Microchemical Journal 207 (2024) 112176

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Hybrid micellar liquid chromatography separation of brimonidine tartrate and brinzolamide. Retention study and quantification in fixed dose ophthalmic suspensions

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ARTICLE INFO

Keywords:
Absorbance spectroscopy
Green
Retention Mechanism
Simbrinza
Validation

ABSTRACT

Background: Brinzolamide/brimonidine 1%/0.2% fixed-dose combination medication is an ophthalmic suspension widely used to treat eye disorders related to elevated intraocular pressure. An analytical procedure based on hybrid micellar liquid chromatography has been developed to quantify the active components in pharmaceutical preparations.

Results: Samples were diluted, up to a target concentration of 50 mg/L brinzolamide and 10 mg/L brimonidine tartrate, in a mixture of methanol and mobile phase by stirring and ultrasonication, filtered and directly injected without an extraction step. The drugs were resolved from the matrix using a mobile phase of 0.10 mol/L sodium dodecyl sulfate – 4 % 1-butanol – 0.01 M sodium dihydrogen phosphate, with a pH adjusted to 3 by adding drops of hydrochloric acid solutions. running at 1 mL/min under isocratic mode through a C18 column. Detection UV absorbance wavelength was set to 250 nm. A theoretical study on the retention mechanism was calculated and the constants of the partition equilibria occurring in the column were calculated. The method was successfully validated by the guidelines of the International Conference on Harmonization in terms of: calibration range (from 0.1 to 2 times the target concentration), linearity ($r^2 > 0.995$), trueness (98.3–102.6 %), precision (<2.2 %), limit of detection, limit of quantification (under the lowest level of calibration) and robustness. The procedure was applied to commercial formulations.

Signification: The method was found cost-effective, easy-to-conduct, safe, ecofriendly (low quantity of hazardous reagents were used) and with a high sample throughput, which makes it suitable for routine analysis in pharmaceutical quality control. On the other hand, we deduced this was mainly driven by electrostatics, and the surfactant had more influence in elution strength than the organic modifier.

1. Introduction

Brinzolamide/brimonidine tartrate (Simbrinza®, Alcon Laboratories, Inc., Fort Worth, TX, USA) is a fixed dose combination (BBFC) medication distributed as a whitish sterile, preserved ophthalmic suspension. It contains 10 mg/mL of brinzolamide and 2 mg/mL brimonidine as active components and excipients such as 0.03 mg/mL benzalkonium chloride (as preservative), propylene glycol, Carbopol® 974P NF polymer, mannitol, ambroxol, sodium chloride, boric acid and water, and the pH is close to 6.5 [1–3]. Its main therapeutical effect is the reduction of the intraocular pressure (IOP). In the USA, it is used to treat ocular hypertension and glaucoma in adults with a dosage of one drop three times daily, while in the EU it is prescribed against the same disorders when monotherapy is not enough by a twice-daily regimen [4]. Prophylactic administration after eye surgery has also been envisaged [5]. It was developed by Alcon and approved for use by US Food and Drug Administration and European Medicines Agency in 2013 and 2014, respectively [1,6].

Brinzolamide (((4*R*)-4-(ethylamino)-2-(3-methoxypropyl)-1,1-dioxo-3,4-dihydrothieno[3,2-e]thiazine-6-sulfonamide, a small

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https://doi.org/10.1016/j.microc.2024.112176
Received 11 October 2024; Received in revised form 8 November 2024; Accepted 11 November 2024
Available online 13 November 2024

