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RESEARCH ARTICLE

METHOD DEVELOPMENT AND ITS VALIDATION FOR QUANTITATIVE SIMULTANEOUS DETERMINATION OF LATANOPROST, TIMOLOL AND BENZALKONIUM CHLORIDE IN OPHTHALMIC SOLUTION BY RP-HPLC

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ABSTRACT

Background and the purpose of the study: To develop a simple, rapid and accurate HPLC method for simultaneous quantitative determination of Latanoprost, Timolol and Benzalkonium chloride (BAK) in ophthalmic solution.
Method: Chromatographic separation was achieved with PDA detector using Inertsil C18, 300 x 3.9mm, 5µ reverse phase analytical column. The mobile phase consist of buffer: acetonitrile (40: 60 v/v), was passed through the column at flow rate of 1.0 ml/min. The method was performed at wavelength gradient .The experiment was carried out at 30°C.
Results: The calibration curves were linear in the concentration range of 25% to 150% of the working concentration (r2 > 0.999). The lower limit of quantification was 0.8, 0.9 and 0.6 for Timolol, BAK and Latanoprost respectively.
Conclusion: The developed procedure was used for simultaneous quantitative estimation of Latanoprost, Timolol and Benzalkonium chloride (BAK) in ophthalmic solution. Developed method was validated as per ICH Q2 (R1), and most useful for academic as well as industrial scale.
Key words: Latanoprost, Timolol, Benzalkonium Chloride, RP HPLC, Validation.

INTRODUCTION

Glaucoma is a condition in which the pressure exerted by the liquid within the eyeball (the aqueous humor) is too great. The high pressure damages the optic nerve at the back of the eye. The damage interferes with the ability of the nerve to transmit visual images from the eye to the brain and thus can lead to blindness. Prostaglandins control the flow of the aqueous humor out of the eye. Latanoprost, a derivative of the chemical, prostaglandin F2-alpha, is used for the treatment of glaucoma. [1-4] Latanoprost, by binding to a specific receptor for prostaglandin, increases the flow of aqueous humor out of the eye, thereby reducing the pressure within the eye and reducing the risk of nerve damage and blindness. When Latanoprost and Timolol (a different drug that also is used to treat glaucoma) are used in combination, there is a greater reduction in pressure than when either drug is used alone. Timolol blocks beta-receptors that are found on the ciliary body. [5-7] This action reduces the amount of aqueous humour that is secreted into the eyeball by ciliary body. Timolol also blocks beta-receptors found on the blood vessels that supply the ciliary body. This causes the blood vessels to constrict, and reduces the amount of watery fluid that filters out of the blood vessels to form aqueous humour. Timolol therefore works by reducing the inflow of aqueous humour into the eyeball, which decreases the pressure within the eye. It is used to treat conditions where there is raised pressure in the eye, such as glaucoma. [8-9]

The aim of this study was to develop a RP HPLC method for the quantitative simultaneous determination of Latanoprost, Timolol and Benzalkonium chloride (BAK). The method developed was validated as per ICH Q2 (R1).

EXPERIMENTAL

Chemicals and reagents

HPLC grade acetonitrile, potassium dihydrogen orthophosphate were used to prepare the mobile phase and were purchased from Merck Specialities. The working standards of Latanoprost, Timolol and BAK were purchased from LG Promochem. Deionized and purified water using a Mili-Q system (Millipore) was used for the mobile phase and the standard solutions preparation. All experiment was performed using 'A' class volumetric glassware. All other reagents were of analytical grade.

Instrument and Chromatographic Conditions

Shimadzu LC 2010 C_{HT} HPLC was used for the chromatographic separation equipped with autosampler and Photo diode array (PDA) detector. The software used was LC Solution. The chromatographic separation of Latanoprost, Timolol and BAK was carried out using Inertsil C18 300 x 3.9 mm, 5µ reverse phase analytical column. Mobile phase consisted of Acetonitrile: Buffer (3.4 g potassium di hydrogen phosphate in 1000 ml WFI and pH adjusted to 2.8 with orthophosphoric acid) in the ratio 60: 40. The mobile phase was filtered by passing it through 0.45 µm filter and the filtrate is degassed by using bath sonicator. Mobile phase was used as diluent. Injection volume was 10 µL. Oven temp was set at 30°C. The mobile phase was pumped at 1 ml/min at room temperature. Detection was carried by using wavelength gradient given in Table 1.

