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# Column Performance Study of Different Variants of Liquid Chromatographic Technique: An Application on Pharmaceutical Ternary Mixtures Containing Tetryzoline

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**High-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC) and rapid resolution liquid chromatographic (RRLC) methods have been developed and validated for the separation and quantitation of both or either of two ternary mixtures present in ophthalmic solutions. The first mixture contains chloramphenicol, dexamethasone sodium phosphate and tetryzoline HCl (TZH); while the second one contains ofloxacin, prednisolone acetate and TZH. Both preparations contain benzalkonium chloride as a preservative. The columns used were a HPLC column (C<sub>18</sub> 5 μm particle size), a RRLC column (C<sub>18</sub> 2.6 μm particle size) and a UPLC column (C<sub>18</sub> 1.7 μm particle size). A comparative study was conducted to illustrate the effect of the change in column particle size and dimensions on the other chromatographic conditions, backpressure and the separation of both ternary mixtures. The methods were validated as per ICH guidelines where accuracy, repeatability, interday precision and robustness were found to be within the acceptable limits. The RRLC column provided shorter run time and better resolution than HPLC, while the UPLC column gave the shortest run time for all columns. The RRLC column resulted in minimum backpressure, so it could be used with any HPLC instrument, which makes the method more practical and economic. The results obtained from the proposed methods were statistically compared with official ones where no significant difference was observed.**

## Introduction

High-performance liquid chromatography (HPLC) is critical to the discovery, development and eventual commercialization of pharmaceutical products. HPLC is the benchmark analytical method in the pharmaceutical industry because of its ability to provide best results in analytical validation characteristics; including accuracy, precision, limit of detection (LOD), specificity, ruggedness and robustness. It is proved to be an ideal way for the separation and identification of antipsychotic, antihypertensive, anticoagulant, antibiotics, steroids and countless other organic or inorganic drugs (1–7). No other analytical techniques can consistently achieve similar results on compounds and matrices that are of interest to the pharmaceutical industry (8, 9).

Conventional HPLC columns include the particles with sizes >3.5 μm. As the efficiency and speed of analysis are of great importance, then it is important to increase throughput and reduce analysis costs. By using smaller particles in column packing, speed and peak capacity are extended to new limits, called ultra-performance liquid chromatography (UPLC). UPLC is an effective technique giving new possibilities in liquid chromatography,

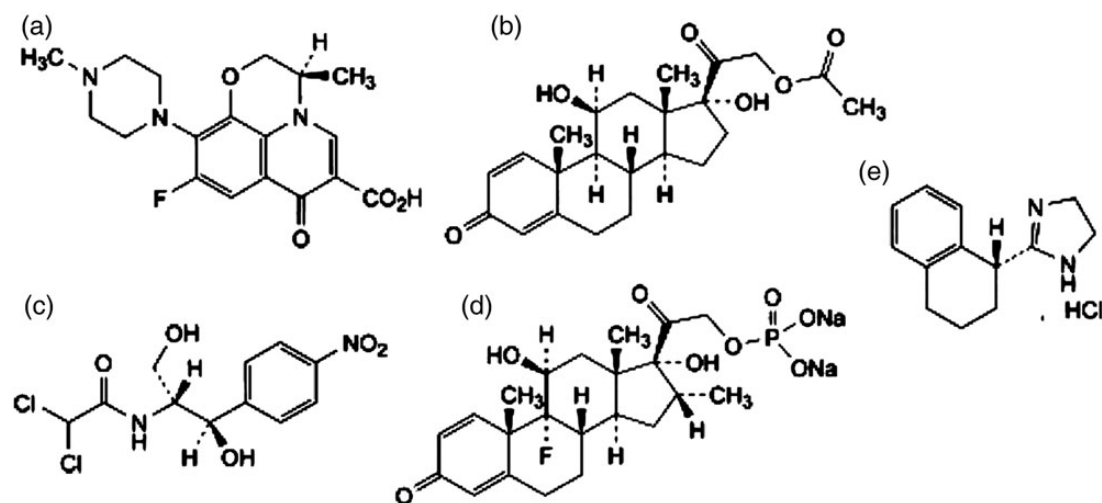
especially concerning decrease of time and solvent consumption (10). It involves the use of sub-2 μm i.d. particles, but it should be used within a developed special UPLC instrumentation capable of resisting backpressure up to 1,500 bar. Nowadays the need for UPLC and rapid resolution is still growing, especially in the pharmaceutical industry, due to the continuous demand for high throughput analysis in research and quality control. Unfortunately, the use of novel UPLC separation media is often restricted to dedicated equipment and not applicable to the large installed base of conventional HPLC systems with a standard operating pressure rating of <400 bar.

