Fast separation and quantification of three anti-glaucoma drugs by high-performance liquid chromatography UV detection

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Abstract

In this study, a simple and accurate high-performance liquid chromatography method was developed and validated for fast separation of three anti-glaucoma drugs: timolol maleate (TM), brimonidine tartrate (BM), and latanoprost (LP). Separation of the three drugs was achieved in < 6 minutes using a BDS Hypersil phenyl column and a mobile phase consisting of acetonitrile: 25 mM phosphate buffer, pH 4.0 (50: 50, v/v) at 1.2 mL/min with UV detection at 210 nm. The method was linear over the concentration ranges of 5.0–200.0 µg/mL, 2.0–80.0 µg/mL and 1.0–25.0 µg/mL with lower detection limits of 0.21 µg/mL, 0.10 µg/mL and 0.11 µg/mL for TM, BM and LP, respectively. The method was applied for the determination of two fixed-dose combination eye drops for the treatment of glaucoma, containing TM together with either BM or LP. Commercial samples of single-ingredient ophthalmic solutions containing the studied drugs were also successfully analyzed. The results obtained by the proposed method were favorably compared with those obtained by the comparison methods using Student's t test and the variance ratio F test.

1. Introduction

Glaucoma is a neurodegenerative ocular disorder associated with distinct changes in the optic nerve head and retinal nerve fiber layer. An increase in the number of patients with glaucoma to ~80 million by 2020 is expected by the World Health Organization [1]. At present, many therapeutic options have been adopted for the treatment of glaucoma, including selective and nonselective β-blockers, carbonic anhydrase inhibitors, prostaglandin analogs, adrenergic agonists, and cholinergic agonists. Among the different drugs for the treatment of glaucoma, timolol maleate (TM), brimonidine tartrate (BM), and latanoprost (LP) are commonly administered either as a single agent or as combined therapy [2].

TM is defined chemically as 2-propanol, 1-(1,1-dimethylethyl)amino-3-[[4-(4-morpholinyl)-1,2,5-thiadiazole-3-yl]-(S)-, (Z)-2-butenedioate (1:1) (salt)). TM is a non-cardioselective β-blocker without intrinsic sympathomimetic or membrane-stabilizing action. It treats glaucoma by inhibition of β-adrenergic receptors in the ciliary epithelium and...
reduction of aqueous humor secretion [3]. BM, 5-bromo-6-(2-imidazolin-2-ylamino) quinoxaline D-tartrate, is an \( \alpha_2 \)-adrenoceptor agonist that decreases the intraocular pressure by reducing the production of aqueous humor [3], while LP (isopropyl(Z)-7-{(1R,2R,3R,5S)-3,5-dihydroxy-2-[(3R)-3-hydroxy-5-phenyl-pentyl]cyclopentyl}-5-heptenoate), is a synthetic prostaglandin \( \text{F}_2 \alpha \) analog that lowers the intraocular pressure by increasing the uveoscleral outflow [3]. The combined therapy of TM with either BM or LP is effective for management of glaucoma and ocular hypertension [3]. The structural formulas of the three compounds are presented in Fig. 1.

The United States Pharmacopoeia [4] and the British Pharmacopoeia [5] recommend titrimetric methods for TM determination in pure form with acetous perchloric acid as a titrant and potentiometric detection of the end point. The United States Pharmacopoeia [4] determined it in tablets and eye drops using HPLC methods, while the British Pharmacopoeia [5] described direct spectrophotometric assay for it in tablets and eye drops, and HPLC for its combination eye drops with dorzolamide. A number of analytical methods determined TM either alone or with other drugs including; spectrophotometry [6–9], high-performance thin layer chromatography (HPTLC) [10], liquid chromatography (LC) [11–19], and capillary electrophoresis [20]. For BM, it was determined by some analytical methods such as spectrophotometry [21–23], spectrofluorimetry [23], LC [24–26], gas chromatography/mass spectrometry [27], and capillary electrophoresis [28]. As for LP, few analytical methods have been published for its determination, such as HPLC [29].

