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A VALIDATED HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF OFLOXACIN AND DEXAMETHASONE SODIUM PHOSPHATE IN OPHTHALMIC FORMULATION

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ABSTRACT

A liquid chromatographic method was developed and validated for the simultaneous determination of Ofloxacin and Dexamethasone sodium phosphate in bulk and ophthalmic formulations. Ophthalmic preparations contain Benzalkonium Chloride as preservative. It was clearly separated by this method. Optimum separation was achieved in less than 5 min using a C18 column (250 mmx4.6 mm i.d, 5µ particle size) by isocratic elution. The mobile phase consisting of Acetonitrile and water (70:30, v/v) adjusted to pH6.08 with 0.1M acetic acid. Column effluents were monitored at 254 nm at a flow rate of 1.5ml/min. Retention times of Dexamethasone sodium phosphate and Ofloxacin were found to be 1.28 and 2.48 min respectively. The linearity of Ofloxacin and Dexamethasone sodium phosphate was in the range of 3-18 µg/ml and 1-6 µg/ml respectively. Developed method was economical in terms of the time taken and amount of solvent consumed for each analysis. The method was validated and successfully applied to the simultaneous determination of Ofloxacin and Dexamethasone sodium phosphate in bulk and ophthalmic formulations.

KEYWORDS: Ofloxacin, Dexamethasone sodium phosphate, HPLC, Ophthalmic formulation, Validation.

INTRODUCTION

Ofloxacin (OFX)(Figure.1) is a second generation fluoroquinolone, broad spectrum antibiotic used in bacterial infections. It is chemically (RS) -7 –fluoro 2 – methyl -6 – (4 –methylpiperazin -1 –yl) -10 –oxo -1 – azatricyclo[7.3.1.0] trideca -5 (13), 6,8,11 –tetraene -11 – carboxylic acid.

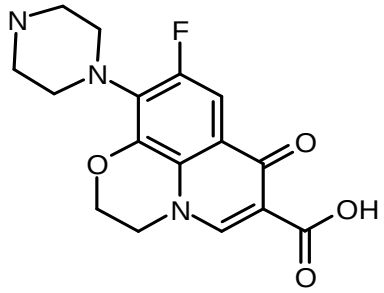

Figure 1: Chemical structure of Ofloxacin (OFX).

Dexamethasone sodium phosphate (DSP)(Figure.2) is a highly selective glucocorticoid which is widely used in ocular inflammatory diseases. Its chemical name is 9-fluoro-11b, 17, 21-trihydroxy-16α- methylpregna-1, 4-diene-3, 20-dione 21-(dihydrogen phosphate) disodium salt.

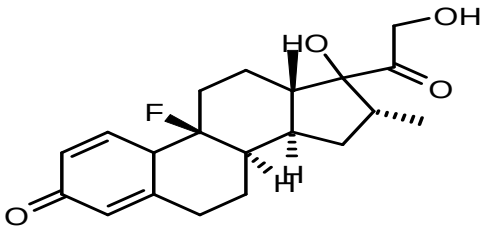

Figure 2: Chemical Structure of Dexamethasone Sodium Phosphate (DSP).

Dexamethasone in combination with ofloxacin is used in several anti-infective eye preparations to treat acute and sub-acute conjunctivitis, keratitis and corneal ulcers caused by susceptible strains of the following aerobic gram positive and negative bacteria such as S. aureus, S. epidermidis, S. pneumonia and haemophilus influenza.

In the literature, there are methods described for the individual estimation of Fluoroquinolones and Dexamethasone in aqueous samples and biological fluids by liquid chromatography, liquid chromatography-fluorescence detection, and UV- Visible spectroscopy. A few methods have also been described for the simultaneous determination of Dexamethasone with other drugs such as Chloramphenicol, Ciprofloxacin. But no such methods have been proposed for simultaneous

determination of OFX and DSP in Ophthalmic formulation with Benzalkonium chloride as preservative.