For this reason rapid resolution liquid chromatography (RRLC), using superficially porous particles or shell particles columns of 2–3 μm inner diameter, were designed to provide powerful chromatographic improvements, in terms of velocity and resolution as for UPLC system, with conventional HPLC operating conditions (11, 12). Accordingly, in the laboratory there is no need to replace the conventional HPLC unit with a new UPLC unit in order to achieve fast chromatographic analysis; just replacing the column will do, which saves time and cost in the manufacture process and provides similar separation efficiencies compared with that of sub-2 μm particles but at much lower pressures (13–15).

This work presents a study on the performance HPLC, RRLC and UPLC methods using different sized chromatographic columns applied for the separation of two ternary mixtures. The proposed methods used the same mobile phase for each mixture to study the efficiency of separation of the three chromatographic columns. This work discussed the advantage and limitations of each method and how to adjust different chromatographic variants, which might help in the development or enhancement of similar methods.

The ternary mixtures used for this study were mixture A [chloramphenicol (CHL), dexamethasone sodium phosphate (DXM) and tetryzoline HCl (TZH)] and mixture B [Ofloxacin (OFX), prednisolone acetate (PRD) and TZH]. The chemical structures for those drugs are shown in Figure 1. These combinations are used as anti-infective eye preparations to treat acute and sub-acute conjunctivitis, keratitis and corneal ulcers.

Different spectroscopic and liquid chromatographic methods have been previously reported for the determination of the cited drugs: OFX (16–20), PRD (21–25), CHL (23, 25, 26), DXM (22, 27, 28) and TZH (29, 30). Lotfy *et al.* described a spectrophotometric method for the analysis of mixture A (31), but no chromatographic method has been reported for this mixture. The HPLC method has been reported for the analysis of mixture B (32) using Waters Spherisorb ODS (5 μm, 4.6 × 150 mm) as a



**Figure 1.** The structural formulae of (a) OFX, (b) PRD, (c) CHL, (d) DXM and (e) TZH.

stationary phase and isocratic elution of 0.05 M phosphate buffer–acetonitrile (65 : 35, v/v), and the pH was adjusted to 2.7 with *o*-phosphoric acid, where the separation exceeded 10 min.

The aim of this work was first, developing two novel liquid chromatographic methods for the separation of mixture A for the first time; second, enhance the separation of mixture B through decreasing its runtime to 6 min. All the proposed methods are considered to be cost-effective and eco-friendly through the short runtime presented, which will save time and reagents. The proposed work will represent a great difference for the quality control laboratories, especially those with limited facilities.

## Experimental

### Apparatus

Agilent 1290 infinity series chromatographic system equipped with a binary pump, thermostated column compartment, and variable wavelength UV–VIS detector was used, and the data were processed using Chemstation software (Agilent Technologies, USA). The columns used were Zorbax SB-C<sub>18</sub> (150 mm × 4.6 mm i.d., 5 μm particle size; Agilent Technologies) for the HPLC method and Kinetex C<sub>18</sub> (100 mm × 4.6 mm i.d., 2.6 μm particle size; Phenomenex, USA) for the RRLC methods.

Waters Acquity UPLC system (Waters, Manchester, UK) with Kinetex C<sub>18</sub> (50 mm × 2.1 mm, 1.7 μm particle size; Phenomenex) was used for the UPLC method. The apparatus was equipped with a binary solvent delivery pump, an autosampler and a tunable UV detector. A 'Jenway 3505' pH-meter (Jenway, UK) equipped with a combined glass electrode was used for pH adjustment.

### Materials

#### Samples

OFX and DXM reference standards were kindly supplied by Egyptian International Pharmaceutical Industries Co. (EIPICO), Cairo, Egypt. The purities were found to be  $99.68 \pm 1.21$  and

$99.94 \pm 0.42$  for OFX and DXM, respectively, by the official methods. The CHL and PRD reference standards were kindly supplied by Sigma Pharmaceutical Industries Limited, Al-Monofeya, Egypt. The purities were found to be  $100.17 \pm 0.62$  and  $100.26 \pm 0.83$  for CHL and PRD, respectively. The TZH reference standard was supplied by Sigma-Aldrich, USA, with a purity of  $100.06 \pm 0.59$ . The purity testing was done according to the BP official methods (33).

Loxtra<sup>®</sup> eye drops, labeled to contain 3 mg of OFX, 2 mg of PRD, 0.4 mg of TZH and 0.05 mg of benzalkonium chloride per 1 mL, were manufactured by Jamjoom Pharma, Kingdom of Saudi Arabia. Orchardexoline<sup>®</sup> eye drops, labeled to contain 5 mg of CHL, 1 mg of DXM, 0.25 mg of TZH and 0.02 mg BNZ per 1 mL, was manufactured by Orchidia Pharmaceutical Industries, Al-Obour city, Egypt. Both samples were purchased from the local market.

