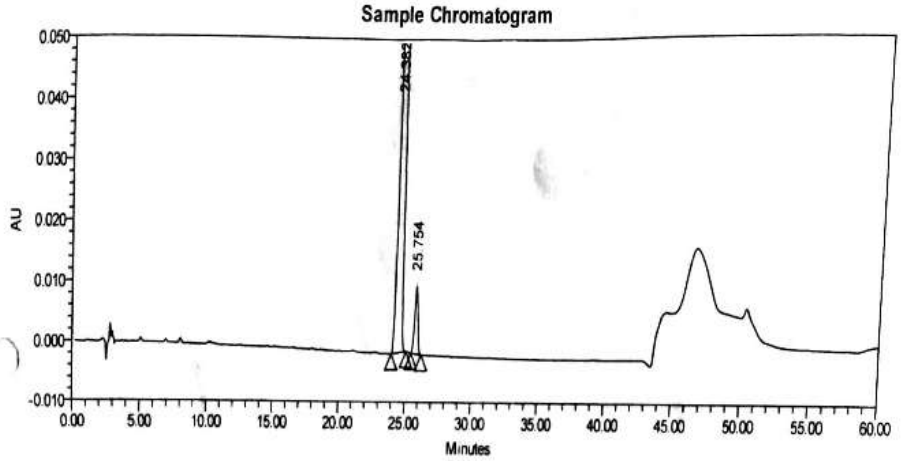

Fig 5: Chromatogram of Isopilocarpine

METHOD VALIDATION:

Specificity:

Prepared and injected standard, sample placebo and impurities as per methodology.

Table 2: Sample chromatogram results

Sample	RT	Area	RT Ratio
Blank	Not detected	NA	NA
Placebo	Not detected	NA	NA
Pilocarpine	25.50	21391097	1.00
Pilocarpic acid	5.26	40755	0.21
Isopilocarpic acid	8.09	NA	0.30
Isopilocarpine	24.24	73764	0.95

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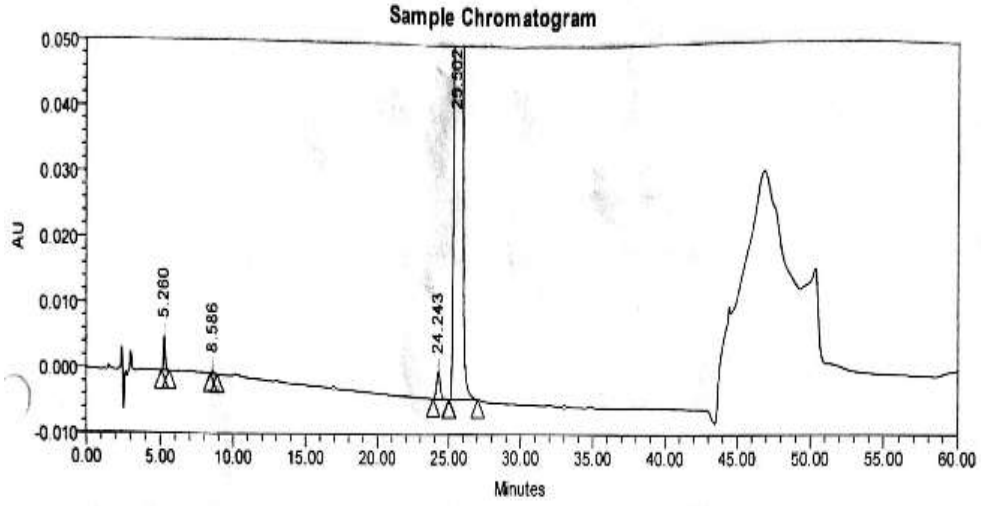

Fig 6: Specificity sample chromatogram

Linearity:

Dilutions were prepared using pilocarpine impurity stock solution at different level of concentrations from 0.05%-200% of 1% level of pilocarpine and each solution was analysed [8].