Methods were also described for simultaneous determination of Ofloxacin with other drugs such as Tetrazoline hydrochloride, Tinidazole, Cefexime. Simultaneous determination of OFN and DSP has been reported in the literature using a Shimpack ODS column using a mixture of methanol citric acid solution (0 05mol/L), acetonitrile, ammonium acetate solution(0.5mol/L), 10g/L phosphoric acid solution (100:75:22:1:2) as mobile phase, the flow rate 1.0ml/min, and the detection wavelength being 242nm. A number of reservations about the conditions used in this method and the mobile phase ratio and composition seem rather critical. The robustness of the method could therefore be significantly affected. So an attempt was made to develop a simple, robust HPLC method for the estimation of these drugs available as eye drops.

The purpose of the present study was to develop a simple, sensitive and economical HPLC method for simultaneous determination of OFX and DSP in bulk and ophthalmic formulations in presence of preservative like benzalkonium chloride. The developed method has been validated by evaluation of the system suitability, specificity, linearity, limit of detection and quantification, precision, accuracy and recovery. The validated method was applied to the commercially available pharmaceutical formulations containing both the drugs.

MATERIAL AND METHODS

Materials

DSP and OFX were obtained as gift samples from Chethana pharmaceuticals Ltd, Perinthalmanna. HPLC grade Acetonitrile was purchased from Merck, Trivandrum, HPLC grade water from Merck was used during the study. The pharmaceutical formulations containing 3mg/ml of OFN and 1mg/ml DSP (Oflacin-DX eye/ ear drops, FLOJODEX manufactured by Chethana pharmaceuticals was validated).

Instrumentation

A Shimadzu Ultra Fast liquid chromatograph (Prominence) equipped with two pumps (Model-LC 20 AD) and Shimadzu Photo Diode Array detector (SPD-M20A), ultrasonic bath **(**labtech Pvt. Ltd, India).

Chromatographic conditions

For chromatographic analysis, a Chromosil C18 column (250 mmx4.6 mm i.d, 5µ particle size) was used. Separation was carried out by isocratic elution. The mobile phase consisting Acetonitrile (ACN) and Water in the ratio of 70:30 v/v, pH corrected to 6.08 by 0.1M acetic acid was used. Mobile phase was degassed in ultrasonic bath for 30 min before passing through the instrument. The injection volume was 20µl and the flow rate was 1.5ml/min. UV detection was carried out at 254 nm. Chromatographic separations were carried out at room temperature (25-30°C).

Preparation of solutions

(a) Preparation of standard solution

Stock standard solutions of OFX and DSP were prepared in the mobile phase at a concentration of 600 µg /ml and 200µg/ml. working standard solutions was prepared by serial dilution of stock solutions with the mobile phase.

(b) Preparation of sample solution

Sample solutions of OFX and DSP were prepared at a concentration of 600 µg /ml and 200µg/ml by diluting 5 ml of the ophthalmic solution to 25 ml with the mobile phase. From this 20µl was taken to get a concentration of 6µg /ml and 1µg /ml of OFX and DSP respectively.

METHOD VALIDATION

The developed analytical method was validated as per ICH and USP guidelines for the parameters like linearity, limit of detection (LOD) limit of quantification (LOQ), precision, specificity, accuracy, robustness, and system suitability.

Linearity Six working standard solutions of each analyte in the concentration range of 3-20 µg/ml for OFX and 1-12µg/ml for DSP were prepared in triplicate and injected. Calibration curves were constructed by plotting concentration versus mean peak area.

Limits of detection and Quantification

According to ICH, limit of detection (LOD) is the lowest concentration of the analyte that can be detected and limit of quantification (LOQ) is the lowest concentration of analyte that can be detected with acceptable accuracy and precision. LOD and LOQ are calculated from the formulae $3.3\sigma/s$ and $10\sigma/s$ respectively. Where σ is the standard deviation of y-intercepts of the regression line and s is the slope of the calibration curve.