### Chemicals

Sodium dihydrogen phosphate and ortho-phosphoric acid (85%) were supplied by Adwic-El Nasr pharmaceutical Chemicals Co., Egypt.; and methanol and acetonitrile (HPLC grade) were obtained from LabScan Limited) Dublin, Ireland. Distilled water was also used.

### Standard solutions

Stock solutions of OFX, PRD, CHL, DXM and TZH (concentration 1 mg mL<sup>-1</sup>) were prepared using a solvent mixture of methanol : water (30 : 70v/v). Further dilution was done using the corresponding mobile phase to prepare working solutions of concentration 40 μg mL<sup>-1</sup>. All solutions were stored at 4°C and were stable for 3 weeks.

### Procedure

#### Chromatographic conditions

Mixture A. the mobile phase consisted of acetonitrile and phosphate buffer (pH 3.5) in the ratio of 15 : 85 (v/v). The mobile

phases were filtered using 0.45- $\mu\text{m}$  Millipore membrane filter (Billerica, MA, USA). The injection volume was 5  $\mu\text{L}$ , and the flow rate was 1  $\text{mL min}^{-1}$  at ambient temperature on the RRLC column (2.6  $\mu\text{m}$  particle size), whereas the injection volume was 2  $\mu\text{L}$  and the flow rate was 0.4  $\text{mL min}^{-1}$  at 35°C on the UPLC column (1.7  $\mu\text{m}$  particle size). UV detection was done at 230 nm.

Mixture B: the mobile phase consisted of acetonitrile and 0.1% *o*-phosphoric acid 85% in the ratio of 25 : 75 (v/v). The injection volume was 20  $\mu\text{L}$  and the flow rate was 1.5  $\text{mL min}^{-1}$  at 40°C on the HPLC column (5  $\mu\text{m}$  particle size), whereas the injection volume was 5  $\mu\text{L}$  and the flow rate was 1  $\text{mL min}^{-1}$  at ambient temperature on the RRLC column (2.6  $\mu\text{m}$  particle size). UV detection was done at 230 nm.

### System suitability

The mentioned injection volumes of the working solutions were injected and applied to the chromatographic conditions. The system suitability parameters were calculated according to the USP guidelines (34).

**Table I**

The Specifications of the Chromatographic Columns Used in the Proposed Chromatographic Methods

	HPLC	RRLC	UPLC
Particle size	Fully porous 5 $\mu\text{m}$	Superficially porous 2.6 $\mu\text{m}$	Superficially porous 1.7 $\mu\text{m}$
Apparatus	Agilent 1290 infinity series	Agilent 1290 infinity series	Waters Acquity UPLC system
Column (length $\times$ internal diameter in mm)	Zorbax SB-C <sub>18</sub> (150 $\times$ 4.6)	Kinetex C <sub>18</sub> (100 $\times$ 4.6)	Kinetex C <sub>18</sub> (50 $\times$ 2.1)
Column temperature	40°C	25°C	35°C
Injection volume ( $\mu\text{L}$ )	20	5	2
Flow rate ( $\text{mL min}^{-1}$ )	1.5	1	0.4
Operating pressure (bar)	150	220	550
Total run time (min)	6.5 <sup>a</sup>	5.5 <sup>a</sup> , 7.0 <sup>b</sup>	4.0 <sup>b</sup>

<sup>a</sup>Runtime for mixture B.

<sup>b</sup>Runtime for mixture A.