Some analytical methods are available for the assay of coformulated ophthalmic solutions containing TM/BM or TM/LP mixtures. LC [30–32], spectrophotometry [32–34], and HPTLC [35] methods are reported for the simultaneous assay of TM and BM in eye drops. Some HPLC methods [36–38] are also reported for the determination of TM/LP mixtures. These methods have some weaknesses such as poor sensitivity [30,31,33–37], narrow linearity ranges [30,31,33–35,38], poor column efficiency [32], and need for time-programmed UV detection [32,37,38], column-temperature control [37], or gradient elution [38].

Hence, we initiated the present study to develop and validate a simple, rapid and sensitive HPLC method for the separation and quantification of TM, BM and LP. Simultaneous assay of the commonly prescribed anti-glaucoma drugs using the same separation conditions is suitable for routine pharmaceutical analysis in quality control laboratories.

2. Materials and Methods

2.1. Instrumentation

The HPLC system (Shimadzu Corporation, Kyoto, Japan) was fitted with an LC-20AD chromatograph, a Rheodyne injector valve with 20-\( \mu \)L sample loop, a SPD-20A UV-Visible detector, and a DGU-20A5 online solvent degasser. The instrument was interfaced to a computer for data acquisition with a CBM-20A communication bus module. A Consort P-901 pH-meter (Turnhout, Belgium) and a Sonix IV SS 101 H 230 ultrasonic bath (Charleston, SC, USA) were used.

2.2. Chemicals and reagents

TM (batch #TML0334201205) and BM (batch #RK12BRT007) pure samples were gifts from EIPICO (Tenth of Ramadan City, Egypt). LP pure solution (10.0 mg oil) was purchased from Tokyo Chemical Industries (Tokyo, Japan). Purities of the samples were found to be 99.45%, 100.18% and 99.25% for TM, BM and LP, respectively, as determined by the comparison methods [30,36]. Maleic acid (99.0%) and potassium dihydrogen phosphate were obtained from Adwic (Cairo, Egypt). Acetonitrile and methanol (HPLC grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Orthophosphoric acid (85%, w/v) was obtained from Riedel-deHäen (Seelze,
Germany). Water purified by filtration with 0.45-μm Millipore membrane filter was used in this study.

2.3. Pharmaceutical samples

The following pharmaceutical formulations were purchased from Egyptian pharmacies: Combigan eye drops (labeled to contain 2 mg/mL BM + 5 mg/mL timolol equivalent to 6.8 mg/mL TM) (Allergan Pharmaceuticals, Westport, Ireland); Xalacom eye drops (labeled to contain 50 mg/mL LP + 5 mg/mL timolol equivalent to 6.8 mg/mL TM) (Pfizer Manufacturing, Belgium); Timolol eye drops (labeled to contain 0.5% timolol equivalent to 0.68% TM) (EIPICO); Alphagan eye drops (labeled to contain 0.15% BM) (Allergan Pharmaceuticals); and Ioprost eye drops (labeled to contain 50 mg/mL LP) (Orchidia Pharmaceuticals, Cairo, Egypt).

2.4. HPLC conditions

A BDS Hypersil phenyl column (4.6 mm × 250 mm, 5-μm particle size), from Thermo Electron Corporation (Runcorn, UK), was used with a mobile phase consisting of acetonitrile and 25mM phosphate buffer, pH 4.0, in the ratio of 50:50, v/v. The mobile phase was filtered with a 0.45-μm Millipore membrane filter and degassed by sonication for 30 minutes before pumping at 1.2 mL/min. UV detection was set at 210 nm.

2.5. Standard solutions

An amount of 20.0 mg TM and BM were individually weighed, transferred to 100-mL volumetric flasks, and dissolved in methanol. The volumes were completed to the mark with the same solvent to prepare the standard solutions (200.0 μg/mL). LP pure solution was diluted with methanol to obtain a standard solution with a concentration of 100.0 μg/mL. The standard solutions were stable for at least 7 days when kept in a refrigerator at 4°C.