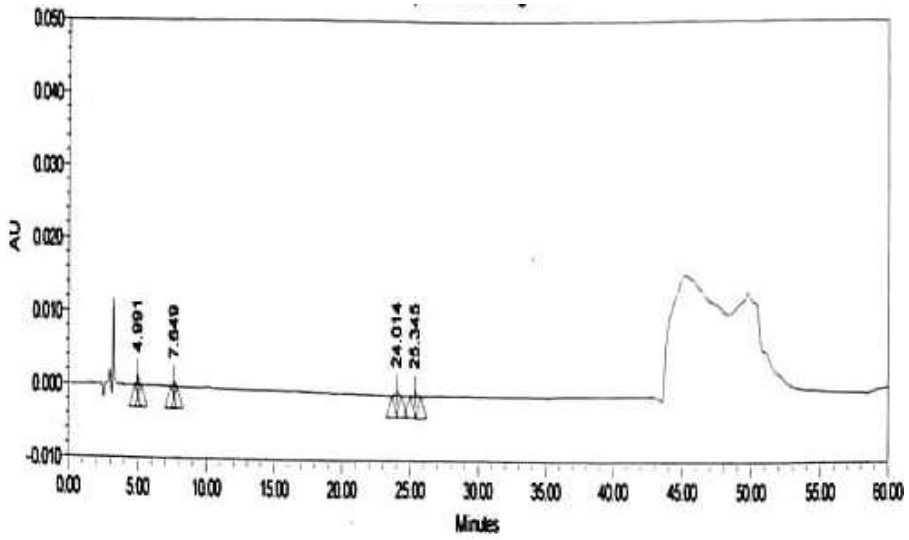

Fig 7: 5%-linearity chromatogram

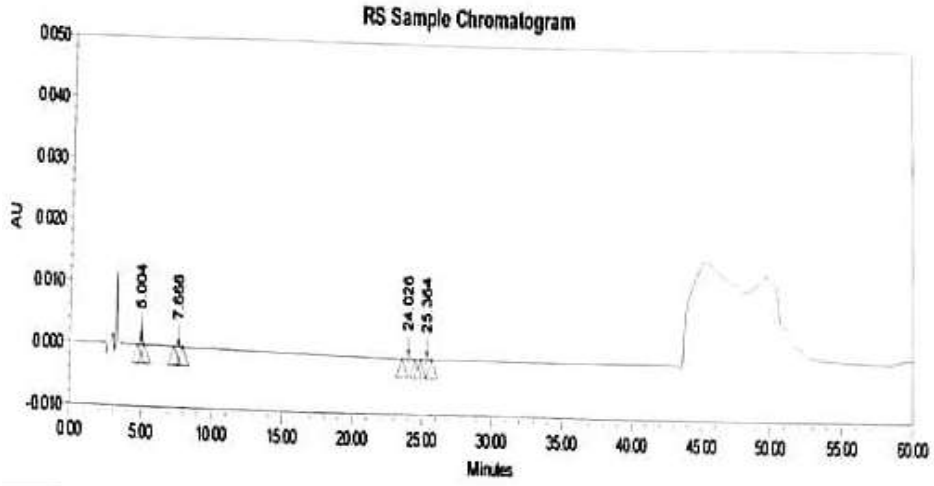

Fig 8: 10%-linearity chromatogram

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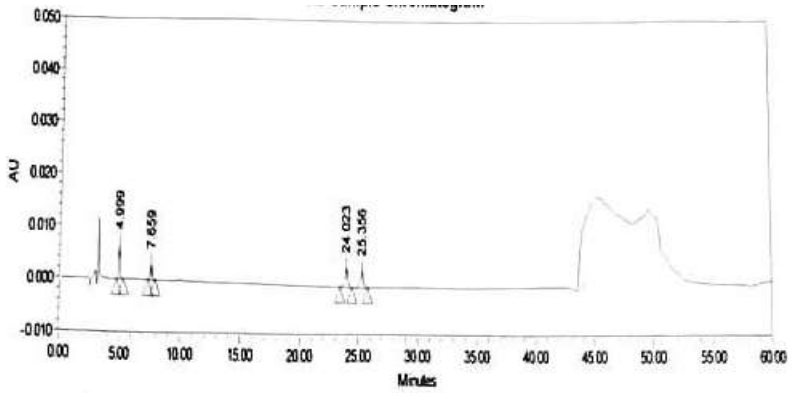