### Construction of calibration curves

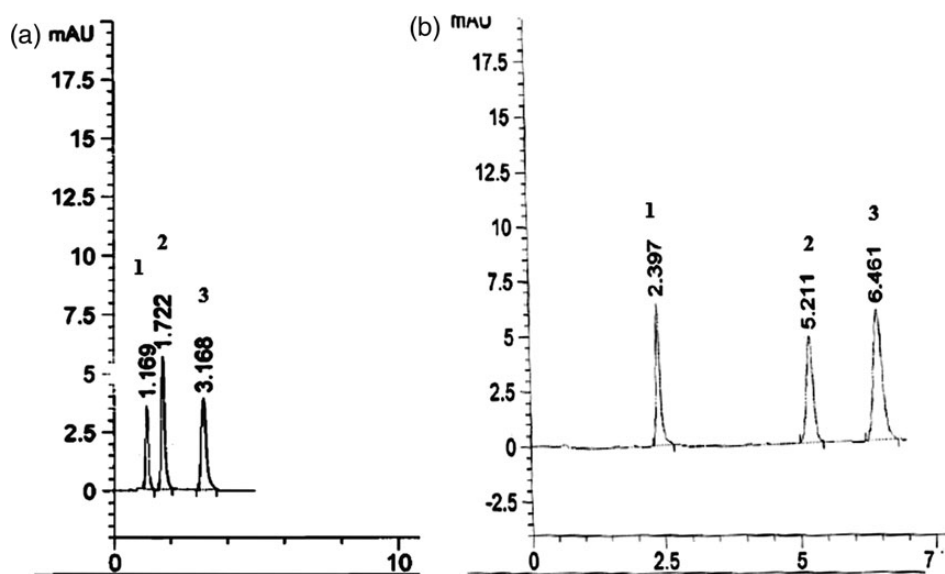
Separate aliquots were transferred from the working solution of each drug to prepare solutions of different concentrations. The corresponding chromatographic conditions of each method were adopted for these solutions and the chromatograms were recorded. The calibration curve of each drug was constructed by plotting the relative peak area (the peak area found to be that of an external standard of the same drug) against the corresponding concentration, from which the regression equations were calculated. The external standard was chosen to be the lowest concentration in the linearity range for each method. The calibration curves were constructed using the average of three experiments.

### Application to pharmaceutical dosage forms

**Orchadexoline<sup>®</sup> eye drops:** One milliliter of the eye drops was transferred into a 25-mL volumetric flask, and the volume was completed with mobile phase to get 200  $\mu\text{g mL}^{-1}$  of CHL, 40  $\mu\text{g mL}^{-1}$  of DXM and 10  $\mu\text{g mL}^{-1}$  of TZH. An appropriate dilution was made with the corresponding mobile phase to prepare the working solution to obtain a solution of 20  $\mu\text{g mL}^{-1}$  of CHL, 4  $\mu\text{g mL}^{-1}$  of DXM and 1  $\mu\text{g mL}^{-1}$  of TZH.

**Loxtra<sup>®</sup> eye drops:** Two milliliters of the eye drops were transferred into a 25-mL volumetric flask, and the volume was completed with mobile phase to get 240  $\mu\text{g mL}^{-1}$  of OFX, 160  $\mu\text{g mL}^{-1}$  of PRD and 32  $\mu\text{g mL}^{-1}$  of TZH. An appropriate dilution was made with the corresponding mobile phase to prepare the working solution to obtain a solution of 12  $\mu\text{g mL}^{-1}$  of OFX, 8  $\mu\text{g mL}^{-1}$  of PRD and 1.6  $\mu\text{g mL}^{-1}$  of TZH.

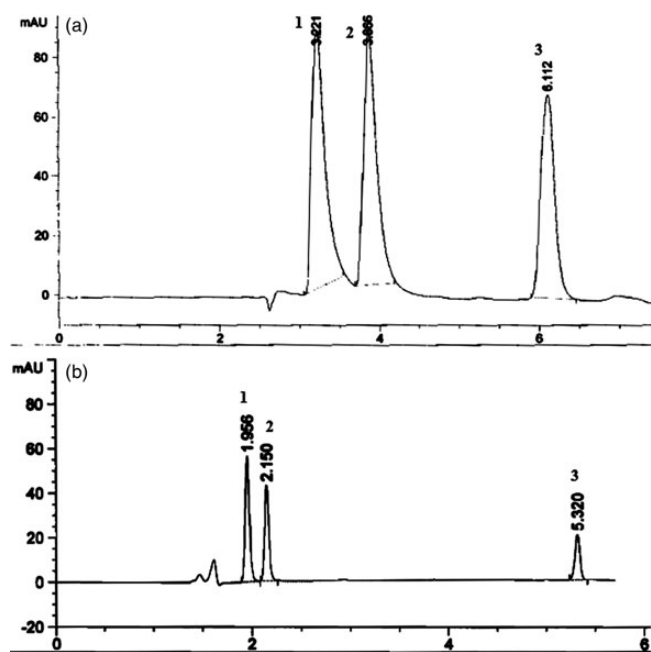
The corresponding chromatographic conditions for mixtures A and B were applied for the previously prepared working solutions. Six replicates of each experiment were done. The concentration of each drug was calculated from its corresponding regression equation. The standard addition technique was applied by adding different known concentrations of pure standard drugs to the pharmaceutical formulation before proceeding in the previously mentioned methods.



**Figure 2.** The chromatograms of separation of mixture A: (a) using UPLC method and (b) using RRLC method. 1, TZH; 2, CHL; 3, DXM.