Table 1: Wavelength Gradient

Time (Min.)	0.01	5.00	5.01	12.00	15.00
λ max	254 nm	254 nm	210 nm	210 nm	Stop

Method Development [8-12]

Different columns containing L1 and L7 stationary phase were tried for separation and resolution. Inertsil-3V column was found satisfactory over the other columns. The UV spectrum of Timolol, BAK and Latanoprost were scanned on photo diode array detector for selecting the optimum wavelength. Wavelength gradient was used in order to optimize the response of Timolol as its concentration was higher than Latanoprost in the sample. A typical HPLC chromatogram for simultaneous determination of latanoprost and timolol maleate from pharmaceutical formulation is shown in figure 1 and 2.

Preparation of standard and test solution

Prepare standard solution and test solution having concentration of Latanoprost (0.01 mg/ml), BAK (0.04 mg/ml) and Timolol (1 mg/ml) dilute to the mark by diluent (mobile phase).

RESULTS AND DISCUSSION

Method Validation

Specificity

The test was carried out by injecting 10 µl standard solutions of Latanoprost (0.01 mg/ml), Timolol (1 mg/ml) and BAK (0.04 mg/ml) in five replicates. The RSD values for areas of Latanoprost, Timolol and BAK standard were found 0.09 %, 0.12%, 0.15 % respectively. Resolution, Theoretical plates and Tailing factor were determined. Results are shown in table 2.

Table 2: System Suitability Parameters

	Resolution	**Tailing factor**	**Theoretical plates**
Timolol	-	1.2	2803.45
BAK	12.9	1.5	7364.09
Latanoprost	7.486	1.066	11254.11

Linearity

The linearity of an analytical procedure within a given range is its ability to obtain test results, which are directly proportional to the concentration of analyte in the standard. The range is derived from the linearity studies. A linearity standard solution was prepared at about 25%, 50%, 75%, 100%, 125% and 150% of the standard solution concentration and then linearity correlation coefficient of Timolol, BAK and Latanoprost obtained from the graph obtained by plotting area count on Y axis and concentration on X axis. Correlation coefficient of Timolol, BAK and Latanoprost are shown in table 3.

Table 3: Correlation Coefficient

Timolol	**BAK**	**Latanoprost**
0.9995	0.9998	0.9994

Precision

System precision

The six injections of standard solutions were injected to the chromatographic system. The relative standard deviation for area and retention time of Timolol, BAK and Latanoprost peak was determined and shown in table 4.

Method Precision

Six sample of a single batch of Timolol, BAK and Latanoprost peak were analyzed by proposed method and their assay was calculated and results are shown in table 4.

Table 4: System Precision and Method Precision

System Precision			
% RSD	**Timolol**	**BAK**	**Latanoprost**
AREA	0.12%	0.15 %	0.09 %,
RT	0.08%	0.05%	0.06%
Method Precision			
% RSD of assay	0.43	0.59	0.34

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Accuracy (Recovery)

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the found value. Recovery samples were prepared in triplicate and injected each sample in duplicate to the chromatography system. Timolol, BAK and Latanoprost peak working standard was added with placebo and recovery solutions were prepared so that, the final concentration contains 50%, 100% and 150 % of the recovery levels of Timolol, BAK and Latanoprost and results are shown in table 5.

Table 5: Accuracy (Recovery)

Analyte	Conc. Added (ppm)	RSD (%)	Mean (%) Recovery
Latanoprost	5	0.502	100.33
	10	0.706	100.43
	15	0.436	100.31
Timolol	500	0.209	100.06
	1000	0.598	100.28
	1500	0.334	100.13
BAK	20	0.325	100.18
	40	0.666	100.01
	60	0.525	100.33

Limit of Detection and Quantification

The limit of detection and Quantification were calculated as per formulas given below

$$LOD = \frac{3\sigma}{S}$$

$$LOQ = \frac{10\sigma}{S}$$

Where σ is standard deviation and S is the slope of the calibration curve. The LOD and LOQ values of Timolol, BAK and Latanoprost are shown in table 6.

Table 6: LOD and LOQ

	Timolol	BAK	Latanoprost
LOD (ppm)	0.4	0.6	0.2
LOQ (ppm)	0.8	0.9	0.6

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The analysis was carried out used the method outlined in the method of analysis and by carried out the following alterations and results are shown in table 7 and 8.

a) By changing the flow rate of the HPLC System by ±0.1 mL/min.

b) By changing the column oven temperature by ± 5°.

