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Article

A Two-Step Optimization Approach: Validated RP-HPLC Method for Determination of Gatifloxacin and Dexamethasone in Ophthalmic Formulation

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Abstract

The growing technology of stationary phase chemistry has a great impact on the chromatographic system performance and analysis economics. In this context, a simple rapid reversed phase high-performance liquid chromatography method development is presented for the analysis of gatifloxacin (GFN) and dexamethasone sodium phosphate (DSP) in their ophthalmic formulation. A two-step optimization approach has been conducted using optimum chromatographic conditions as well as proper selection of stationary phase. The chromatographic separation was carried out using sodium phosphate buffer pH 3.0 ± 0.1 and acetonitrile 72:28 v/v, respectively, with flow rate 1 mL min^{-1} and simultaneous detection at 243 nm. Three different column technologies were investigated at the optimum set of the chromatographic conditions: Xbridge® bridged ethylene hybrid silica, Kinetex™ Core-Shell and the Onyx™ Monolithic stationary phase. The monolithic column has shown better chromatographic separation, based on system suitability testing as well as shorter analysis time and sensitivity. The proposed method was validated according to International Conference on Harmonization guidelines. The linearity was achieved for GFN and DSP in the range 0.58–120 μg mL^{-1} and 0.50–120 μg mL^{-1}, respectively, with acceptable accuracy, precision and selectivity.

Introduction

High-performance liquid chromatography (HPLC) columns are the heart of the chromatographic method. The continuous advances in packing technology should help the analysts to find more selective, durable, cost-saving and efficient stationary phase to be used in a certain application (1–3). Each of the emerging packing modules has proven advantages and facilitation to the chemical, pharmaceutical analysis and method development (4, 5). Analysts and method developers could get confused choosing from the huge variety of stationary phase types that are available today. Comparative studies and real applications using different types of packing technologies are of growing importance to give clues to analysts on which packing technology could be chosen for similar or related applications (3, 6–8). The most commonly and recently used technologies in chromatographic analysis are Xbridge® bridged ethylene hybrid silica (9), Kinetex™ Core-Shell (10) and Onyx™ Monolithic (4). First, Xbridge® C_{18} column (Waters) is categorized as fully porous stationary phase. It offers superior pH stability over wide range (pH 2–12), which is enormously relevant in HPLC method development for pharmaceutical compounds, especially for weak acidic or basic analytes. Also, its mechanical stability increases the column efficiency and improves the column reliability and reproducibility. It shows excellent performance, lifetime and separation while changing instruments and vendors (9). Second technology presented is Kinetex™ Core-Shell column (Phenomenex), sometimes called superficially porous or fused silica material in which homogeneous

porous nano-structure shell is grown on a solid silica core to create a core–shell particle. This particle morphology results in less band broadening compared with fully porous particles and thus delivers high efficiencies. The high separation efficiency of core–shell particles is a result of a faster analyte mass transfer from the mobile phase into and out of the porous layer of the particle (10). Finally, Onyx^{TM} Monolithic column (Phenomenex) is a silica-based monolithic HPLC column. This technology creates highly porous rods of silica with a revolutionary bimodal pore structure. The main advantages of monoliths are their hydrodynamic properties (11) where a fast convective flow of the mobile phase can be provided through the monolith due to macropores (with size of ~2 µm), which increase the mass transfer between mobile and stationary phases. It provides a large surface of the monolith owing to mesopores (with size of ~13 nm) (12). It supply shorter run time and saving in solvent consumption. Owing to the effect of internal diameter and physical dimensions on the performance of columns, care must be taken when comparing different ones (13). Due to the effect of particle size, monolithic columns are expected to give better performance compared with packed columns because they are made of a single piece of porous silica, which is also called a "silica rod" (12).