Precision

The precision of the method was evaluated in terms of intermediate precision i.e., intra-day and inter-day precision and precision by different analysts. For intra-day precision three different concentrations of OFX and DSP in the linearity range was prepared in triplicate and was analyzed during the same day. For inter-day precision the same concentrations were analyzed on three consecutive days and RSD values were calculated. Instrument precision was analyzed by injection repeatability. This was examined by analyzing six injections of the mixture containing 6 and 1µg /ml of OFX and DSP, respectively. RSD values were calculated from the peak areas and retention times of OFX and DSP.

Accuracy

Accuracy of the method was determined by recovery studies. These studies were carried out by addition of known amounts of OFX and DSP to a sample solution of

known concentration and comparing calculated and measured concentrations. A sample solution containing OFX and DSP (0.6 and 0.2 mg/ml, respectively) was prepared by diluting 5 ml of the ophthalmic solution to 25 ml in a volumetric flask, and make up the solution with the mobile phase. Samples (0.1ml) of the filtered solution were transferred to 10 ml volumetric flasks containing 0.1, 0.15, and 0.2 ml of OFN and DSP standard solution, and analyzed.

Specificity

Specificity of an analytical method may be defined as the ability of the method to measure accurately and specifically the analyte in presence of additional components such as matrix, impurities, degradation products and other related substances. The chief excipient present in the eye drops is benzalkonium chloride which is used as preservative. Sample solution containing benzalkonium chloride was injected into the system and chromatogram was recorded.

Robustness

Robustness of the method was evaluated by deliberately varying method parameters such as detection wavelength and flow rate. Detection wavelength was changed from 254 nm to 254±2 nm and flow rate was changed from 1.5ml/min to 1±0.1ml/min. Effect of these changed parameters was studied by injecting the sample in to the system.

System suitability

System suitability was established in order to determine the adequate resolution and reproducibility of the proposed method. Suitability parameters including retention factor, resolution, asymmetry factor, plate number were investigated.

Assay of the marketed formulation

The developed method was applied to the simultaneous determination of OFX and DSP in pharmaceutical formulations. Sample was analyzed by performing six independent determinations and each series was injected in triplicate.

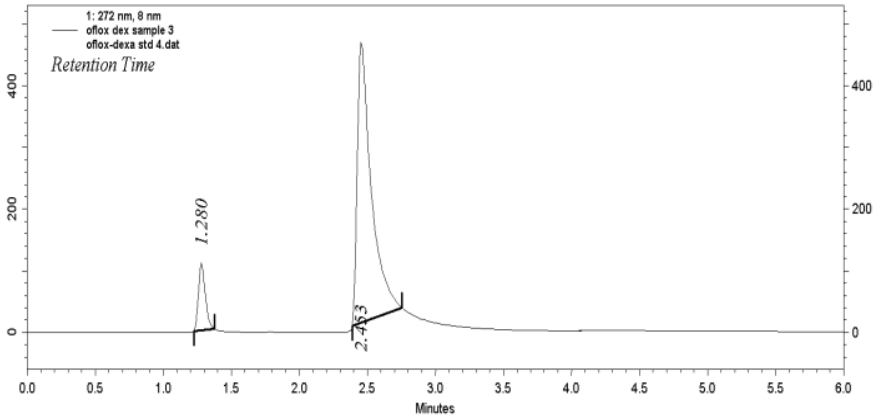

Fig. 3: HPLC chromatogram of sample containing Ofloxacin and Dexamethasone.

RESULTS AND DISCUSSION

Mobile phase optimization

Chromatographic parameters were optimized to develop a HPLC method for simultaneous determination of OFX and DSP with short analysis time (< 10 min), and acceptable resolution (RS>2). Various compositions of mobile phases like methanol: buffer and ACN: buffer in different ratios were tried. But with Acetonitrile (ACN) and Water in the ratio of 70:30, v/v pH corrected to 6.08 by 0.1M acetic acid, symmetrical peaks with good resolution were obtained. The optimum wavelength for detection was set at 254 nm at which better detector response for both drugs was obtained. The retention times were 1.28 and 2.48 min for OFX and DSP respectively.