2.6. Calibration graphs

The standard solutions of the studied drugs were diluted with the mobile phase to prepare working solutions containing 5.0–200.0 μg/mL, 2.0–80.0 μg/mL and 1.0–25.0 μg/mL TM, BM and LP, respectively. The solutions were well mixed, and 20-μL injections were made in triplicate and eluted under the optimum chromatographic conditions. The calibration graphs were obtained by plotting the average peak areas of each drug versus the corresponding concentrations and the regression equations were derived.

2.7. Analysis of laboratory-prepared mixtures of TM/BM and TM/LP

Laboratory-prepared mixtures containing TM/BM and TM/LP mixtures in the recommended pharmaceutical ratios of 6.8:2 and 136:1, respectively (as in their coformulated eye drops), were prepared in the mobile phase. Triplicate 20-μL injections of each solution were made. The average percentage found for each drug was determined using the corresponding regression equation.

2.8. Analysis of ophthalmic solutions

One milliliter of each eye drop formulation was transferred to 10-mL volumetric flasks and diluted to a final volume with high-purity water. Appropriate volumes of each eye drop solution were transferred into a set of 10-mL volumetric flasks and made up to the final volume with the mobile phase. Solutions were well mixed, triplicate 20-μL injections were made, and eluted under the optimum chromatographic conditions. The nominal concentration of each drug was calculated from the regression equation.

3. Results

3.1. Method development and optimization

Different chromatographic conditions were studied for separation of TM, BM and LP. The most important aspects in HPLC method development are the achievement of good resolution and peak symmetry in a reasonable analysis time with appropriate sensitivity. Detection wavelength, mobile phase composition, pH, and flow rate were carefully optimized. Good separation of TM, BM, LP and maleic acid, which is the salt part of TM, was attained within a short run time (< 6 minutes) using a mobile phase consisting of acetonitrile: 25mM phosphate buffer, pH 4.0, (50:50, v/v) at 1.2 mL/min with UV detection at 210 nm. Fig. 2 illustrates a typical chromatogram for the separation of the three analytes, where LP, TM and BM were eluted at 3.1, 4.1 and 4.9 minutes, respectively, without interference from maleic acid (tR = 2.1 min).

3.2. Method validation

Validation procedure was carried out according to International Conference on Harmonization (ICH) Guidelines [39].
3.2.1. Linearity and concentration range
To establish the linearity of the proposed method, calibration graphs were constructed using sets of standard solutions at seven concentration levels for each drug. The data were statistically analyzed [40] and the results are illustrated in Table 1.

### Table 1 – Collective calibration data for the studied drugs by the proposed method.

| Parameter                      | TM  | BM  | LP  |
|--------------------------------|-----|-----|-----|
| Concentration range (µg/mL)    | 5.0–200.0 | 2.0–80.0 | 1.0–25.0 |
| Limit of detection (µg/mL)     | 0.21 | 0.10 | 0.06 |
| Limit of quantification (µg/mL) | 0.65 | 0.29 | 0.19 |
| Correlation coefficient (r)    | 0.9999 | 0.9999 | 0.9999 |
| Slope                          | 9.64 × 10^4 | 6.51 × 10^4 | 3.97 × 10^4 |
| Intercept                      | −3.93 × 10^4 | −3.70 × 10^4 | 966 |
| Standard deviation of the residuals (Sₚₑ) | 4.03 × 10^3 | 1.23 × 10^3 | 261 |
| Standard deviation of the intercept (Sₙ) | 622 | 1.90 × 10^3 | 77 |
| Standard deviation of the slope (Sₘ) | 23.97 | 183.30 | 11.92 |
| %RSD                           | 1.19 | 1.50 | 1.31 |
| % Error                        | 0.45 | 0.57 | 0.50 |

BM = brimonidine tartrate; LP = latanoprost; TM = timolol maleate.