## Results

By using smaller particles in column packing, speed and peak capacity are extended to new limits. Upon using UPLC, the method will be more efficient with smaller particle-sized columns, and it is now possible to run higher resolution methods, using shorter columns and smaller size of packing particles, with higher flow rates under high pressure. Because most pharmaceutical manufacturers try to reduce the research and development costs and time, faster and higher UPLC separation can decrease the time for method development in research and development laboratories. A dramatic decrease in solvent consumption is also achieved by using UPLC systems with the decrease in column re-equilibration time. The injection volume is nearly 10 times smaller in UPLC than HPLC. Unfortunately, many laboratory budgets do not allow the purchase of new UPLC systems. The RRLC method is considered to be a solution for this problem, as it acquires some of UPLC's advantages, such as shortening run time, saving solvents and giving higher resolution than traditional HPLC methods. Yet it can be operated using the occasional HPLC systems with a maximum backpressure of 400 bar.



**Figure 3.** The chromatograms for separation of mixture B: (a) using HPLC method and (b) using RRLC method. 1, OFX; 2, TZH; 3, PRD.

The aim of this work was, first, developing two novel liquid chromatographic methods for the separation of mixture A for the first time; second, enhancing the separation of mixture B through decreasing its runtime.

This work illustrated the effect of the column dimensions and particle size on the efficiency of resolution of different chromatographic columns on the separation of two ternary mixtures. To optimize the proposed methods, it was necessary to test the effect of different variables. The mobile phases of different compositions, flow rates and change in column temperature have been tested. The specification of the chromatographic columns used is listed in Table I.

### Mixture A

No HPLC method has been reported for the separation of this mixture. In order to separate this mixture, the HPLC column has been tested using the same mobile phase, but the run time exceeded 15 min and poor separation was achieved for DXM. Therefore, RRLC was developed and a mobile phase of acetonitrile and phosphate buffer (pH 3.5) in the ratio of 15 : 85 (v/v) was delivered at 1 mL min<sup>-1</sup> at ambient temperature. By applying the previous conditions, the mixture was separated with acceptable resolution and robustness, where the retention times for TZH, CHL and DXM were 2.40, 5.21 and 6.50 min, respectively, as shown in Figure 2b.

When the UPLC column was attached to the Agilent HPLC instrument, and by running the mobile phase through the system at 0.2 mL min<sup>-1</sup>, the backpressure was raised up to 400 bar, which lead to the automatic stop of the system. Therefore, in order to develop the UPLC method, the UPLC column was attached to special Waters UPLC instrument and the same mobile phase was delivered at a maximum flow rate of 0.4 mL min<sup>-1</sup> at 35°C, where the retention times for TZH, CHL and DXM were 1.17, 1.72 and 3.17 min, respectively, as shown in Figure 2a. The run time is considered to be half the time taken by RRLC, but the disadvantage is the need of a special UPLC instrumentation capable of resisting higher backpressure.

### Mixture B

The HPLC method (32) was reported for the separation of this mixture, but the run took 10 min. In order to enhance the separation of this mixture, another HPLC was developed with shorter run time. The HPLC column was thermostated at 40°C and the mobile phase used was acetonitrile and 0.1% *o*-phosphoric acid

**Table II**  
Statistical Analysis of Parameters Required for System Suitability of the Proposed Chromatographic Methods

Parameter	Mixture A						Mixture B						Reference USP value
	UPLC			RRLC			HPLC			RRLC			
	TZH	CHL	DXM	TZH	CHL	DXM	OFX	TZH	PRD	OFX	TZH	PRD	
$t_R$ (retention time)	1.17	1.72	3.17	2.40	5.21	6.50	3.21	3.87	6.11	1.96	2.15	5.32	
$n$ (column efficiency)	2,009	2,800	4,019	4,926	7,960	7,073	2,093	3,042	6,084	9,573	11,219	48,829	$n > 2,000$
HETP (height equivalent to theoretical plates)	0.025	0.018	0.012	0.020	0.013	0.014	0.07	0.05	0.02	0.01	0.008	0.002	The smaller the value, the higher the column efficiency
$K'$ (capacity factor)	1.13	2.13	4.76	1.47	4.37	5.7	1.14	1.59	3.07	1.02	1.22	4.51	1–10 acceptable
$\alpha$ (separation factor)		1.88	2.23		2.97	1.30		1.39	1.93		1.20	3.70	$> 1$
$T$ (tailing factor)	1.01	0.91	0.95	0.85	0.96	0.88	0.72	1.00	0.82	0.85	0.86	0.99	$T \leq 2$ $T = 1$ for symmetric peak
$R_s$ (experimental resolution)		4.58	8.79		15.13	4.66		1.73	5.72		2.41	35.66	$R_s > 1.5$



85% in the ratio of 25 : 75 (v/v) delivered at 1.5 mL min<sup>-1</sup>. By applying the previous conditions, the retention times for OFX, THZ and PRD were found to be 3.21, 3.87 and 6.11 min, respectively, which is considered to be time saving than the reported method with 35%. Any minor change in the acetonitrile proportion or increasing the flow rate affected the robustness of the method.