molecule with a molecular mass of 383.5 g/mol) is a highly non-competitive, specific, reversible carbonic anhydrase II (CA-II) inhibitor. It is a sulfonamide and a thienothiazine moderately polar (log P = −0.6) and with a solubility in water of nearly 700 mg/L. It is a weak base, the pKa of its conjugated base is 6.5. It is neutral at pH = 7 and monocationic at pH = 3. Brimonidine (5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)quinoxalin-6-amine, a small molecule with a molecular mass of 383.5 g/mol) is a third-generation alpha-2 adrenergic receptor agonist, which inhibits the enzyme adenylyl cyclase. It is a quinoxaline derivative, a secondary amine and an imidazole moderately hydrophobic (log P = 1.4) and water soluble. It does not exhibit acidic/alkaline activity in the interval pH = 2.5–7.5 and has a formal charge of + 1. Their structures can be seen in [7,8]. After topical administration, both drugs are primarily absorbed through the cornea and then reach the systemic circulation [9]. The mixture exerts IOP-lowering efficacy via two complementary mechanisms: decreased aqueous humor production by brimonidine and brinzolamide and increased aqueous outflow with brimonidine [10]. BBFC displays a favorable benefit-risk balance and is generally well tolerated [1,3]. The major ocular adverse effects are conjunctival hyperemia, visual disturbances, conjunctivitis, irritation and eye discomfort, while the most systemic ones are altered taste sensation, mouth dryness, fatigue, somnolence, and decreased awareness [1,10]. This fixed-combination therapy exhibits a higher IOP-efficacy than the concomitant administration of both medications, and offers several advantages, like lower cost, simplified regimen, less systemic effects, improved adherence, decreased risk of drug washout, and lower damage associated with exposure to preservatives [10].

The quantification of the active components in the finished product is an important step in pharmaceutical quality control and to avoid the distribution of low-quality counterfeit formulations [11]. Several methods based on UV spectroscopy [12,13], TLC [14], voltammetry [15] have been developed to determine brinzolamide/timolol [12,14], brinzolamide/brimonidine [13], and brimonidine/timolol [15] in ophthalmic formulations. However, HPLC-UV is preferred for these sorts of analyses, for it exhibits ability to discriminate the analyte from matrix compounds, automatization capability, versatility and sensitivity [16]. Several analytical methods based on this technique have been developed to analyze fixed dose combination eye drop suspension medications of brinzolamide/timolol [14], Brinzolamide/Brimonidine Tartrate [13,17,18], brinzolamide/timolol and brimonidine/timolol in the same run [19,20], brimonidine/timolol [21] and brimonidine alone [22]. The interaction between the cationic analytes with the free silanols was prevented by the addition of triethylamine as a sacrificial base [13,14], using a pH less than 4.5 [18,19,21] and by ion pairing [22]. However, these methods employes solutions containing high proportions of organic solvent, like acetonitrile or methanol, as mobile phases (25 – 80 %) and to dissolve the sample (25–100 %). This represents a threat to workplace safety and the environment, and increases the cost associated with waste treatment. Besides, one important aim of White Analytical Chemistry (WAC) is the minimization of the quantity of toxic reagents used, while maintaining the analytical performance and affordability of the method [16]. In fact, an attempt has been made to develop a greener method by using isopropanol as an organic modifier, which is eco-friendlier [23].

Hybrid Micellar Liquid Chromatography (HMLC) has been widely used in the analysis of pharmaceutical preparations [24]. It is based on the use of mobile phases containing solutions of a surfactant, mainly the harmless and aerially biodegradable anionic sodium dodecyl sufate (SDS) forming micelles and a low volume (<15 %) of organic modifier, being the most common ones 1-propanol, 1-butanol and 1-pentanol, less toxic and volatile than acetonitrile and methanol. The same solution is used to dissolve the pharmaceutical samples. Therefore, resulting methods are far greener than the usual hydroorganic-RP-HPLC ones [25]. These solutions have an increased solubilization power, what simplifies sample treatment. In HMLC, a secondary partition equilibrium, bulk mobile phase and micelle, occurs together with the stationary phase – bulk mobile phase one. Besides, SDS monomers adsorb on the stationary phase with the sulfate group oriented outwards, which generates a negative layer able to interact with positively charged compounds. Retention is ruled by the three-phase theory, and it is highly reproducible and can be predicted using well-established models. HMLC offers several advantages face to hydroorganic RP-HPLC like greenness, better retention of cationic analytes and separation of mixtures of analytes with a large range of hydrophobicity under isocratic mode [26,27]. Besides, the spectrophotometric activity is also enhanced when the chromogen is located in an organized media [28].