Validation

Calibration graphs were constructed by plotting the peak area versus their corresponding concentrations. Good linearity was obtained in the range of 3-20 µg/ml and 1-12 µg/ml for OFX and DSP. The results are shown in table 1. Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the slope and standard deviation of y-intercepts of the regression line of the calibration curve. For OFX it was found to be 0.043 and 0.029 µg/ml and for DSP 0.018 and 0.08 µg/ml respectively. The precision of the method and instrument precision was evaluated and relative standard deviation (RSD) values were calculated. The RSD values for OFX and DSP showed that the precision of the method was satisfactory. The results are shown in table 5. The accuracy of the method was determined by recovery studies. The recoveries were close to 100% for OFX and DSP; the results are given in the Table 3. Developed method was found to be robust when the detection wavelength and flow rate was changed from 254 nm to 254±2 nm and 1.5ml/min to 1±0.1ml/min.

There was no considerable change in the peak areas and retention times. Using 0.9 ml/min flow rate, the retention time for OFX and DSP were found to be 1.51 and 2.5 min respectively and with 1.6 ml/min flow rate, retention times for OFX and DSP were found to be 1.51 and 2.53 min, respectively without affecting the resolution of the drugs. When detection wavelength was changed to 254±2 nm, the retention time for OFX and DSP were not changed from the normal. System suitability parameters are shown in Table 9.

Validated and found % RSD for Accuracy, Precision, Intermediate precision, Repeatability, Linearity and System suitability was within acceptance range. This method is also specific. Benzalkonium chloride, a preservative used in this formulation did not interfere with this method. Hence this method can be routinely employed for the quantification of Ofloxacin and Dexamethasone Sodium Phosphate in Ophthalmic preparations.

1. Linearity

Standard solutions prepared at five concentrations, typically 80, 100, 120, 180 and 200% of target concentration. Three individually prepared replicates at each concentration was analyzed.

The method was evaluated by determination of the correlation coefficient and intercept values. The results are given in Table-1. The representative calibration equation for DSP and OFX were Y=322915.4X+66083.47 and Y=755107.82x-1304036 respectively. The correlation coefficient was greater than 0.9912 and 0.9850. From the calibration plots it was clear that the response was linear in the studied range (4 - 12µg/20µl for OFN and 1-12µg/20µl for DSP) (**Table: 1 & Table: 2**).

Table 1: Linearity data of standard Dexamethasone.

Linearity-Data Sheet	**Dexamethasone**		**Electronic file name: D\Spinco\12-march\15\Dexamethasone standard**	
Concentration (µg/20 µl)	**Concentration as % Of Analyte Target**	**Peak Area**	**Peak Area(mean of three injections)**	**Peak Area RSD (%)**
1	100	377023 385253 395924	386066.6	2.45
1.5	150	407254 414050 393061	404788.3	2.65
3	300	1252954 1257955 1258069	1256326	2.14
6	600	1964239 1836855 1993788	1931627.3	3.59
12	1200	4009597 3890050 3920720	3940122	0.02
Equation for regression line= Y=322915.4X+66083.47			Correlation coefficient(r^2)=0.9912	

Table 2: Linearity data of standard Ofloxacin.

Linearity-Data Sheet	**Ofloxacin**		**Electronic file name: D\Spinco\12-march-15\Ofloxacin standard**	
Concentration (µg/20µl)	**Concentration as % Of Analyte Target**	**Peak Area**	**Peak Area(mean of three injections)**	**Peak Area RSD (%)**
4	80	1713633 1714233 1802312	1743392.6	0.81
6	100	3336976 2985255 3110188	3144139.6	2.22
7	120	3982397 3769400 3773180	3841659	3.32
10	180	6642054 6642054	6736614.5	1.63

		6831175		
12	200	7490488	7463218.3	1.56
		7563886		
		7335281		
Equation for regression line= Y=755107.82x-1304036			Correlation coefficient(r^2) =0.9850	

3. Range

The data obtained during the linearity and accuracy studies will be used to assess the range of the method. The precision data used for this assessment is the precision of the three replicate samples analyzed at each level in the accuracy studies. The range for DSP is 1-12µg and OFX is 4-12µg per 20µl of mobile Phase. (**Table: 3**)

Table 3: Range showing data.

