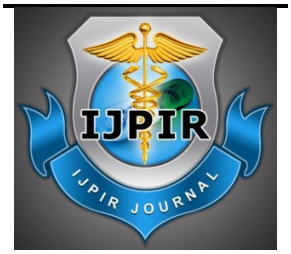
Research Article



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Method development and validation for simultaneous estimation of fluorescein and benoxinate by RP-HPLC

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ABSTRACT

A short selective, precise, accurate and sensitive method for the estimation of benoxinate and fluorescein was done by RP-HPLC. The chromatographic separation was clear at the flow rate of 1 ml/min, at UV detection of 358 nm. The assay for benoxinate and fluorescein were found to be 101.9 and 99.7 respectively. which shows that the method is useful for routine analysis. The linearity of benoxinate and fluoresceinwas found to be direct with a relationship coefficient of 0.9968 and 0.9962, which appears that the strategy is competent of creating great affectability. The vigor restrain for versatile stage variety and stream rate variety are well inside the constrain, the % debasement comes about are in limits. Which appears that the strategy is having great framework reasonableness and accuracy beneath given set of conditions. From the recovery and the studies, showing acceptable limits, it can be concluded that this can be employed for estimation of benoxinate and fluorescein in its dosage forms.

Keywords:benoxinate, fluorescein, Validation, RP-HPLC.

INTRODUCTION

Fluress® (Fluorescein Sodium and Benoxinate Hydrochloride Ophthalmic Solution USP) is a sterile ophthalmic solution combining a disclosing agent with an anesthetic agent.[1]

Fluorescein sodium isa phthalic indicator dye that appears yellow-green in normal tear film and bright green in a more alkaline medium, such as the aqueous humor, and is used therapeutically as a diagnostic aid in corneal injuries and corneal trauma. It has been approved by FDA for use in externally applied drugs and cosmetics.[2]Fluorescein sodium is a disclosing agent. Fluorescein appears as yellow amorphous solid or orange-red crystals. Latter have greenish-yellow fluorescence by reflected light. It is insoluble in water, soluble in dilute aqueous bases and very dilute alkaline solutions exhibit intense, greenish-yellow fluorescence by reflected light with low toxicity, may be sensitive to prolonged exposure to light.[3]Fluorescein sodium is highly water-soluble.Fluorescein sodium is used extensively as a diagnostic tool in the field of ophthalmology. Fluorescein is a fluorescent compound or fluorophore having a maximum absorbance of 494 m and an emission maximum of 521 nm. The yellowish-green fluorescence of the compound can be used to demarcate the vascular area under observation, distinguishing it from

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adjacent areas. It is applied topically in the form of a drop or it can be injected intravenously to produce a fluorescein angiogram. Topical fluorescein is a useful tool in the diagnosis of corneal abrasions, corneal ulcers, herpetic corneal infections, and dry eye. Fluorescein angiography is used to diagnose and categorize macular degeneration, diabetic retinopathy, inflammatory intraocular conditions, and intraocular tumors.[4, 5]

Benoxinate Hydrochloride is an anesthetic agent, is an ester-type local anesthetic, which is used especially in ophthalmology and otolaryngology.[6 - 8]Oxybuprocaine (also known as Benoxinate) is a local anesthetic, which is used especially in ophthalmology and otolaryngology. Oxybuprocaine binds to sodium channels and reversibly stabilizes the neuronal membrane which decreases its permeability to sodium ions.Water solubility: Very soluble (hydrochloride salt). Oxybuprocaine binds to sodium channel and reversibly stabilizes the neuronal membrane which decreases its permeability to sodium ions. Depolarization of the neuronal membrane is inhibited thereby blocking the initiation and conduction of nerve impulses.[9]

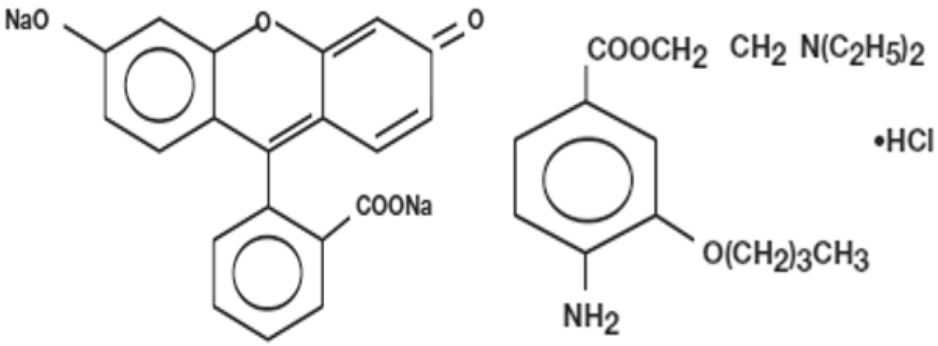

Fig.1: Structure of fluorescein and benoxinate

Only few methods were reported for the simultaneous estimation of benoxinate and fluorescein by HPLC.[10-13] Hence we had made an attempt to develop a simple, accurate and precise RP-HPLC method for the simultaneous estimation of benoxinate and fluorescein.