Through using the RRLC column with the previous chromatographic conditions of the HPLC column, poor resolution was achieved for the three drugs. So a modification was done to the chromatographic conditions with the same mobile phase delivered at 1 mL min<sup>-1</sup> at ambient temperature, the retention times for OFX, THZ and PRD were 1.96, 2.15 and 5.32 min, respectively. This method is considered to be time saving than the reported method by 55%. This method showed higher efficiency values (*n*) and better robustness by applying minor changes to the mobile phase composition, where the resolution between the peaks of OFX and TZH was kept constant. Any further increase in the flow rate caused the backpressure to exceed the accepted limit. The chromatograms for both methods are shown in Figure 3.

## Discussion

The resolution of the separated peaks and the run time are related to the column particle size and column length. Shorter columns are expected to give short run time but with poorer resolution, and smaller particle sizes are expected to give better resolution but with higher backpressure. So, RRLC methods compromised between the conventional HPLC and UPLC methods to give good resolution with relatively short run time. In contrast to UPLC, the RRLC column was performed with the conventional HPLC instruments. Accordingly, in the laboratory, there is no need to replace the conventional HPLC unit with a new UPLC unit in order to achieve fast chromatographic analysis; just replacing the column will do, which makes the RRLC more practical, economic and yet of good resolving power than the conventional HPLC methods.

The detection was done at 230 nm for both mixtures to overcome the interference of the added preservative benzalkonium chloride ( $\lambda_{\max}$  210 nm). The temperatures were adjusted to the mentioned values in the HPLC and UPLC method to obtain optimum separation. System suitability parameters for the proposed methods adopted for mixtures A and B were calculated, as shown in Table II. The assay sheet and validation parameters are listed in Table III.

### Application to pharmaceutical dosage forms

The suggested proposed methods were valid and applicable for the analysis of the ternary mixtures present in both eye drop preparations. The validity of the proposed methods was further assessed by applying the standard addition technique, which showed accurate results. The results confirm the suitability of the proposed methods for the routine determination of these components in their combined formulations. The results are shown in Table IV.

### Methods validation

Method validation was performed according to the ICH guidelines (35) for all the proposed methods.

**Table III**  
Assay Parameters and Validation Sheet Obtained by Applying the Proposed Chromatographic Methods

Parameters	Mixture A					Mixture B							
	UPLC			RRLC		HPLC			RRLC				
	CHL	DXM	TZH	TZH	CHL	DXM	TZH	OFX	PRD	TZH	OFX	PRD	TZH
Calibration range <sup>a</sup> (µg/mL)	4–30	1–15	1–12	1–15	4–30	1–15	1–15	2–15	3–15	1–15	2–15	1–14	1–12
Slope	0.2198	1.3346	1.0184	1.0971	0.2199	1.0971	0.8043	0.8043	0.2776	0.7133	0.1806	1.1596	0.7274
Intercept	0.1489	0.1988	0.1820	-0.1700	0.2106	-0.1700	-0.6974	-0.6974	0.3813	0.0273	0.6897	-0.1813	0.6087
Correlation coefficient ( <i>r</i> )	0.9999	0.9998	0.9998	0.9998	0.9998	0.9998	0.9999	0.9999	0.9998	0.9998	0.9996	0.9999	0.9998
Mean <sup>a</sup>	99.88	100.36	100.52	99.86	100.32	99.86	99.90	99.90	100.06	100.29	100.16	99.88	100.09
RSD (%)	1.059	1.214	1.324	0.625	1.116	0.625	0.650	0.650	0.796	0.765	1.213	0.519	1.337
Accuracy <sup>a,b</sup>	100.33 ± 1.270	100.35 ± 0.891	100.48 ± 1.375	99.68 ± 0.733	99.58 ± 0.818	99.68 ± 0.733	99.46 ± 0.343	100.58 ± 1.000	100.28 ± 0.718	100.58 ± 1.000	100.36 ± 0.683	99.37 ± 0.804	100.18 ± 1.287
Repeatability <sup>a,c</sup>	0.765	0.911	0.823	0.614	0.766	0.614	0.763	0.611	0.921	0.592	0.611	0.811	0.915
Interday precision <sup>a,c</sup>	0.691	0.839	0.744	0.864	1.119	0.864	0.812	0.693	1.003	1.001	0.693	1.130	0.955
Robustness <sup>a,c</sup>	101.12–0.923	100.66–0.599	101.21–1.697	99.99–1.081	99.81–0.712	99.99–1.081	97.03–1.723	96.83–1.852	100.21–1.751	96.83–1.852	100.39–0.612	99.91–0.712	100.65–1.117

<sup>a</sup>Average of three experiments.