3.2.2. Limits of quantification and detection
Limit of quantification and limit of detection were calculated according to ICH Guidelines [39] using the method of standard deviation (SD) of the intercept of the regression line and the slope. Table 1 illustrates the obtained results.

3.2.3. Accuracy
The developed method was tested for the accuracy by analyzing pure samples of TM, BM and LP in triplicate over the working concentration ranges of 5.0–200.0 µg/mL, 2.0–80.0 µg/mL and 1.0–25.0 µg/mL, respectively (n = 7 for each compound). The average percentage found (± SD) were 100.26 ± 1.20%, 100.51 ± 1.51% and 100.09 ± 1.31% for TM, BM and LP, respectively. The results were compared with those obtained using the comparison methods [30,36] (99.45 ± 1.25%, 100.18 ± 1.02% and 99.25 ± 0.85% for TM, BM and LP, respectively) by applying Student’s t test and the variance ratio F test [40]. In all cases, the calculated t and F values were lower than the tabulated values.

3.2.4. Precision
Intra-day precision was tested by the analysis of three concentrations of each compound three times within the same day. Inter-day precision was also considered by the analysis of three concentrations of each compound in three successive days. The results of precision study are shown in Table 2.

3.2.5. Selectivity
The selectivity of the method was tested by the analysis of laboratory-prepared mixtures of the studied drugs in the ratio of 6.8:2 and 136:1 for TM/BM and TM/LP, respectively. The average percentages found ± SD for TM and BM in their mixture were 100.14 ± 0.28% and 100.44 ± 0.70%, respectively, and those for TM and LP in their mixture were 100.12 ± 0.34% and 100.66 ± 0.94%, respectively.

3.2.6. Robustness
To prove the robustness of the method, small changes were made in the percentage of acetonitrile (50 ± 1%, v/v), molar concentration of phosphate buffer (25 ± 1mM) and the flow rate (1.2 ± 0.1 mL/min). No significant changes in theoretical plates count (NTP), resolution factor (Rₛ), or tailing factor (T) were observed under these conditions.

3.2.7. System suitability testing
System suitability parameters were evaluated so as to prove the system performance using working solutions of TM, BM and LP. Parameters including NTP, Rₛ and T were calculated and illustrated in Table 3.

### 3.3. Pharmaceutical application
Applicability of the method was confirmed by the analysis of commercially available coformulated ophthalmic solutions containing fixed-dose combinations of TM/BM and TM/LP (Table 4). Additionally, single-component ophthalmic solutions containing the three compounds were analyzed (Table 4). Fig. 3 shows typical chromatograms for the determination of the three drugs in different ophthalmic solutions.

## 4. Discussion
### 4.1. Method development and optimization
For the choice of optimum detection wavelength, different wavelengths were investigated (210, 254 and 295 nm). LP is a weak UV-absorbing compound that exhibits considerable absorbance only in the middle UV region. In addition, it exists in low concentration in formulation (50 µg/mL eye drops). As a consequence, 210 nm was a suitable wavelength to record all chromatographic peaks showed increased retention in addition to poor resolution. A mobile phase composed of acetonitrile and water (75% v/v) was selected for this wavelength because it has a sharp cutoff of < 210 nm [41].

Several mobile phases were tested using various proportions of different aqueous phases and organic modifiers. Methanol and acetonitrile were tried as organic modifiers, and water and phosphate buffer were investigated as aqueous phases. When using mobile phases containing methanol as an organic modifier or water as an aqueous phase, the chromatographic peaks showed increased retention in addition to poor resolution. A mobile phase composed of acetonitrile and phosphate buffer was selected in further studies.

The ratio of acetonitrile in the mobile phase was studied over the range of 40–65%, v/v. Increasing the concentration of acetonitrile by > 50%, v/v leads to inadequate separation of TM
from BM and LP from maleic acid. So, the proportion of acetonitrile in the mobile phase was kept at 50%, v/v to achieve the best separation within a short time.