The aim of the work is the development of an analytical procedure based on HMLC for the analysis of the two active components of the medication Simbrinza, brinzolamide and brimonidine. It has to be safe, ecofriendly, easy-to-handle, cost-effective and practical for routine analysis. Its analytical performance will be evaluated by an appropriate and official guideline [29], and then applied to commercial samples. Besides, the effect of the mobile phase composition on retention and the partition constants of the different equilibria involved in retention mechanism will be determined, for a better understanding of it.

2. Experimental

2.1. Standard and chemicals

Powdered standard of brinzolamide (purity > 98.0 %) and brimonidine tartrate (Ph Eur reference standard) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Solid sodium dodecyl sulfate was bought from Chemical Industry (Tokyo, Japan). Sodium dihydrogen phosphate monohydrate (>99 %) and the solvents methanol and 1-butanol (HPLC grade) came from Sharlab (Barcelona, Spain). Hydrochloric acid (37 %) was from Panreac (Barcelona, Spain). Ultrapure water was in-lab generated using a water purification system Adrona HPLC Connect (Adrona, Riga, Latvia) from deionized water supplied by the University of Valencia as tap water. This ultrapure water was employed to prepare all aqueous solutions.

Individual stock solutions of brinzolamide 500 mg/L and brimonidine tartrate 100 mg/L in methanol were made. Working solutions (individual or combined) were prepared by proper dilution in mobile phase. These solutions were stored at 4 degrees Celsius. Solutions in mobile phase were discarded after a week, while in methanol were kept for a long time. The preparation of micellar solutions can be seen in [16].

2.2. Chromatographic conditions

Chromatographic analyses were performed using a HP1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, a degasser, an autosampler and an UV–Visible absorbance diode array detector (DAD), connected to a PC. The software Agilent Chemstation (Rev. B.04.03(16) 2010) was employed to control the instrumentation, as well as to visualize and process the signals. The column was a C18 Kromasil (AkzoNobel, Amsterdam, The Netherlands) with the following characteristics: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 μm; pore size, 10 nm. The mobile phase was a solution of 0.10 mol/L SDS – 4 % 1-butanol – 0.01 M sodium dihydrogen phosphate, with a pH adjusted to 3 by adding drops of hydrochloric acid solutions. It runs under isocratic mode at 1 mL/min. Injection volume and detection wavelength were 20 μL and 250 nm, respectively.

The solutions to be injected were filtered through a 0.45 μm-Nylon membrane filters (Micron Separations, Westboro, MA, USA) by hand pushing with a 3-mL plastic syringe before introduced in the chromatographic vials, which were sealed and placed in the autosampler tray.

The working and care specifications regarding the HPLC instrumentation when dealing with micellar mobile phases is detailed in [30]. The thorough following of these recommendations is necessary to prevent column and narrow connections and tubes clogging, considering

the high amount of salt in the mobile phase.

2.3. Preparations of solutions

A sample of Simbrinza 10 mg/mL (brinzolamide) + 2 mg/mL (brimonidine tartrate) suspended eye drop was purchased from a local chemist. This is the only formulation containing these two active components permitted to be distributed in Spain [2].

A volume of 10 mL of methanol and an aliquot of 250 μL of sample were placed in a glass beaker and hand shaken. Afterwards, 30 mL of mobile phase were added, and this mixture was shaken using a magnetic stirrer and then by ultrasonication, 15 min each one, to achieve the maximal solubilization of the matrix. Finally, it was introduced in a 50-mL volumetric flask, which was filled up to the mark with mobile phase and filtered before injection. Therefore, the target concentration of brinzolamide and brimonidine tartrate were 50 and 10 mg/L. Processed samples were discarded after analysis.

3. Results and discussion

3.1. Optimization of the sample treatment

Sample treatment conditions were taken from Ref. [24], and then adapted to the characteristics of the sample.

Sample was found to form a doughy mixture with mobile phase when diluted 1:20, because of the low solubility of the matrix. Therefore, the dilution ratio was increased and then the sample volume was 0.250 mL, which led to the preparation of a solution containing 50 mg/L of brinzolamide and 10 mg/L of brimonidine tartrate. A higher dilution ratio was not considered to excessively reduce the sensitivity of the method and to avoid an increase of the uncertainty associated to sampling. Therefore, the target concentrations were set to these values. However, even at this dilution, some oily shape and turbidity were noticed. Consequently, the sample was first mixed with 10 mL of methanol to better solubilize the matrix, and then the mobile phase was added. In this case, the final mixture was a purely transparent solution. Therefore, the sample was quantitatively injected without any clean-up step.