METHODOLOGY

Gift samples of benoxinate and fluorescein were received from Startech lab, Hyderabad, whereas water, methanol for HPLC, acetonitrile for HPLC and phosphoric acid were purchased from Merck.

Instrumentation

Waters HPLC was used for the separation of benoxinate and fluorescein. UV/VIS spectrophotometer (LABINDIA UV 12.500^{+}) was used for detection. Instruments such as; pH meter used was of Adwa — AD 10100 and weighing machine was of Afcoset ER-1000A.

Preparation of buffer

Accurately weighed 20.214 g of Disodium hydrogen phosphate taken in 1000 ml of HPLC water, add 3.394 g of Monosodium phosphate to the solution and the volume was adjusted to pH 8.0 with HCl. Last arrangement was sifted through 0.44 µm Film channel and sonicate it for 10 mins.

Preparation of mobile phase

Precisely measured 150 ml (15%) of water, 350 ml (35%) acetonitrile and 500 ml (50%) of buffer were blended and degassed in an ultrasonic water shower for 10 minutes and after that sifted through 0.45 µ channel beneath vacuum filtration and used as the diluent.

Standard Solution Preparation

Precisely weigh and add 80 mg of benoxinate and 50 mg of fluorescein working standard into a 100 ml clean dry volumetric jar, to this 70mL of mobile phase was added, sonicated and the volume was made up with the mobile phase. Pipette 5 mL of the clear solution in to 50 mL volumetric flask and make up volume with mobile phase.

Sample Solution Preparation

Precisely weigh a quantity of liquid equivalent to 80 mg of benoxinate and 50 mg of fluorescein into a 100 mL clean dry volumetric jar and add 70mL of mobile phase then sonicated it for 30min intermittent shaking after 30min make up volume with mobile phase. Pipette out 5 mL of the clear solution in to 50 mL volumetric flask and make up volume with mobile phase. Filter the solution through 0.45µm filter paper. The resulting solution is used to record the chromatogram.

Method development and optimization

Due to the significant difference in the physical and chemical properties of benoxinate and fluorescein, several mobile phases and columns were initially trialed in order to have both eluents on the same chromatogram. The suitability of the column and the mobile phase used in the optimized method have been decided based upon the basis of the selectivity, sensitivity as well as acceptable chromatographic parameters of the produced peaks in terms of peak sharpness, peak symmetry, tailing factor and resolution between the two peaks. We used the mobile phase as a solvent for all samples to ensure minimum noise and to eliminate any unwanted solvent peaks.

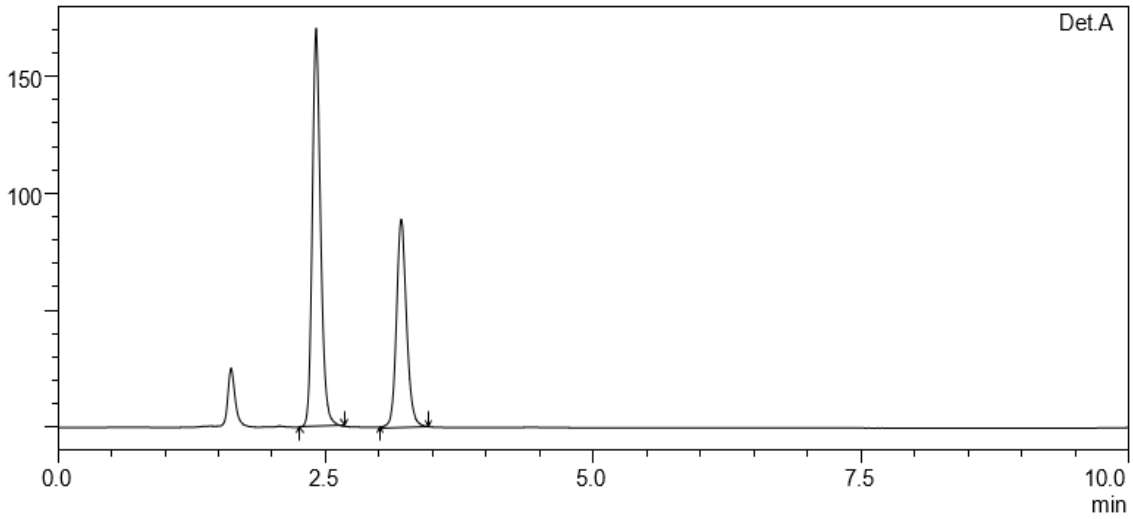

Fig.2: Standard Chromatogram of benoxinate and fluorescein

RESULTS AND DISCUSSION

Method Validation

The optimized method for simultaneous determination of benoxinate and fluorescein has been validated as per International Conference of Harmonisation (ICH) guidelines Q2 (R1) for evaluating system suitability, specificity, precision, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ) and robustness.[13]

Optimized chromatographic conditions

Instrument used	:	Waters HPLC with auto sampler and UV detector.
Temperature	:	Ambient
Column	:	Zorbax RX C8 (150x4.6mm ID) 5.0µm
Mobile phase	:	Water: Acetonitrile: Phosphate Buffer (15:35:50) %v/v/v
Flow rate	:	1 ml per min
Wavelength	:	358 nm
Injection volume	:	10 µl
Run time	:	10 min.