The ionic strength of phosphate buffer was also investigated over the concentration range of 10–50mM. With buffer concentrations < 25mM, TM and BM showed peak broadening and increased retention times, whereas, using buffer solutions of concentrations > 25mM resulted in poor resolution between the three drugs. Phosphate buffer at 25mM was finally selected as the optimum concentration.

Furthermore, the pH of phosphate buffer was studied over the range of 3.0–6.5. It was observed that the change in the pH of the buffer solution had an insignificant effect on the retention of the three compounds. This behavior was probably due to the high pKₐ of the three compounds (pKₐ = 9.21, 7.4 [30], and 14.47 [42] for TM, BM and LP, respectively). So, the three compounds are in the cationic forms over the working pH range. Eventually, phosphate buffer at pH 4.0 was used in this study to maintain the durability and lifetime of the column.

For the flow rate optimization, it was studied over the range of 0.8–1.2 mL/min. For rapid routine analysis, a flow rate of 1.2 mL/min was adopted, allowing a total chromatographic run of < 6 minutes, with good resolution of the studied compounds.

It is well known that the selectivity of phenyl column differs from that of the alkyl-silica columns. The retention on phenyl column increases as the 

**Table 2** – Precision data for the three studied drugs by the proposed method.

| Compound | Concentration (µg/mL) | Intra-day precision | Inter-day precision |
|----------|-----------------------|---------------------|---------------------|
|          | % found ± SD | % RSD | % Error | % found ± SD | % RSD | % Error |
| TM       | 5.0         | 100.79±1.03 | 1.03 | 0.59 | 100.10±1.25 | 1.25 | 0.72 |
|          | 25.0        | 99.79±1.44  | 1.45 | 0.84 | 98.84±1.39  | 1.40 | 0.81 |
|          | 100.0       | 100.12±1.14 | 1.14 | 0.66 | 100.17±1.35 | 1.35 | 0.78 |
| BM       | 2.0         | 100.50±0.88 | 0.87 | 0.51 | 99.46±1.33  | 1.33 | 0.77 |
|          | 15.0        | 101.17±1.26 | 1.25 | 0.72 | 99.13±1.44  | 1.45 | 0.84 |
|          | 40.0        | 101.51±0.74 | 0.72 | 0.42 | 100.46±1.33 | 1.32 | 0.76 |
| LP       | 1.0         | 100.84±0.62 | 0.61 | 0.35 | 100.80±0.87 | 0.86 | 0.50 |
|          | 10.0        | 101.46±0.68 | 0.67 | 0.38 | 100.13±0.35 | 0.35 | 0.20 |
|          | 20.0        | 100.80±1.33 | 1.33 | 0.76 | 99.46±0.87  | 0.87 | 0.51 |

BM = brimonidine tartrate; LP = latanoprost; RSD = relative standard deviation; SD = standard deviation; TM = timolol maleate.

| Compound | No. of theoretical plates (NTP) | Tailing factor (T) |
|----------|---------------------------------|-------------------|
| TM       | 2574                            | 1.40              |
| BM       | 2610                            | 1.47              |
| LP       | 2780                            | 1.23              |
| Maleic acid/LP | Resolution (Rₛ) | 1.81 |
| LP/TM    | 3.35                            | 1.75              |
| TM/BM    | 3.39                            | 1.75              |

BM = brimonidine tartrate; LP = latanoprost; RSD = relative standard deviation; SD = standard deviation; TM = timolol maleate.

The performance of the developed method was validated following the ICH Guidelines [39]. Results of the statistical analysis of the data [40] point out to the linearity of the method (Table 1). In addition, the ICH Guidelines were used to calculate the limit of detection and the limit of quantification for the three studied drugs (Table 1).