3.2. Optimization of the chromatographic conditions

In the frame of the current work, the composition of the mobile phase, *i.e.*, SDS concentration, nature and proportion of organic solvent and pH, the detection conditions were optimized to achieve a reasonable level of resolution, analysis time and sensitivity. The surfactant, column and mobile phase running were directly taken as most common in HMLC [31].

Considering the polarity of brinzolamide (log P = −0.6), low retention is expected by pure hydrophobic interaction. Therefore, the pH was set to 3, in order to obtain the protonated form of brinzolamide (pKa = 6.5) and increase the interaction with the modified stationary phase by electrostatics [32]. The union of brinzolamide with the micelles in the mobile phase would be increased by the same process. At this pH, free silanols of the silica particles are protonated, thus preventing their interaction with the positively charged analytes [33].

We envisage the addition of 1-propanol, 1-butanol or 1-pentanol, as an organic modifier to reduce retention, ameliorate peak shape and improve the interaction of mobile and stationary phase [34]. The higher expected retention, the more hydrophobic alcohol has to be chosen [35]. The use of 1-pentanol was discarded, because the elution strength of the mobile phase would be too high. Therefore, a mixture of both drugs at their target concentration was analyzed using mobile phases of at 0.1 mol/L SDS – 4 % 1-butanol and 0.1 mol/L SDS – 7.5 % 1-propanol. This last one provides too long retention for brimonidine; thus 1-butanol was selected as organic modifier.

The optimization of the concentration of SDS and 1-butanol was performed by studying their influence on retention using an interpretative approach. A solution containing both drugs was analyzed by only five mobile phases, selected using a full factorial design plus the central point. The extreme values were selected as the minimal and maximal concentration values commonly employed for SDS and 1-butanol, 0.05–0.15 mol/L and 1 – 7 %, respectively [36]. The assayed mobile phases and the results can be seen in Table 1.

Regardless of the mobile phase, brinzolamide undergoes a higher retention than brimonidine. Both drugs have a binding behavior face to the micelles, as the elution strength of the mobile phase augmented at increasing SDS concentration, mainly by electrostatic attraction. As usual in HMLC and RP-HPLC, the retention time diminishes at higher values organic solvent proportion.

The operating mobile phase was selected from those tested. Those with [SDS] = 0.05 mol/L were discarded because of their excessive analysis time, while 0.15/7 because the two peaks were too close in the chromatogram. The other two mobile phases provide similar retention times, and then the less salty was selected to reduce the deterioration of the column and the pump backpressure. Consequently, the optimal mobile phase was made of 0.10 mol/L SDS − 4 % 1-butanol buffered to pH 3 with 0.01 mol/L phosphate salt. Both drugs were adequately resolved in a reasonable time and enough far from the dead time to prevent overlapping with matrix compounds from the formulation, using an organic solvent less hazardous and at less proportion than commonly used in RP-HPLC.

To optimize the detection conditions, the UV spectrum of both compounds were in-time registered during their elution at the retention time (Fig. 1). Both spectra were similar to those obtained using hydro-organic mobile phases [18]. Both drugs exhibit a band between 230 and 270 nm. Maximal absorbances were found at 248 and 254 nm for brinzolamide and brimonidine, respectively. The detection absorbance wavelength was set to 250 nm for both compounds, because both maxima were close, both drugs display an adequate absorbance at this value and to avoid baseline disturbance for wavelength change. The spectra were also measured at taken at 50 % and 5 % height tailing and fronting for peak purity studies.

A system suitability test was successfully carried out to prove the consistency of the responses and the suitability of the chromatographic conditions and instrumentation by the six consecutive injections of a solution of brinzolamide and brimonidine tartrate 50 and 10 mg/L, respectively (Fig. 2A). The evaluated parameters, results for brimonidine and brinzolamide, and acceptance criteria were [37]: relative standard deviation (RSD) of peak area, <0.8 %, <0.9 %, <2.0 %; RSD% of retention time, <0.5, <0.4, <2.0 %; retention factor, 6.4, 8.8, >2.0; number of theoretical plates, 2235, 2073, > 2000; and asymmetry: 1.1, 1.0, 0.8–1.6.