Fig 9: 25%-linearity chromatogram

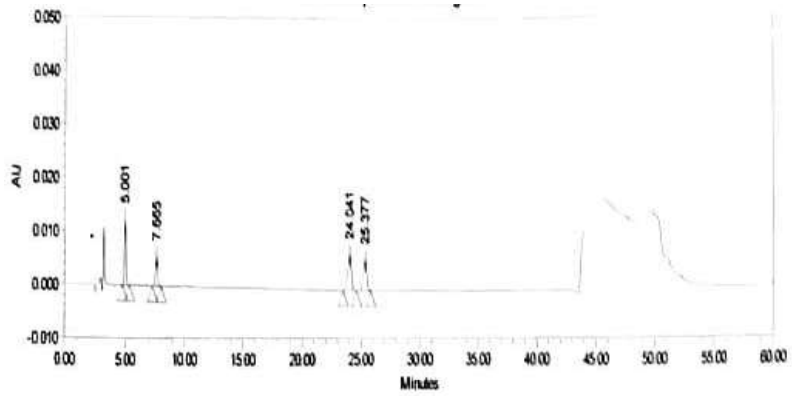
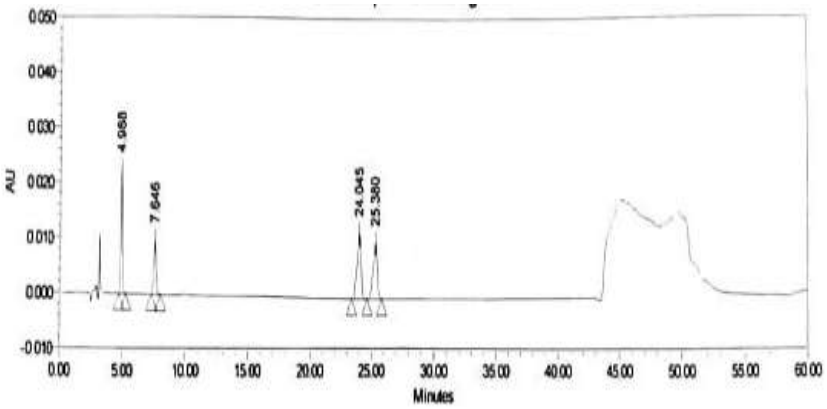