<sup>b</sup>Mean ± SD of five concentrations of each drug.

<sup>c</sup>FSD of three concentrations of each drug (4, 6 and 8 µg/mL).

**Table IV**Application of the Standard Addition Technique to the Analysis of Loxtra<sup>®</sup> and Orhadexoline<sup>®</sup> Eye Drops by the Proposed Chromatographic Methods

Orhadexoline <sup>®</sup>	CHL				DXM				TZH												
	Found in $\mu\text{g mL}^{-1\text{a}}$	Recovery (%)	Added in $\mu\text{g mL}^{-1}$	Recovery (%)	Found in $\mu\text{g mL}^{-1\text{b}}$	Recovery (%)	Added in $\mu\text{g mL}^{-1}$	Recovery (%)	Found in $\mu\text{g mL}^{-1\text{c}}$	Recovery (%)	Added in $\mu\text{g mL}^{-1}$	Recovery (%)									
UPLC	20.05	$100.25 \pm 0.300$	4	101.50	4.05	$101.33 \pm 1.665$	4	100.5	1.00	$99.97 \pm 0.896$	4	101.50									
			6	101.67				6				99.83	6	100.17							
			8	99.88				8				101.50	8	100.13							
			$101.01 \pm 0.989$					$100.61 \pm 0.839$													
			RRLC	20.02				$100.08 \pm 0.451$				4	99.50	4.03	$100.75 \pm 1.250$	4	99.50	0.99	$99.23 \pm 0.666$	4	100.25
												6	100.83			6	101.50			6	100.17
8	101.38	8			100.25	8	101.38														
$100.57 \pm 0.965$				$100.42 \pm 1.010$				$100.59 \pm 0.675$													
Loxtra <sup>®</sup>	OFX				PRD				TZH												
	Found in $\mu\text{g mL}^{-1\text{d}}$	Recovery (%)	Added in $\mu\text{g mL}^{-1}$	Recovery (%)	Found in $\mu\text{g mL}^{-1\text{e}}$	Recovery (%)	Added in $\mu\text{g mL}^{-1}$	Recovery (%)	Found in $\mu\text{g mL}^{-1\text{f}}$	Recovery (%)	Added in $\mu\text{g mL}^{-1}$	Recovery (%)									
HPLC	12.04	$100.29 \pm 0.959$	2	99.50	8.02	$100.29 \pm 0.959$	2	99.50	1.58	$99.06 \pm 1.654$	2	101.50									
			2.5	101.20			4	101.00				4	99.75								
			3	99.67			6	99.17				6	101.50								
			$100.12 \pm 0.937$				$99.89 \pm 0.977$					$100.92 \pm 1.010$									
			RRLC	12.10			$100.81 \pm 0.875$	2				100.50	8.01	$100.08 \pm 0.315$	2	99.01	1.59	$99.46 \pm 1.170$	2	102.00	
								2.5				99.60			4	99.50			4	100.50	
3	99.67	6			101.83	6		101.83													
$99.92 \pm 0.501$				$100.11 \pm 1.512$				$101.44 \pm 0.822$													

<sup>a</sup>CHL claimed to be equivalent to 20  $\mu\text{g mL}^{-1}$ .<sup>b</sup>DXM claimed to be equivalent to 4  $\mu\text{g mL}^{-1}$ .<sup>c</sup>TZH claimed to be equivalent to 1  $\mu\text{g mL}^{-1}$ .<sup>d</sup>OFX claimed to be equivalent to 12  $\mu\text{g mL}^{-1}$ .<sup>e</sup>PRD claimed to be equivalent to 8  $\mu\text{g mL}^{-1}$ .<sup>f</sup>TZH claimed to be equivalent to 1.6  $\mu\text{g mL}^{-1}$ .

### Range and linearity

The linearity of the proposed methods was evaluated by processing the different calibration curves on three different days. The calibration curves were constructed within concentration ranges that were selected on the basis of the anticipated drug concentration during the assay of the dosage form. The corresponding assay parameters and validation sheet for the proposed methods are listed in Table III.

### Limit of detection and limit of quantification

The LOD and limit of quantification were calculated for each drug using the proposed methods with a ratio of 3.3 and 10 of standard deviations of the blank, respectively, and the slope of the calibration line, as shown in Table III.