3.3. Theoretical study on retention

3.3.1. Determination of the partition physico-chemical constants

The retention in HMLC depends on the partition equilibria of the solute between the bulk mobile phase and the modified-stationary phase (main interaction) and between the bulk mobile phase and the pseudomicellar phase (secondary interaction). Several mechanistic models have been proposed to predict the retention factor from the concentra-

Table 1
Retention times of the analytes when analyzed by the tested mobile phases.

[SDS] (mol/L)	[1-butanol] (%, v/v)	Retention time for brimonidine tartrate (min)	Retention time for brinzolamide (min)
0.05	1	17.7	24.7
0.05	7	9.1	13.5
0.10	4	6.6	8.8
0.15	1	6.8	9.2
0.15	7	4.1	5.3

Experimental dead time = 0.9 min

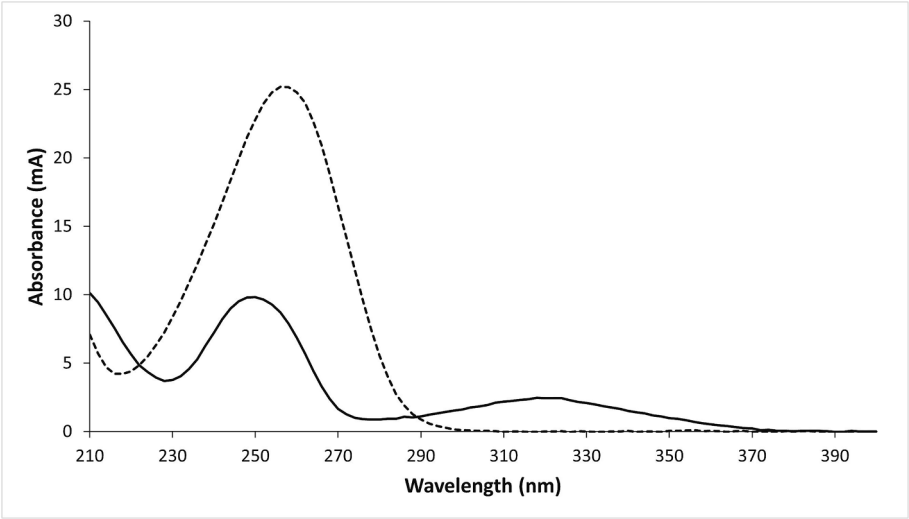

Fig. 1. Absorption spectra of brimonidine (—) and brinzolamide (———) at their respective target concentrations taken at the retention time during elution using the selected conditions.