Fig 10: 50%-linearity chromatogram



*

Fig 11: 100%-linearity chromatogram

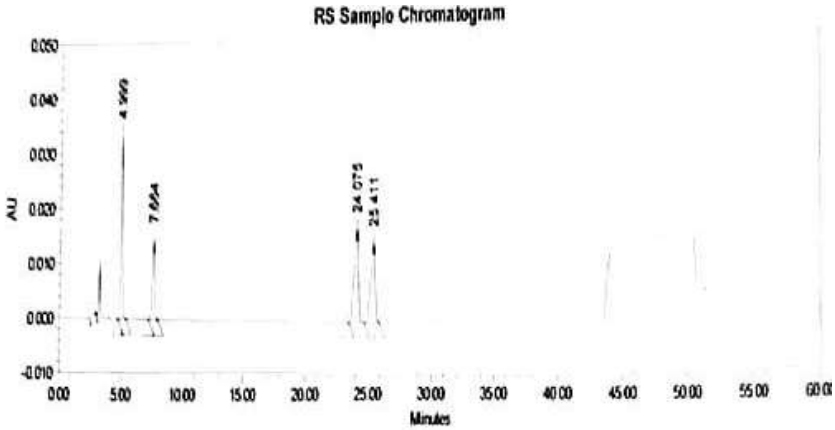


Fig 12: 150% linearity chromatogram

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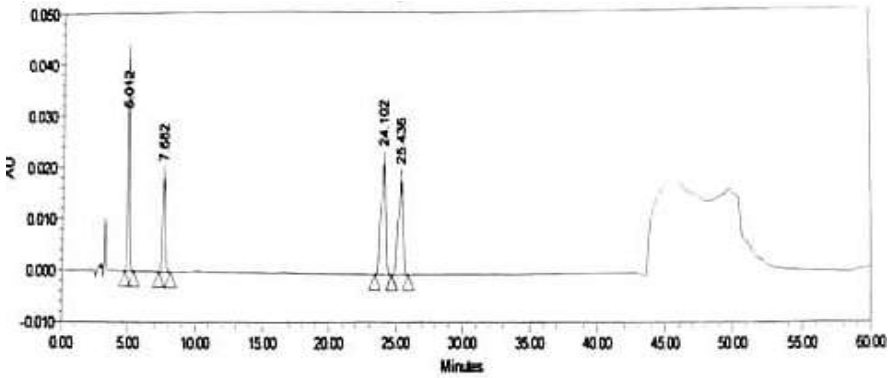

Fig 13: 200%-linearity chromatograms

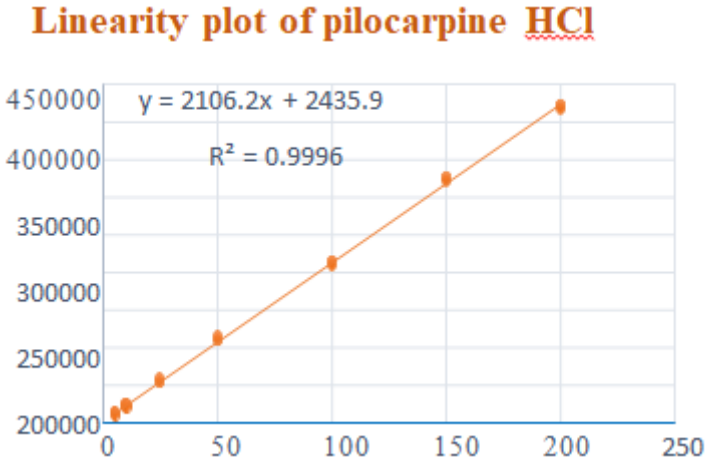

Fig 14: Linearity calibration curve of pilocarpine Hcl

Table 3: Linearity data of pilocarpine Hcl

Volume of stock solution taken(ml)	Area	Concentration (µg/ml)	% of linearity solution to test concentration
0.050	12370	0.249	5.00
0.050	24347	0.497	10.00
0.125	60145	1.243	25.00
0.250	122372	2.485	50.00
0.500	233988	4.970	100.00
0.750	345792	7.455	150.00
1.000	461982	9.940	200.00

LOD & LOQ:

The concentration of LOD & LOQ were calculated from linearity data.

Table 4: LOD & LOQ values from linearity dat

Name	Slope	Standard deviation	Predicted value of LOQ	Predicted value of LOD
Pilocarpic acid	38518.21087	3468.21087	0.900	0.297
Isopilocarpic acid	30470.5	1813.98062	0.595	0.196
Isopilocarpine	61621.7	3226.86312	0.524	0.173

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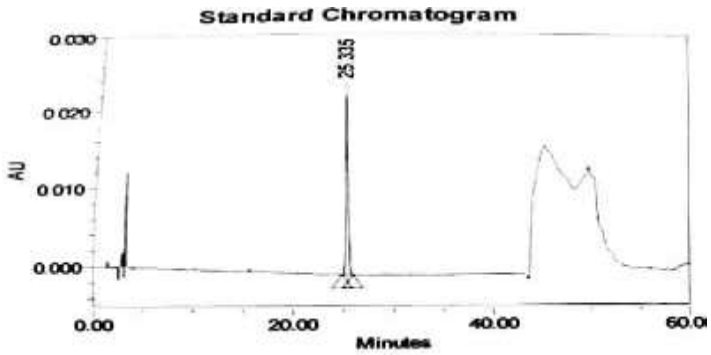
Pilocarpine	46227.1	2527.60868	0.547	0.180

System suitability:

It is carried to verify that the analytical system was working properly and can give accurate results [9]. Working standard of pilocarpine was prepared as standard solution and injected 6 times into the HPLC system. System suitability parameters were evaluated.