Accuracy of the proposed method was also assessed. The average percentages found and SD values were satisfactory. By comparing the results obtained by the developed method with those of the comparison HPLC methods [30,36], the accuracy of the proposed method was confirmed since the calculated t and F values were lower than the tabulated ones [40], which indicated no significant differences between the two methods regarding the accuracy and precision, respectively. Results of intra- and inter-day precision showed small values of percentage relative standard deviation (%RSD) not exceeding 1.45%, confirming the precision of the method (Table 2).

Selectivity of the method was confirmed by its ability to separate the drugs in their binary mixtures with satisfactory percentage found and small SD. Moreover, there were no interferences from common excipients with the peaks of the studied drugs or from maleic acid.

Deliberate minor variation in the optimum chromatographic conditions did not significantly affect the NTP, Rs or T of the chromatographic peaks, demonstrating the robustness of the proposed method. The finally calculated system suitability test parameters were satisfactory and within the acceptance values (Table 3).

In comparison with the reported methods for TM/BM mixture, the proposed method was 2 times more sensitive for TM and 5, 2 and 2.5 times more sensitive for BM than the reported HPLC methods [30,31,33, respectively]. While the solutes increase according to the following order: aliphatic < substituted benzenes < polyaromatic hydrocarbons [43]. In addition, the introduction of heteroatoms into the aromatic rings has a pronounced enhancing effect on their π activity [44]. Maleic acid is an aliphatic molecule, thus, it has the lowest π activity and was eluted first, followed by LP (substituted benzene), then TM (aromatic compound with heteroatoms) and BM (polyaromatic hydrocarbon with heteroatoms) (Fig. 2).

### 4.2. Method validation
The proposed method was 100 times more sensitive for both TM and BM than the reported HPTLC method [35]. Although some of the reported spectrophotometric [32,34] and HPLC [34] methods exhibited comparable sensitivities for TM and BM, these methods had narrow linearity ranges. Moreover, some of the reported methods need additional manipulation steps such as column-temperature control [30] and wavelength gradient [31,32], which may limit their widespread use in routine quality control.

Regarding the published literature for the determination of TM/LP, the proposed method was 50 and 1.2 times more sensitive for TM, and 2.5 and 3 times more sensitive for LP than the published HPLC methods [36,37, respectively]. Although there is a reported HPLC method [38] for TM/LP mixture exhibiting better sensitivity than our method, the reported linearity ranges for these compounds is narrow and does not practically permit the determination of the two compounds simultaneously in their coformulated eye drops. Also, the long retention times are

**Table 4 - Application of the proposed and comparison methods for determination of the studied drugs in different dosage forms.**