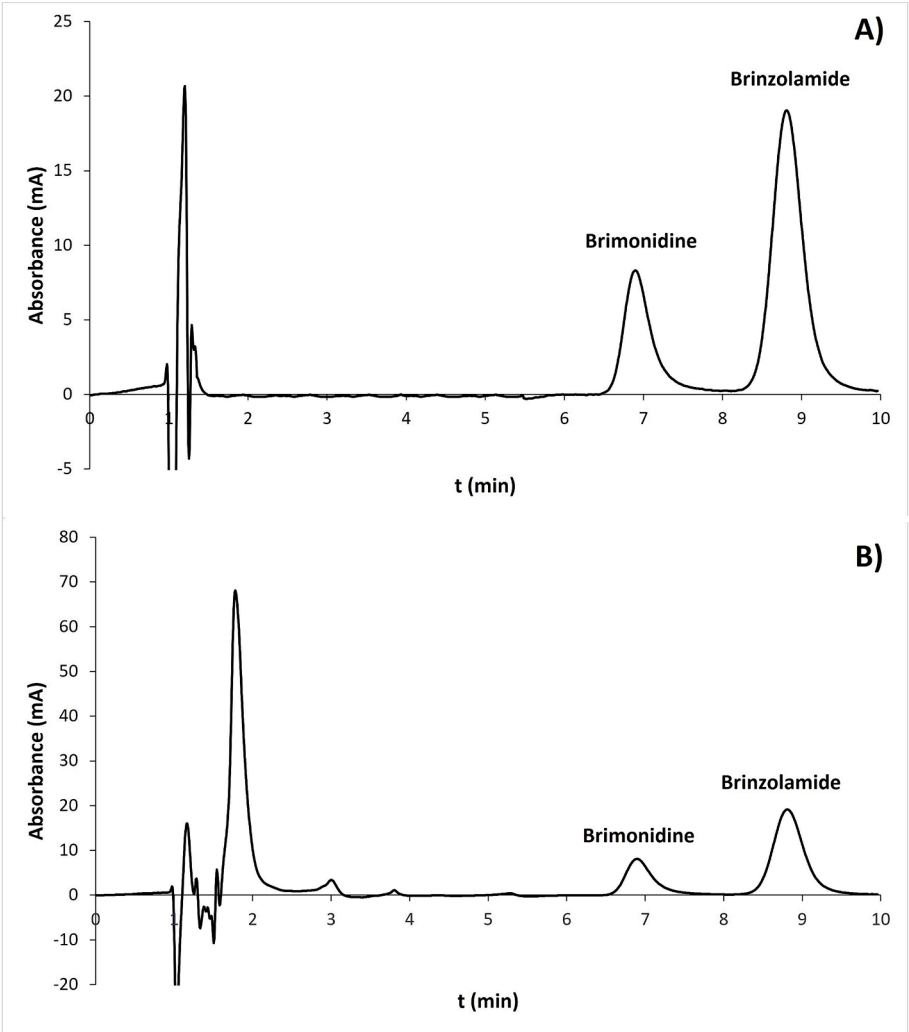

Fig. 2. Chromatogram obtained by the analysis of: A) a standard solution of brinzolamide 50 mg/L and brimonidine tartrate 10 mg/L and B) sample 1.

tion of SDS and organic solvent, and that shown in Eq. (1) has been demonstrated to provide reliable results for moderately hydrophobic compounds at proportions of 1-butanol < 10 % [38]:

$$k = \frac{FK_{AS}\frac{1}{1+K_{AD}[1-butanol]}}{1 + K_{AM}\frac{1+K_{MD}[1-butanol]}{1+K_{AD}[1-butanol]}[SDS]} \quad (1)$$

The main and secondary equilibria are quantified by the constants K_{AS} and K_{AM}, when the organic solvent is absent. These equilibria are affected by the organic modifier, which increases the solubility of the solute in the mobile phase (K_{AD}) and in the micelle (K_{MD}) by a change in its configuration. Therefore, retention is increased by augmenting K_{AS} and at decreasing values of the other constants. F is the quotient between the stationary and mobile phases (phase ratio). This equation was fitted using the experimental data obtained by the study described in section 3.1. Results are shown in Table 2. The models provide reliable results for both drugs, according to the values of multiple coefficients of determination and calibrator errors.

In both cases, we found a moderately-strong interaction between the solute and the micelles and the stationary phase, higher for brimonidine than for brinzolamide. That points to the electrostatic attraction as the main cause of the interaction, which is common to both compounds. This interaction is reinforced for brimonidine by the larger contribution of the hydrophobic forces, according to its superior log P. On the other hand, brinzolamide relative interaction with the stationary phase compared to that with the micelles (K_{AS}/K_{AM}) is larger than that of brimonidine, what explains its later retention time, despite its lower hydrophobicity. The solubility of brimonidine in the bulk mobile phase is strongly increased because of the presence of 1-butanol, while barely affects brinzolamide, for its higher hydrophobicity. According to the values of K_{MD}, the effect of 1-butanol in the association of the drugs to the micelle is not relevant.