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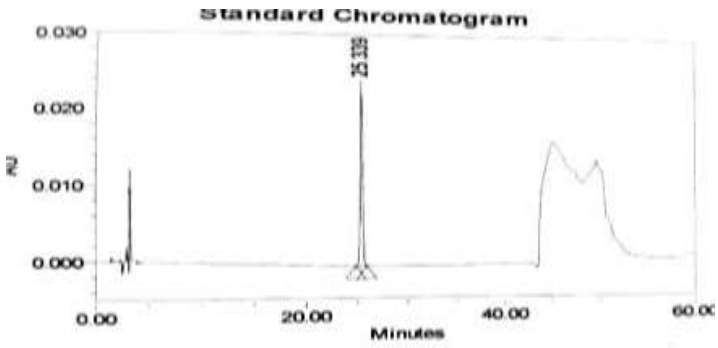

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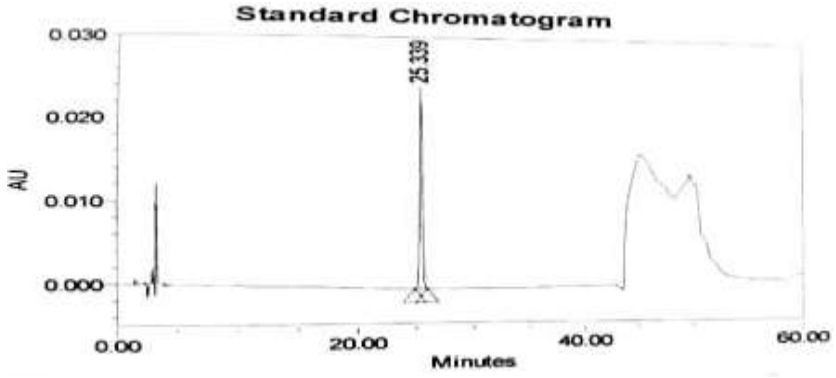

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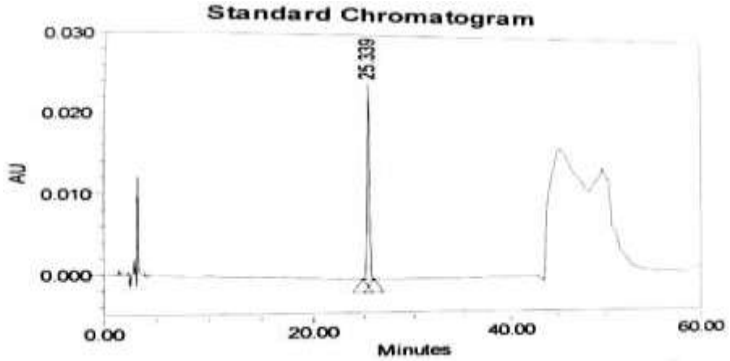

d)

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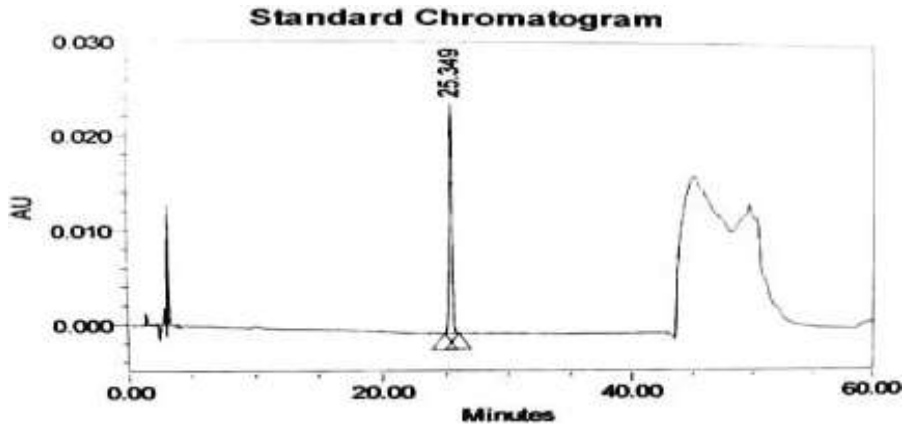