Range-Data sheet	**Electronic file name: Electronic file name: D\Spinco\12-march\15\range**
Record range: Dexamethasone-1-12µg/20µl	
Ofloxacin- 4-12µg/20µl	

4. ACCURACY

Spiked samples prepared at three concentrations over the range of 50 to 150% of the target concentration**. (Table: 4).** Average Recovery was above 100.4%

Table 4: Accuracy table.

Accuracy-Data sheet						
Sample	**Standards**	**Total Area**	**Amount Of Sample**	**Amount of Standard (Spiked)**	**Amount of Standard (Found)**	**Recovery (%)**
1	Dexamethasone	1772407	1µg	2µg	2.97	99
2	Dexamethasone	1870816	1µg	3µg	2.95	98
3	Dexamethasone	2885050	1µg	4µg	5.02	100.4
4	Ofloxacin	5620018	6µg	2µg	7.92	99
5	Ofloxacin	4200756	6µg	3µg	8.89	98.8
6	Ofloxacin	6450767	6µg	4µg	9.92	99.2

5. Precision - Repeatability

One sample solution containing the target level of analyte was prepared. Six replicates made from this sample solution according to the final method procedure. Retention time, Peak Area, Peak height and SD and %RSD were calculated**(Table: 5).** For OFX and DSP, %RSD for peak area was 1.2% and 1.6%. Result was within the limit.

Table 5: Repeatability data.

Repeatability					**Electronic file name: D\Spinco\12-march-15\Repeat**	
Injection No	**Retention time**		**Peak Area**		**Peak Height**	
	DSP	**OFX**	**DSP**	**OFX**	**DSP**	**OFX**
1	1.280	2.475	349590	2462727	104253	308262
2	1.280	2.475	419523	2551206	109765	311645
3	1.280	2.453	383676	3227371	108879	454153
4	1.280	2.453	383676	3324551	108879	455690
5	1.280	2.475	347233	2462727	102213	308262
6	1.280	2.475	347233	2527576	102213	309174
Mean	1.280	2.464	384234	3063455	106667.7	369172.2
SD			21752	386851.3	2617.639	68749.61
RSD%			1.6%	1.8%	1.2%	1.8%

6. Intermediate Precision

Intermediate precision (within-laboratory variation) demonstrated by two analysts, using same HPLC systems on different days at three concentration levels (50%, 100%, and 150%) that cover the analyte assay method range 80 to 120%. The relative % purity (% area) of each concentration on the datasheet was recorded. Calculated the mean, standard deviation, and RSD for

the operators (**Table: 6**). Precision was found to be in acceptable limit.

Table 6: Intermediate Precision data.

Sample	Dexamethasone			Ofloxacin		
Percentage	S1 (50%)	S2 (100%)	S3 (150%)	S1 (50%)	S2 (100%)	S3 (150%)
Operator 1 Day1	377023	407254	1252954	1713633	3336976	3982397
Operator 1 Day 2	395924	414050	1257955	1714233	2985255	3769400
Operator2 Day1	385253	393061	1258069	1802312	3110188	3773180
Operator2, Day2	395062	414050	1257231	1714241	2985234	3846212
Mean						
	388315.5	407103.8	1256552	1350401	3104413	3842797
%RSD						
	1.6	1.4	0.19	1.5	1.7	1.6

7. Specificity

The Specificity of the method was checked. Specificity of an analytical method may be defined as the ability of the method to measure accurately and specifically the analyte in presence of additional components such as matrix, impurities, degradation products and other related substances. The chief excipient present in the eye drops is Benzalkonium chloride which is used as preservative. Sample solution containing 1µg/20µl of Benzalkonium chloride was injected into the system and chromatogram was recorded. The method is specific.

8. Limit of Detection

The lowest concentration of the standard solution wasdetermined by using the formula 3.3 * σ/s (where σ is standard deviation of the Linearity curve and s is the slope). (**Table: 8**).

Table 8: Limit of detection.