Dexamethasone sodium phosphate (DSP) (9-fluoro-11β,17-dihydroxy-16 α-methyl-3,20-dioxopregna-1,4-dien-21-yl disodium phosphate) is frequently used to suppress inflammation in the eye and can preserve sight when used properly (Figure 1A) (14). It is administered topically for diseases of the outer eye and anterior segment and attains therapeutic concentrations in the aqueous humor after instillation into the conjunctival cul-de-sac (15). Gatifloxacin (GFN) ((RS) 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methylpiperazin-l-yl)-4-oxo-3-quinolinecarboxylic acid) (Figure 1B) (16, 17) has broad antimicrobial activity and is effective for the treatment of a wide variety of infectious diseases (15). The combination between fluoroquinolones (GFN) and glucocorticoids (DSP) is used for allergic conjunctivitis and other ophthalmic microbial inflammations (18). Owing to moderate-to-low stability of gatifloxacin in ophthalmic solutions, few commercial ophthalmic formulations are available (19, 20). A critical review of literature of this pharmaceutical combination had revealed some chromatographic methods using single column technology in applications (21–25) and two spectrophotometric assays (26, 27). However, the reported chromatographic methods had some limitations that needs to be addressed (21–25) such as a relatively long run time, a gradient elution which requires re-equilibration (21), moderate linearity range and sensitivity (21, 22, 24, 25). The aim of this study is to develop a rapid, reproducible, sensitive and cost-effective reversed phase HPLC (RP-HPLC) method for quantitation of GFN and DSP in their bulk and pharmaceutical formulation by a two-step optimization approach. The first step is to investigate and optimize the chromatographic conditions such as the mobile phase composition, pH and flow rate, while the second step aims to compare between three different column technologies, based upon system suitability parameters testing using the same analytes, instrument and chromatographic conditions including mobile phase.

Experimental

Chemicals and solvents

Pure gatifloxacin was kindly supplied by Global Napi Pharmaceuticals, Cairo, Egypt, and its purity was certified to be 99.89 ± 0.691%. Pure DSP was kindly supplied by Orchidia Pharmaceutical Company, Cairo, Egypt, and its purity was certified to be 99.69 ± 0.231%. Gatilox DM® Eye Drops were purchased from Akums Drugs and Pharmaceuticals Ltd (India). Labeled to contain GFN and DSP in concentrations of 3:1 mg mL^{-1}, respectively (batch number FTP 3033). Acetonitrile (HPLC grade) and sodium dihydrogen phosphate were purchased from Sigma-Aldrich (Germany). A water purification system (New Human Power I, Korea) was used to obtain ultra-pure water.

Instruments

Chromatographic separations were carried out using an Agilent 1100 Series liquid chromatograph consisted of a dual pneumatic pumping system (model G1310A), UV variable wavelength detector (model G1314A) and a Rheodyne manual injector (model 7725 I) equipped with 20-µL injector loop (Agilent, USA). System control and data analysis were achieved using Chemstation software (Agilent, USA).

Chromatographic conditions

Chromatographic separations were investigated using three columns: (i) regular Xbridge® C_{18} 5 µm, 250 mm × 4.6 mm I.D (column 1), (ii) regular Kinetex^{TM} Core-Shell C_{18} 5 µm, 250 mm × 4.6 mm I.D (column 2) and (iii) Onyx^{TM} Monolithic C_{18} 100 mm × 4.6 mm I.D (column 3). Phosphate buffer 0.02 M was prepared according to British Pharmacopoeia (14), pH was adjusted to 3.0 ± 0.1 using orthophosphoric acid. Optimum mobile phase composition was acetonitrile: sodium dihydrogen phosphate buffer pH 3.0 ± 0.1 (28:72, v/v). The mobile phase was degassed for 30 min in ultrasonic bath prior to use. Isocratic elution was employed at a flow rate of 1 mL min^{-1} and detection was achieved at 243 nm. All chromatographic separations were carried out in an air-conditioned room maintained at 22 ± 2°C.

Stock solutions

Standard stock solutions of GFN and DSP 1 mg mL^{-1} were prepared separately using methanol as solvent. Working standard solutions for HPLC 100 µg mL^{-1} of GFN and DSP were prepared from stock solutions by appropriate dilution with the mobile phase.

Methods

Construction of calibration curves. Suitable aliquots of GFN and DSP were accurately transferred from their respective standard stock solutions (1 mg mL^{-1}) into two separate series of 10-mL volumetric flasks. The volume was then completed with the mobile phase to prepare concentration in the range of 1–120 µg mL^{-1} for GFN and 2–120 µg mL^{-1} for DSP. Solutions were then filtered through a 0.22-µm syringe membrane filter, injected in volumes of 20 µL in triplicate and chromatographed using the previously mentioned chromatographic conditions. The average peak areas obtained for each concentration of GFN and DSP were plotted versus the corresponding concentrations. The regression equations were computed. Validation was performed according to International Conference on Harmonization (ICH) guidelines and the following parameters were determined: linearity, range, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) (28).