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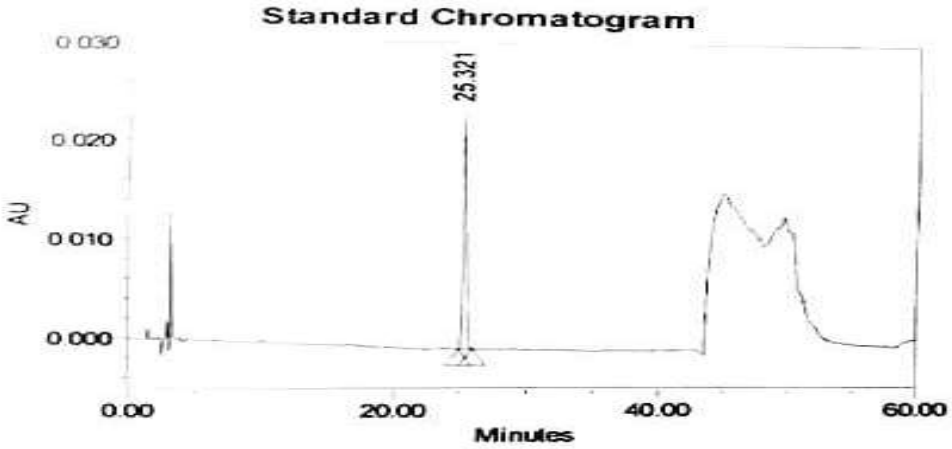

f)

Fig 15: Above six (a, b, c, d, e &f) chromatograms of pilocarpine standard solution.

Table 5: %RSD for standard areas

Pilocarpine standard injection	Area	USP Plate count	USP tailing
1	406356	49581	1.1
2	405168	49382	1.1
3	406326	49113	1.1
4	404834	49319	1.1
5	405587	49403	1.1
6	405884	49807	1.1
Mean	405693		
&RSD	0.15		

FORCED DEGRADATION STUDIES:

This specificity of this method was determined through the forced degradation studies by conducting acid, alkaline, peroxide and thermal degradation of sample. Sample was exposed to these conditions thus, indicating that this method effectively separates the degradation products from the main Active pharmaceutical ingredient.

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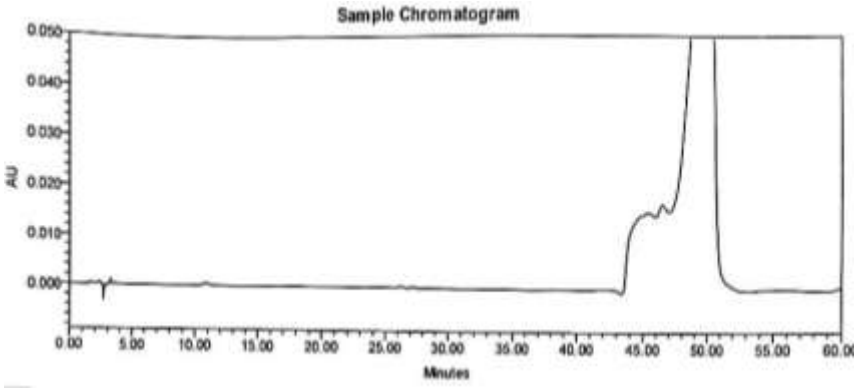