System Suitability

Following figure 2 for the crests due to benoxinate and fluorescein in Standard arrangement ought to not be more than 2.0 Theoretical plates for the benoxinate and fluorescein crests in Standard arrangement ought to not be less than 2000. Resolution for the benoxinate and fluorescein crests in standard arrangement ought to not be less than 2**.**

Table 1: Results of system suitability parameters

S.No	Name	Retention time(min)	Area (µV sec)	Tailing factor (TF)	Theoretical plates (TP)	Resolution
1	Benoxinate	2.404	924225	1.358	2588.8	
2	Fluorescein	3.199	577339	1.202	3370.6	4.75

Linearity

Standard stock solutions of benoxinate (800µg/mL) and fluorescein (500mg/mL) were prepared by dissolving 80 mg of benoxinate and 50 mg of fluorescein in 100 mL of mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min. (Fig.3,4) The standard stock solution of benoxinate is diluted in the concentration range of (40–120 µg/ml). Triplicates of such concentration range were prepared and plotted on a calibration curve. (Fig.3,4) The standard stock solution of fluorescein is diluted in the concentration range of (25–75 µg/ml). Triplicates of such concentration range were prepared and plotted on a calibration curve. Slope, intercept and correlation coefficient of the calibration curves (peak area versus concentration) were determined to ensure linearity of the analytical method. **(**Table 2)

Table 2: Results of Linearity of benoxinate and fluorescein

S. No.	Benoxinate		Fluorescein	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	40	483927	25	294145
2	64	792394	40	474171
3	80	998257	50	615087
4	96	1196914	60	785915
5	120	1437461	75	1002772

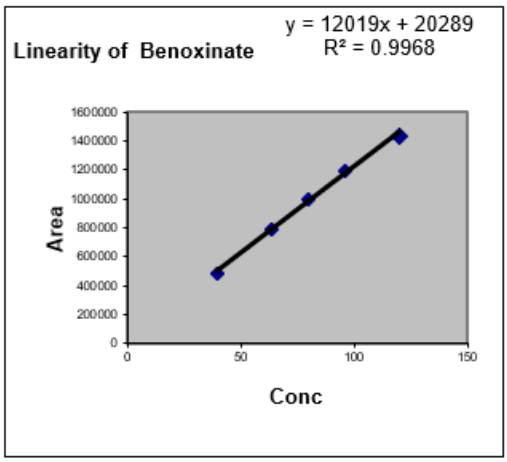

Fig 3: Calibration graph for benoxinate

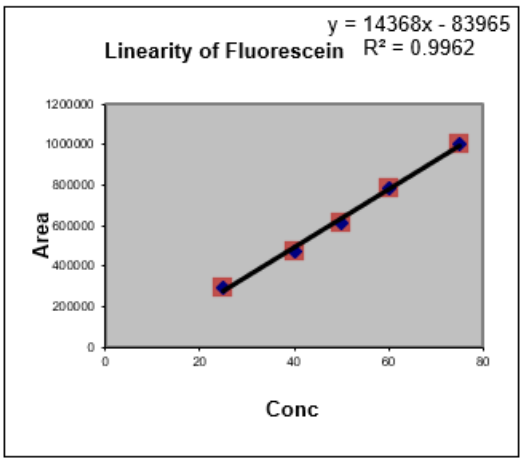

Fig 4: Calibration graph for fluorescein

Accuracy

Accuracy of the proposed method was confirmed with benoxinate and fluorescein separately at 3 different levels 50%, 100% and 150%, the determinations of these 3 levels have been recorded to obtain the mean and % recovery. **(**Table 3,4)

Table-3: Accuracy (recovery) data for benoxinate

%Recovery	Amount present (µg/mL)	Amount found (µg/mL)*	Percent Recovery *	% Mean Recovery
50%	40	40.39	100.9	
100%	80	80.45	100.5	101.0
150%	120	121.15	101.6	

Table-4: Accuracy (recovery) data for fluorescein

%Recovery	Amount present (µg/mL)	Amount found (µg/mL)*	Percent Recovery *	% Mean Recovery
50%	25	25.37	101.6	
100%	50	50.84	101.7	101.0
150%	75	74.77	99.7	

LOD and LOQ

The LOD and LOQ arrangements was arranged infused, for three times and measured the region for all three infusions in HPLC. The %RSD for the zone of six reproduce infusions was found to be inside the required limits. **(**Table 5,6).

Table-5: Results of LOD

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
benoxinate	52	152	2.9
fluorescein	52	156	3

Table-6: Results of LOQ

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
benoxinate	52	522	10.03
Fluorescein	52	524	10.1

CONCLUSION

The presented validated method is rapid, economic, simple, accurate, sensitive, robust, specific and linear. It can be used for routine analysis of benoxinate and fluorescein in combination products.

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