Determination of GFN and DSP in their pharmaceutical formulation. An accurate volume of the eye drops equivalent to 8 mg DSP, 24 mg GFN was pipetted into 10-mL measuring flask, the volume was

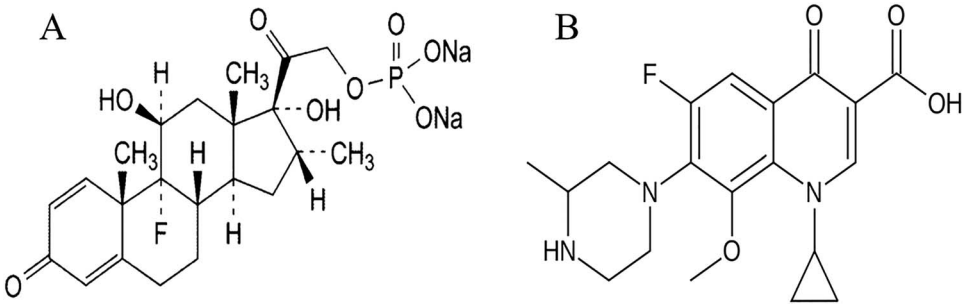

Figure 1. Chemical structure of (A) dexamethasone sodium phosphate (DSP) and (B) gatifloxacin (GFN).

completed with the mobile phase and analyzed under the previously mentioned chromatographic conditions.

Results

The main criteria for development of a successful HPLC method are its ability to determine the analyzed drugs in the studied matrix with sufficient resolution in short analysis time. In addition, it should be accurate, reproducible, robust, cost-effective and simple enough for routine use in the quality control laboratory as well (7, 29).

Method development and optimization

Mobile phase optimization. To develop and optimize a suitable mobile phase for the separation of GFN and DSP, the chromatographic behavior was examined using mobile phase mixtures of different polarity using the Xbridge® C_{18} column. Initially, green solvents, such as aqueous isopropyl alcohol and aqueous ethanol, were trialed but very poor resolution and selectivity were obtained. A preliminary study using different mobile phase ratios of methanol/water and acetonitrile/water were employed. The mobile phase had resolved the analytes into broad peaks with long retention time.

A combination of acetonitrile/phosphate buffer pH 3.0 was expected to give better results, so the next factor to optimize was the ratio between acetonitrile and the buffer. Different combinations were tried, increasing acetonitrile up to 70%. Shortened the retention time while increasing the buffer up to 90% delayed the retention time and could affect the column lifetime. The limiting factor was the resolution between the two peaks, and the ratio (28:72, v/v) achieved the best results. Maximum sensitivity was obtained at wavelength 243 nm which is considered a center point between λ_{max} of both drugs. In addition, different flow rates (0.6, 0.8, 1.0, 1.2 and 1.5 mL min^{-1}) were tested. Flow rates of 0.6 and 0.8 mL min^{-1} had increased the peak broadening and run time by 30%, while increasing the flow rate to 1.5 mL min^{-1} had decreased resolution by 50%. Optimum resolution, peak symmetry and elution time were obtained by adjusting the flow rate at 1 mL min^{-1}. Under optimized conditions, good separation of the two analytes was obtained.

Choice of stationary phase. The second growing interest step of optimization was the stationary phase or the packing technology of the HPLC column (25). Three types of columns were investigated, regular Xbridge® C_{18} 5 µm, 250 mm × 4.6 mm I.D, regular Kinetex™ Core-Shell C_{18} 5 µm, 250 mm × 4.6 mm I.D and Onyx™ Monolithic C_{18} 100 mm × 4.6 mm I.D.

The Xbridge® column has shown very good pH stability during the trials over pH range 2–12. Good peak shape and better symmetry than the Kinetex™ Core-Shell. However, relative longer run time, higher pack pressure and longer conditioning time and mobile phase consumption relative to the Onyx™ Monolithic (Figure 2). Core–shell packing material exhibited comparable resolution, efficiency and worse peak shape and symmetry relative to Xbridge and Onyx™ Monolithic columns as shown in Table I. Also poor sensitivity was observed.

Onyx™ Monolithic resulted in a perfect separation of the drugs with shorter retention times, excellent resolution (Figure 2) and highest number of theoretical plates which means better efficiency plus high permeability, which is nearly twice as high as that of packed columns. This gave possibility to work under higher flow rates without the doubt of increasing column back pressure and also the ability to withstand various sample matrices such as plasma with easy washing procedure with minimal cost of analysis due to decrease in the volume needed of mobile phase and enormously reduced conditioning time.