Fig 16: Chromatogram of blank

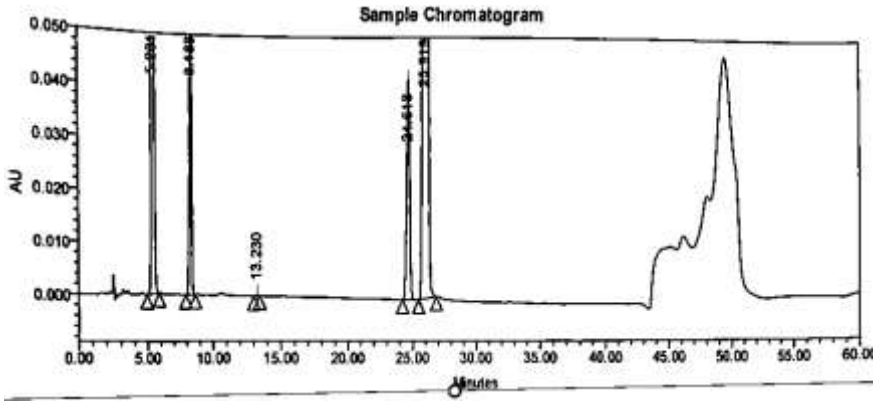

Fig 17: Chromatogram of acid degradation

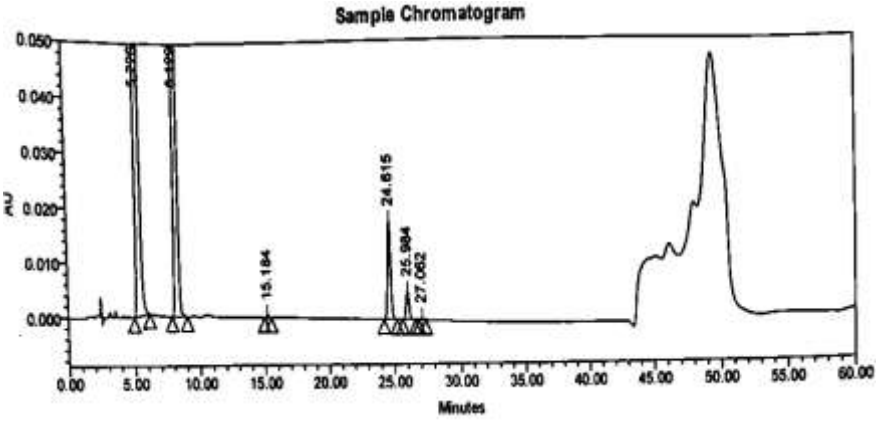

Fig 18: Chromatogram of alkaline degradation

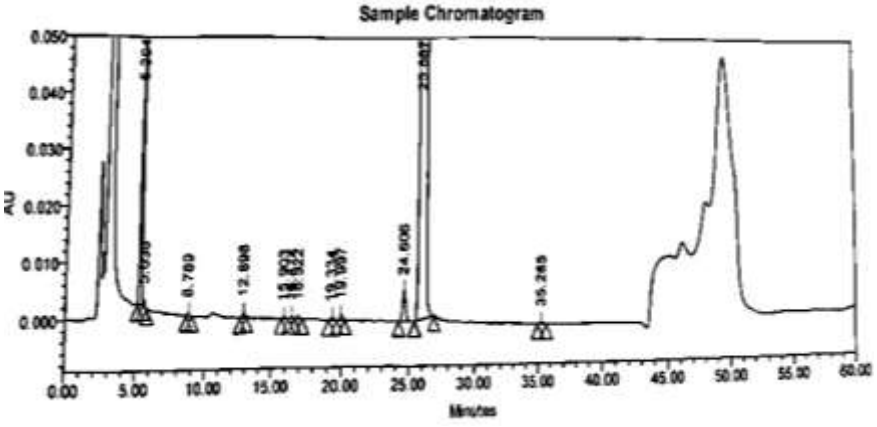

Fig 19: Chromatogram of 30% peroxide degradation

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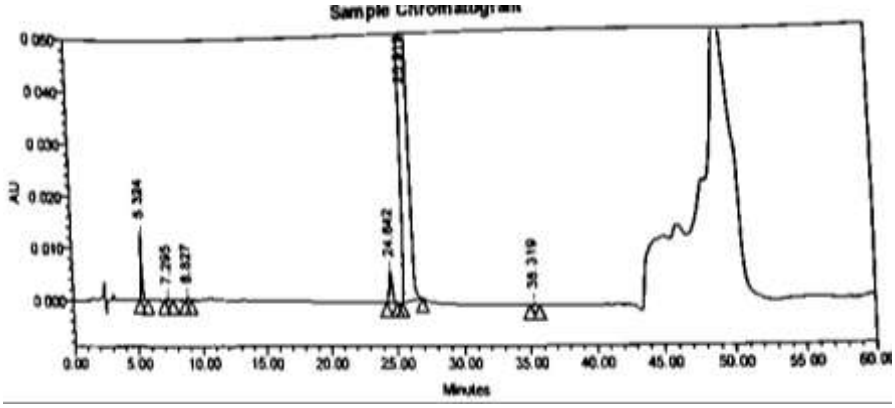

Fig 20: Chromatogram of thermal degradation

Table 6: Results of degradation for pilocarpine hydrochloride

S. No	Conditions	Peak area of pilocarpine	% Assay	Total Impurities formed(w/)	Mass balance
1	Undegraded sample	22506707	102.6	0.50	-
2	Acid degradation	19691490	87.4	14.92	99.2
3	Base degradation	26143461	100.0	4.60	98.9
4	Peroxide degradation	21742641	98.3	4.31	97.0
5	Thermal degradation	23053163	99.4	1.29	97.66

Table 7: Summary of validation data

Parameter	Pilocarpine HCl	Pilocarpic acid	Isopilocarpine	Isopilocarpic acid	Limit
Specificity	specific	specific	specific	specific	No interference of any peak
Method precision % RSD	0.4	0.15	0.17	0.14	NMT 2.0%
Accuracy % recovery		98.67	97.92	103.72	(98-102) %
Linearity range (µg/mL)	0.24-9.94	0.27-10.93	0.21-8.61	0.21-8.54	-
Slope(m)	46227.1	38518.1	61621.7	30470.5	-
Intercept(c)	2933.4494	2435.9189	3733.8510	1534.4117	-
Regression equation (y=mx+c)	y = 2106.2x + 2435.9	Y=2106.2x+ 2435.9	y = 1301x +1534.4	y = 1301x + 1534.4	-
R^2	0.9996	0.9996	0.9997	0.9997	NLT 0.999
LOD (µg/mL)	0.180	0.297	0.173	0.196	-
LOQ(µg/mL)	0.547	0.900	0.524	0.595	-