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Table 7: Robustness by changing flow rate

At flow rate 1.1 ml/ min			
	Timolol	**BAK**	**Latanoprost**
% RSD	0.14	0.16	0.09
Tailing factor	1.21	1.53	1.081
Theoretical plates	2818.36	7357.13	11269.11
At flow rate 0.9 ml/ min			
% RSD	0.15	0.17	0.12
Tailing factor	1.31	1.64	1.18
Theoretical plates	2829.31	7321.01	11245.18

Table 8: Robustness by changing temperature

At Temp 25°C			
	Timolol	**BAK**	**Latanoprost**
% RSD	0.16	0.20	0.09
Tailing factor	1.34	1.67	1.10
Theoretical plates	2865.29	7312.16	11231.09
At Temp 35°C			
% RSD	0.21	0.39	0.24
Tailing factor	1.12	1.87	1.29
Theoretical plates	2953.63	7419.26	11210.49

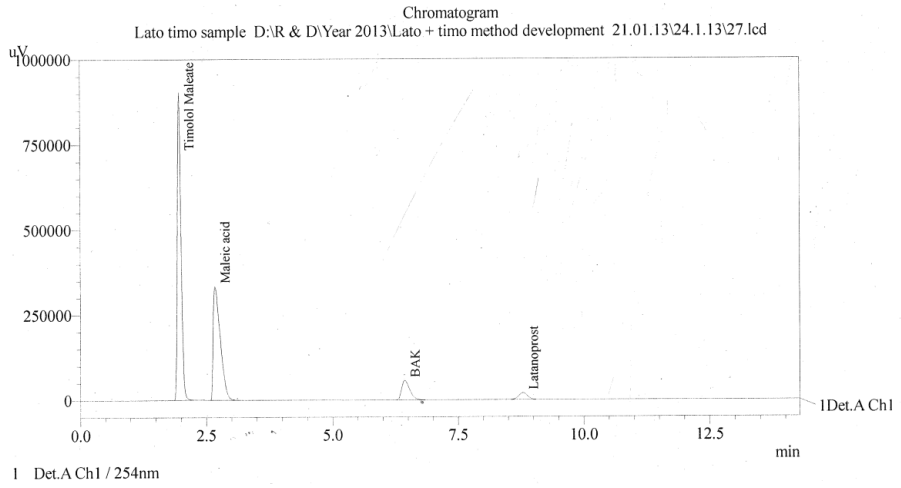

Figure 1: Chromatogram of Latanoprost, Timolol and BAK in standard preparation

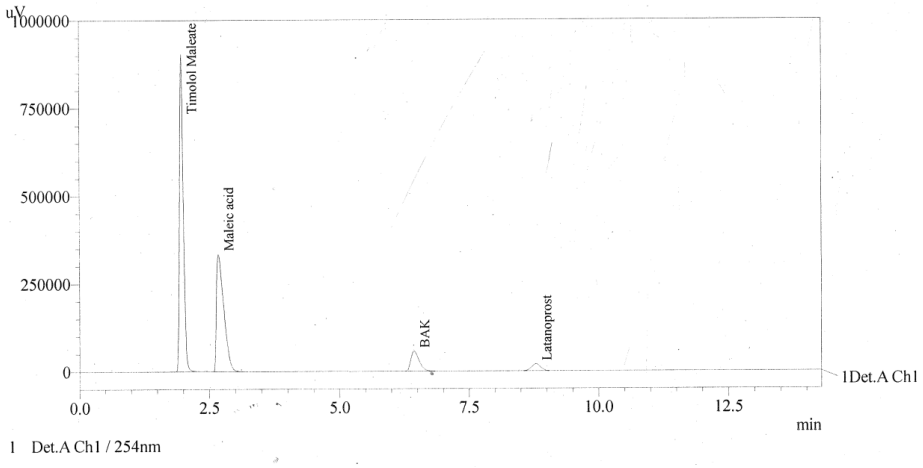

Figure 2: Chromatogram of Latanoprost, Timolol and BAK in sample preparation

CONCLUSION

The present study shows that the method developed for the determination of Timolol, BAK and Latanoprost was specific, linear, accurate, precise and robust. Wavelength gradient was used in order to optimize the response of Timolol as its concentration was higher than Latanoprost in the sample. The method clearly shows that all the peaks had tailing factor less than 2. The RSD for areas and theoretical plates (> 2500) was also found to be satisfactory. Validation parameters were performed according to ICH Q2 (R1) guidelines. The recoveries achieved were highly significant in the developed method. Hence it can be concluded that the method developed can be effectively used in the industries as well as research purposes.

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