Method validation and application to pharmaceutical formulation

Under the specified experimental conditions, a linear relationship was obtained between the relative peak areas at the selected wavelength and the corresponding concentrations of the drugs in the range of 1–120 µg mL^{-1} and 2–120 µg mL^{-1} for GFN and DSP, respectively. These ranges allowed the analysis of the dosage form in a single run.

The regression equations were computed to confirm the linearity claims and found to be

$$P\ \text{GFN} = 38.763x–0.6024\ r = 0.9999$$

$$P\ \text{DSP} = 37.909x–13.99\ r = 0.9999$$

where P is the relative peak area, C is the concentration in µg mL^{-1} and r is the correlation coefficient. The suggested method was validated according to ICH guidelines (28) and the calculated validation parameters are shown in Table II. Accuracy was evaluated by mean percentage recoveries obtained as shown in Table II. Repeatability was assessed through the relative standard deviation values. Satisfactory results were obtained for the proposed methods. Our newly developed method had showed better sensitivity relative to previously reported methods with LOD of 0.19 µg mL^{-1} for GFN and 0.17 µg mL^{-1} for DSP (21, 22, 24). Robustness of the proposed method was assessed with respect to the effect of small but deliberate variation in chromatographic conditions. The robustness was conducted at three different concentrations for each analyte

Table I. System Suitability Parameters of the Proposed HPLC Method Using Xbridge Column, Kinetex Column and Onyx Monolithic Column

Parameters[a]	Xbridge®		Kinetex™ Core-Shell		Onyx™ Monolithic	
	GFN	DSP	GFN	DSP	GFN	DSP
Retention time	3.47	4.76	3.15	4.58	1.87	2.66
Retention factor (K')	2.86	4.29	2.50	4.09	3.69	5.65
Selectivity factor (α)	1.50		1.64		1.53	
Asymmetric factor (T)	1.05	1.12	1.05	1.20	1.06	1.01
Resolution (Rs)	6.70		6.28		7.60	
Number of theoretical plates (N)	6,487	8,628	3,936	5,208	5,696	9,399
Height equivalent theoretical plates (HETP) (cm)	0.002	0.001	0.003	0.002	0.002	0.001

[a]Reference values for HPLC parameters: Rs ≥ 1.5, $T \leq 2$, $N \geq 2{,}000$ and $K' \geq 2$ (30).