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System suitability (Pilocarpine)		Peak area (n=6) %RSD - 0.15 USP Plate count – 49807 USP Tailing – 1.1				NMT 2% NLT 2000 NMT 2%
Robustness (%RSD)	Low pH	0.1	0.2	0.2	0.1	NMT 2%
	High pH	0.2	0.1	0.1	0.1	NMT 2%
	Column temp +	0.1	0.5	0.2	0.1	NMT 2%
	Column temp -	0.2	0.1	0.2	0.1	NMT 2%
	Flow increase	0.1	0.2	0.4	0.8	NMT 2%
	Flow decrease	0.2	1.5	0.4	0.4	NMT 2%
Forced degradation studies		No interference peak				-

V. CONCLUSION

A Specific, Selective, Accurate, Precise, Robust and Stability Indicating method was developed for the determination of related substance in pilocarpine hydrochloride ophthalmic solution by using RP-HPLC. This method was validated as per ICH guidelines, the results obtained in all parameters were within the acceptance limits, this method was specific, as no interference peaks were observed at the retention of related substances in the drug product. This method was linear for the determination of related substances because R^2 for all the impurities of pilocarpic acid, Isopilocarpine, Isopilocarpic acid and Pilocarpine Hcl were found within the limits. This RP-HPLC method for the determination of related substances in pilocarpine Hcl ophthalmic solution was stable, it was confirmed by the forced degradation conditions under various stress conditions. In all these conditions there were no interference of degraded peaks Hence, it was concluded that this method can be used for the routine analysis.

VI. REFERENCES

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