### Accuracy

To study the accuracy of the proposed methods, procedures under linearity were repeated three times for the determination of different blind concentrations of pure drugs. The accuracy expressed as percentage recoveries  $\pm$  standard deviation is shown in Table III. The interference of excipients in the pharmaceutical formulations was studied by applying the standard addition method to the pharmaceutical formulation. Good accuracy proved that the excipients in pharmaceutical formulations did not interfere in the analysis of these compounds, as shown in Table IV.

### Precision

The precision of the proposed methods, expressed as relative standard deviation (RSD), was determined by the analysis of three different concentrations of pure drugs within the linearity

range. The intraday precision was assessed from the results of three replicate analyses of three pure drug samples on a single day. The interday precision was determined from the same samples analyzed on three consecutive days. The results are illustrated in Table III.

### Robustness

The robustness of the proposed methods was investigated by the analysis of samples under a variety of experimental conditions. A small change in proportions of acetonitrile by up to  $\pm 2\%$  was introduced to the mobile phases. A slight change in the retention time and peak parameters was observed for UPLC and RRLC; however, the peak areas were conserved. For the HPLC method adopted for mixture B, this slight change in acetonitrile proportion led to the overlap of OFX and TZH peaks, where the experimental resolution ( $R_s$ ) was found to be  $< 1.5$ .

The effect of robustness is shown in Table III, which proved that the RRLC method was more robust than the conventional HPLC method for mixture B upon changing the experimental conditions.

### Statistical analysis

The results obtained by the proposed methods for the determination of pure samples of the cited drugs were statistically compared with those obtained by the official methods (33). The values of the calculated  $t$  and  $F$  were less than the corresponding tabulated ones, which revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the official ones as shown in Table V.

**Table V**

Statistical Comparison Between the Results Obtained by the Proposed Method and the Official BP Methods for the Determination of the Cited Drugs in Their Pure Powder Form

Items	OFX			PRD			CHL		
	HPLC	RRLC	Official method <sup>a</sup>	HPLC	RRLC	Official method <sup>a</sup>	UPLC	RRLC	Official method <sup>a</sup>
Mean (%)	99.90	100.16	99.68	100.06	99.88	100.26	99.68	100.32	100.17
Variance	0.422	1.474	0.624	0.634	0.269	0.696	1.121	1.246	0.386
SEM <sup>b</sup>	0.247	0.459	0.352	0.301	0.196	0.373	0.374	0.422	0.277
<i>n</i>	7	7	5	7	7	5	8	7	5
Student's <i>t</i> -test (2.228) <sup>c</sup>	0.279	0.769		0.413	0.977		0.938 (2.201) <sup>c</sup>	0.253	
<i>F</i> -value (6.163) <sup>d</sup>	1.454	2.373		1.101 (4.534) <sup>d</sup>	2.590 (4.534) <sup>d</sup>		2.906 (4.120) <sup>d</sup>	3.227	
	DXM			TZH					
	UPLC	RRLC	Official method <sup>a</sup>	HPLC	RRLC	UPLC	RRLC	Official method <sup>a</sup>	
Mean (%)	100.07	99.86	99.94	100.29	100.09	100.52	100.10	100.06	
Variance	0.904	0.391	0.180	0.585	1.785	1.753	0.584	0.353	
SEM <sup>b</sup>	0.359	0.221	0.189	0.289	0.505	0.500	0.289	0.266	
<i>n</i>	7	8	5	7	7	7	7	5	
Student's <i>t</i> -test (2.228) <sup>c</sup>	0.295	0.249 (2.201) <sup>c</sup>		0.571	0.047	0.715	0.089		
<i>F</i> -value (6.163) <sup>d</sup>	5.035	2.175 (4.120) <sup>d</sup>		1.662	5.066	4.975	1.657		

<sup>a</sup>BP methods for CHL, DXM and PRD are zero-order absorption methods, while for OFX and TZH it is a potentiometric titration method.<sup>b</sup>SEM, Standard error of mean.<sup>c</sup>The corresponding tabulated values of *t* at *P* = 0.05.<sup>d</sup>The corresponding tabulated values of *F* at *P* = 0.05.

## Conclusion

RRLC, UPLC and conventional HPLC were developed and validated for the separation of ternary mixtures present in ophthalmic preparations. RRLC columns compromised between the conventional HPLC and UPLC methods to give good resolution with relatively short run time. In contrast to UPLC, the RRLC column was performed with the conventional HPLC instruments. Accordingly, there is no need to replace the conventional HPLC unit with a new UPLC unit in order to achieve fast chromatographic analysis, which makes the RRLC more practical and economic and yet of good resolving power than the conventional HPLC methods.

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