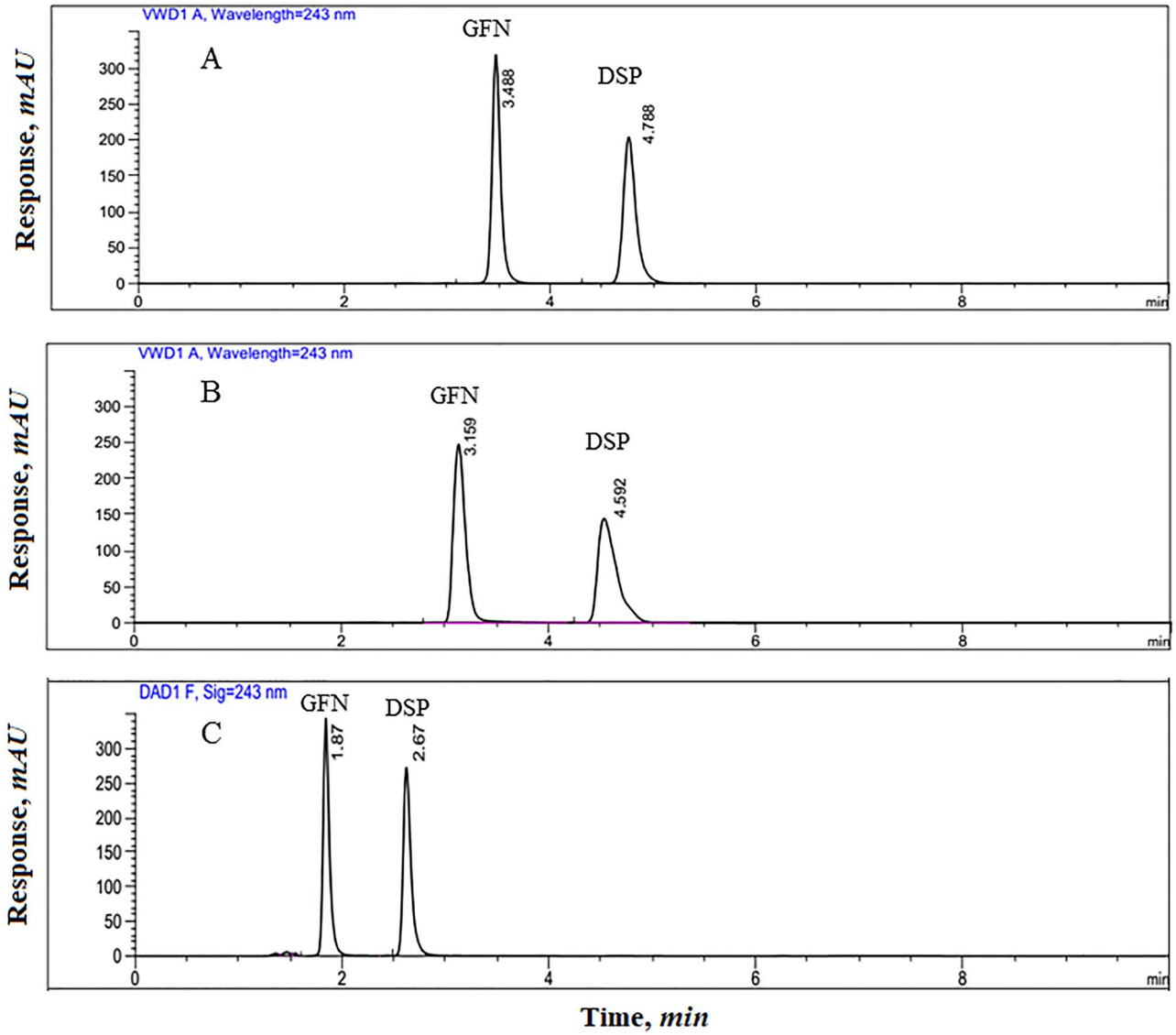

Figure 2. HPLC chromatogram of GFN-DSP using (A) Xbridge column, (B) Kinetex column and (C) Onyx monolithic column.

Table II. Assay Validation Parameters of the Proposed HPLC Method for the Simultaneous Determination of GFN and DSP

Parameter	GFN	DSP
Accuracy[a] (mean recovery % ± SD)	98.83 ± 0.50	99.44 ± 1.12
Precision		
Repeatability[b]	±0.75	±0.71
Intermediate precision[c]	±1.69	±1.79
Linearity		
Slope	38.76	37.90
Intercept	–0.602	–13.99
Correlation coefficient (r)	0.999	0.999
Range (μg mL^{-1})	0.58–120	0.5–120
LOD[d] (μg mL^{-1})	0.19	0.17
LOQ[d] (μg mL^{-1})	0.58	0.50

[a]Average of (n = 3), average five concentrations (5, 60 and 100 μg mL^{-1}) for GFN and DSP.
[b]The intraday (n = 3), average of three concentrations (20, 50 and 60 μg mL^{-1}) for GFN and DSP repeated three times within the day.
[c]The inter-day (n = 3), average of three concentrations (20, 50 and 60 μg mL^{-1}) for GFN and DSP repeated three times in 3 days.
[d]Determined via calculations, LOD = 3.3 (SD of the response/slope), LOQ = 10 (SD of the response/slope).

(30, 40 and 50 μg mL^{-1}). The degree of reproducibility and system suitability parameters of the results were within reasonable range indicating that the methods were robust enough as shown in Table III.

The methods are applicable for the analysis of Gatilox DM® Eye Drops with no interference of the excipients as shown in Table IV. The validity of the proposed procedure was assessed using the standard addition technique. This was achieved by spiking different known concentrations of the pure drug to the formulation followed by applying the procedure described under the determination of pharmaceutical formulations as shown in Table IV. System suitability was investigated according to United States Pharmacopeia by calculating the potential aspects that affect the separation, such as asymmetric factor, retention factor, selectivity factor, resolution, column efficiency (N) and the plate height (HETP), for the three columns Table I (30). Enhanced chromatographic separation and performance were attained using the monolithic column as discussed earlier.

Discussion

Method development and optimization

Mobile phase optimization. Mobile phase optimization was implemented using the Xbridge® C_{18} column. The reason for starting on this column is the great stability and wide pH range applicability that facilitate the trials of varieties of mobile phases and the stability against wide range of pH upon trying different buffers (9). To choose the optimum pH for the buffer, we had to take into consideration the physicochemical properties of the studied drug. It should be stated that DSP is acidic in nature with pKa 1.89 (31) while that of GFN is ~6.18 (32). An effective pH for the mobile phase would depend on the fact that the optimum buffer capacity equals pKa of the analyte ± one pH unit (33, 34). Best separation was attained at pH 3.0 ± 0.1. At this pH, DSP exists in its unionized form in contrast to GFN which has fixed positive charge (ionic molecule) allowing considerable resolution.