| Pharmaceutical preparation | Proposed method | | | Comparison methods [30,36] | |
|----------------------------|-----------------|---|---|--------------------------|---|
|                            | TM              | BM | TM | BM | TM | BM |
| Formulation A              | 17.0            | 5.0 | 100.96 | 98.66 | 100.19 | 98.20 |
|                            | 34.0            | 10.0 | 99.28 | 101.01 | 101.11 | 100.10 |
|                            | 68.0            | 20.0 | 100.12 | 99.83 | 102.15 | 99.55 |
| Mean ± SD                  | 100.12±0.84     | 99.83±1.18 | 101.15±0.98 | 99.28±0.98 |
| t<sup>c</sup>              | 1.38            | 0.623 | 1.36 | 1.44 |
| F<sup>c</sup>              | 6.808±0.06      | 1.997±0.02 | 6.878±0.07 | 1.986±0.02 |
| Nominal content (mg/mL)    | 6.824±0.06      | 49.945±0.30 | 6.690±0.06 | 50.700±0.45 |
| Formulation B              | 136.0           | 1.0 | 99.5 | 100.1 | 98.45 | 101.25 |
|                            | 170.0           | 1.25 | 100.25 | 99.22 | 97.48 | 102.36 |
|                            | 190.4           | 1.4 | 101.31 | 100.36 | 99.22 | 100.58 |
| Mean ± SD                  | 100.35±0.91     | 99.89±0.60 | 98.38±0.87 | 101.40±0.90 |
| t<sup>c</sup>              | 2.708           | 2.412 | 1.088 | 2.264 |
| F<sup>c</sup>              | 6.824±0.06      | 49.945±0.30 | 6.690±0.06 | 50.700±0.45 |
| Nominal content (mg/mL TM and µg/mL LP) | 6.824±0.06 | 49.945±0.30 | 6.690±0.06 | 50.700±0.45 |
| Formulation C              | 17.0            | 98.32 | 98.77 |
|                            | 34.0            | 98.52 | 98.04 |
|                            | 68.0            | 99.79 | 100.04 |
| Mean ± SD                  | 99.79±1.47      | 0.815 | 98.95±1.01 |
| t<sup>c</sup>              | 2.11            | 6.786±0.10 | 6.729±0.07 |
| F<sup>c</sup>              | 1.501±0.01      | 1.498±0.02 |
| Nominal content (mg/mL)    | 6.786±0.10      | 6.729±0.07 |
| Formulation D              | 15.0            | 100.58 | 100.29 |
|                            | 30.0            | 99.42 | 98.11 |
|                            | 45.0            | 100.19 | 101.15 |
| Mean ± SD                  | 100.06±0.59     | 0.221 | 99.85±1.57 |
| t<sup>c</sup>              | 7.05            | 1.001 | 1.001 |
| F<sup>c</sup>              | 1.501±0.01      | 1.498±0.02 |
| Nominal content (mg/mL)    | 1.501±0.01      | 1.498±0.02 |
| Formulation E              | 1.0             | 98.07 | 97.55 |
|                            | 1.5             | 98.56 | 98.05 |
|                            | 2.0             | 99.01 | 100.45 |
| Mean ± SD                  | 98.55±0.47      | 0.146 | 98.68±1.55 |
| t<sup>c</sup>              | 10.87           | 49.275±0.24 | 49.340±0.78 |
| F<sup>c</sup>              | 49.275±0.24     | 49.340±0.78 |

BM = brimonidine tartrate; LP = latanoprost; TM = timolol maleate.
<sup>a</sup> Formulation A: Compigan eye drops (2 mg BT + 6.8 mg/mL TM); Formulation B: Xalacom eye drops (50 mg LP + 6.8 mg/mL TM); Formulation C: Timolol eye drops (0.68% TM); Formulation D: Alphagan eye drops (0.15% BT); Formulation E: Ioprost eye drops (50 mg/mL LP).
<sup>b</sup> Each result is the average of three independent determinations.
<sup>c</sup> Tabulated t and F values at p = 0.05 are 2.776 and 19.00, respectively [40].
a limitation for this method. In addition, gradient elution, wavelength programming, and column-temperature control are needed for most of these methods [37, 38].

The present method is simpler, with no need for multistep procedures like those mentioned earlier, and allowed the determination of the two mixtures (TM/BM and TM/LP) with a single simple procedure providing wide linearity ranges and proper retention times.

4.3. **Pharmaceutical application**

Commercially available coformulated and single-ingredient ophthalmic solutions containing the studied drugs were successfully analyzed. The obtained results indicated the applicability of the proposed method for the routine quality control of different ophthalmic solutions without interferences from common excipients. This was evidenced by the calculated
values of the nominal contents which agreed well with the contents claimed by the manufacturer as well as the small values of SD (Table 4). It is worth noting that the method has the ability to analyze TM and LP in such a challenging ratio (136:1, respectively), with no need for complicated steps such as wavelength programming.

5. Conclusion

A simple, rapid, and accurate HPLC method was established and validated for the separation and determination of three commonly administered anti-glaucoma drugs (TM, BM and LP) simultaneously. Chromatographic separation of the three compounds was successfully achieved within a short analysis time (< 6 minutes), offering a great advantage with respect to quality control analysis. The proposed method was applied for the analysis of ophthalmic solutions containing two binary mixtures of the studied drugs, TM/BM and TM/LP, in addition to single-ingredient ophthalmic solutions containing these compounds.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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