Limit of detection	3.3σ/s
Dexamethasone	$2.39x10^{-4}$µg/20µl
Ofloxacin	$4.83x10^{-6}$µg/20µl

9. Limit of Quantification

Establish the lowest concentration at which an analyte in the sample matrix can be determined with the accuracy and precision required for the method in question. This value may be the lowest concentration in the standard curve. It can be found out using formula 10*σ/s (**Table: 9**)

Table 9: Limit of quantification.

Limit of quantification	10σ/s
Dexamethasone	0.3µg/20µl
Ofloxacin	2.5µg/20µl

10. System Suitability

System suitability tests performed on HPLC systems to determine the accuracy and precision of the system by injecting six injections of a solution containing analyte at 100% of test concentration. The following parameters determined: plate count, tailing factors, Resolution, and reproducibility (percent RSD of retention time, peak area, and height for six injections). (**Table: 10**).

Table 10: System Suitability range.

System Suitability Parameter	Acceptance criteria	Results
Injection precision (Retention time)	RSD ≤ 1%	0.44%
Injection precision (area)	RSD ≤ 1%	0.88%
Injection precision(height)	RSD ≤ 1%	0.92%
Resolution	Rs≥	9.2
USP tailing factor	T ≤ 2	1.4
Capacity factor	K ≥ 2	4.3
Theoretical plates	N ≥ 2000	4532(-DSP),3200(OFX)

***average of six injections (taken from Repeatability data)**

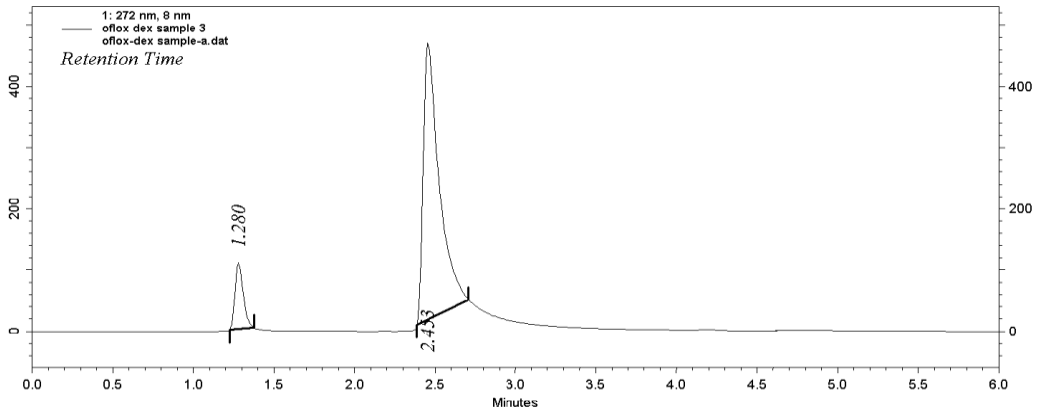

Fig. 2: HPLC chromatogram of standard solution of Ofloxacin and Dexamethasone.

Assay of the marketed formulation

According to ICH in the case of assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. The assay value of the marketed formulation was found to be within the limits. The low RSD value indicated suitability of this method for routine analysis of OFX and DSP in pharmaceutical dosage forms. Chromatogram of the sample shows that there was no interference from the excipients present in the formulation. This indicates the specificity of the method. The results are shown in table 11.

Table 11: Assay of eye drops (n=6).

Drug	Label claim (mg/ml)	Amt found (mg/ml)	Mean %recovery	%RSD
OFX	3	3.05	100.9	1.7
DSP	1	1.00	101.7	1.4

CONCLUSION

The method described in this paper for the simultaneous estimation of OFX and DSP was found to be simple, sensitive, accurate, precise, rapid, robust and economical. The analytical conditions and the solvent system developed provided good resolution within a short analysis time. The RSD for all parameters was found to be within the limits, which indicates the validity of method and assay results obtained by this method are in fair agreement. Thus the developed method can be proposed for routine analysis of OFXand DSP in laboratories and for quality control purposes.

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