Choice of stationary phase. The poor results and peak shape of core–shell Figure 2 may be explained by loadability issues. Although it was reported that the large size core–shell particle columns show better separation power over their fully porous counterparts (1), when both types of support were tried at the same operating pressure, almost same observations were made (13). GFN and DSP have shown the tendency to overload because of being small polar molecules, they experience weak adsorption at the interface between the solvated C18 bonded layer and the bulk eluent, while high energy sites are intercalated deeper within the grafted C18 chains. These results came in accordance with literature (35).

Onyx™ Monolithic shows better resolution and efficiency (Table I). This may be attributed to its higher permeability, greater adsorption resulting from larger effective or accessible surface area (12). In agreement with the reported goals of applying monolithic columns in HPLC (36). Onyx™ Monolithic column provides a 60% lower back pressure than core shell and Xbridge columns. Reported results had showed that monolithic RP column decreased column back pressure three to five times than the particle packed column (37). It could be concluded that monolithic column had proven to show unique results and performance as per Guiochon stated that monoliths are the first original breakthrough to have occurred in this area since Tswett invented chromatography (38). Accordingly, Onyx™ Monolithic column was chosen in the final step of method

Table III. System Suitability Parameters for Robustness of the Proposed HPLC Method Using Monolithic Column

Parameters		Symmetry		Retention factor		Number of theoretical plates		Selectivity	Resolution
		GFN	DSP	GFN	DSP	GFN	DSP	DSP	DSP
Wavelength (nm)	243 + 5	0.97	0.88	3.64	5.80	5,469	9,387	1.59	7.20
	243–5	1.03	0.94	3.64	5.80	5,499	9,393	1.59	7.60
Flow rate (mL min^{-1})	1.10	0.80	1.06	2.78	4.48	5,575	11,727	1.61	8.40
	0.90	0.97	0.85	4.38	6.69	6,049	13,061	1.52	8.45
Mobile phase ratio (v/v)	70–30	0.83	0.92	3.50	4.77	7,569	10,625	1.36	5.92
	75–25	0.91	0.92	3.95	7.04	6,284	11,864	1.78	11.33
pH	3.30	0.97	0.88	3.64	5.80	5,469	9,387	1.59	7.20
	2.70	0.90	1.14	3.90	6.66	5,721	8,464	1.70	9.10

Table IV. Determination of GFN and DSP in Gatilox DM® Eye Drops and Standard Addition Technique by the Proposed Chromatographic Method

Pharmaceutical formulation	HPLC method					
	Drug	Taken (μg mL^{-1})	Found[a] ± SD	Standard addition technique		
				Pure added (μg mL^{-1})	Found	%Recovery[a]
Gatilox DM® Eye Drops Batch number FTP 3033	GFN	24	23.836 ± 1.08	12	11.92	99.36
				24	23.89	99.56
				48	48.44	100.92
					Mean ± SD	
	DSP	8	7.93 ± 0.98	4	3.97	99.20
				8	7.9	98.74
				16	16.19	101.15
					Mean ± SD	

[a] Average of three determinations.

development, which is validation as well as pharmaceutical dosage form analysis.

Conclusion

The proposed HPLC method has the advantage of being simple with no excessive data manipulation, reproducible and accurate according to the study in hands. Under optimized separation conditions of GFN and DSP, monolithic column had showed superior chromatographic performance over core shell and Xbridge columns regarding system suitability parameters, analysis time, analysis cost, conditioning time, back pressure and reduced risk of clogging. The performance of the monolithic column was best for the specific application in the analysis of the pharmaceutical formulation and bulk powder; however, the other columns can show superiority in different applications such as impurities detection or stability studies. We believe that monolithic columns continue to show a promising impact in the future of HPLC analysis, because various structures could be designed and optimized under the evolving research to obtain optimum selectivity and greater performance along with their proved advantages. The proposed method can be used for the routine analysis of GFN and DSP, in pharmaceutical formulations or in bulk powder. The method is characterized by broad applicability, short analysis time and adequate robustness. The suggested method was validated as per ICH guidelines. It could be implemented in QC laboratories for a cost-effective analysis.

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