3.3.2. Effect of the composition of the mobile phase on retention

The influence of each component of the mobile phases was carried out using an empirical model, which used Eq. (2) to predict the retention factor [38]:

$$\frac{1}{k} = c_0 + c_1[SDS] + c_2[1 - butanol] + c_{12}[SDS][1 - butanol] \quad (2)$$

This equation is obtained by rearranging Eq. (1), thus the goodness-of-fit is the same as Eq. (1). Concentration values of SDS and organic modifier were normalized to –1 and + 1, to better compare the constants. c_1, c_2 and c_{12} stand for the effect of SDS concentration, organic modifier proportion and the interaction on the elution strength of the mobile phase, while c_0 is the elution strength at intermediate conditions. The results are in Table 2.

The elution strength of the mobile phase was higher for brimonidine, probably by its higher interaction with the micelles. The effect of the mobile phase components and their order of importance were similar for both compounds: SDS > 1-butanol > the interaction. Therefore, the interaction with the micelles, which is produced mainly by electrostatic interaction, is more relevant for elution than the increase of solubility caused by 1-butanol.

Table 2
Values obtained for mechanistic and empirical model for both drugs (freedom degree = 1).

Parameter	Brimonidine	Brinzolamide
K_{AS}	389.70	256.75
K_{AM}	337.38	153.18
F K_{AS}	1.16	1.68
K_{AD}	1.21	0.10
K_{MD}	0.14	0.18
Multiple regression coefficient (R^2)	0.998	0.997
Maximal absolute value of error in *k* prediction (calibrator points)	3.9 %	6.0 %
c_0	0.151	0.107
c_1	0.068	0.051
c_2	0.046	0.032
c_{12}	0.019	0.016

3.4. Method validation

Validation of the procedure was performed by the guideline Validation of Analytical Procedures Q2(R2) form the International Conference of Harmonization [39], and Methods, Method Verification and Validation, Food and Drug Administration – Office of Regulatory Affairs (FDA-ORA) Laboratory Manual Volume II [40].

3.4.1. Calibration range and linearity

Seven standard solutions of brimonidine tartrate and brinzolamide were prepared in the range 1 – 20 mg/L and 5 – 100 mg/L (calibration range), respectively, and analyzed in triplicate. In both cases, homoscedasticity was proven by comparing the highest and lowest variance by a two-tailed F-test (confidence level of 5 %). The least square regression method was used to linearly relate the average peak area (as a dependent variable) and the corresponding concentration (as a predictor). The results were:

A(brimonidine) = (21.37 ± 0.17) [brimonidine (mg/L)] + (−1.6 ± 1.9) r^2 = 0.9997.

A(brinzolamide) = (12.65 ± 0.12) [brinzolamide (mg/L)] + (1.5 ± 7.1) r^2 = 0.9995.

An adequate linearity was found, as the r^2 were > 0.995 and the graph residual v.s. concentration shows a random pattern. No systematic error was observed, as the y-intercept accounts for < 2.0 % of the target concentration and is significantly undistinguishable from 0. Besides, the calibration range covers 80–120 % of the target concentration.

Limit of detection/limit of quantification were calculated as 3.3 and 10, respectively, times the standard deviation of the y-intercept divided by the slope [41]. The results for brimonidine and brinzolamide were 0.29/0.89 and 1.8/5.6 mg/L, which are sufficiently below the target concentration and are consistent with the calibration range.

3.4.2. Trueness and precision

They were determined at 0.8, 1 and 1.2 times the target concentration of each drug in sample.

Six samples of a pharmaceutical formulation were processed and conveniently diluted to get the desired concentrations and analyzed in the same sequence. Repeatability was the RSD of the six found concentrations, while trueness was quantified as the relative recovery, as the quotient between the found and nominal concentration, respectively. To evaluate the passage of time in the variability of the results, the intermediate precision was determined by repeating this procedure five times over a three-month period, as the RSD% of the 30 found concentrations. Results (Table 3) were within the acceptance criteria, thus proving the reliability and the consistency of the method is adequate for quantification purposes. This was mainly achieved by the solubilization power of micellar solutions. Due to the remarkable

Table 3
Results obtained for trueness and precision (Acceptance criteria: trueness, 97–103 %, repeatability, <2%, intermediate precision, <3%).

Drug	Concentration	Trueness[a] (%)	Repeatability[a] (RSD%)	Intermediate precision[b] (RSD%)
Brimonidine tartrate	8.0	98.7	1.3	2.2
	10	102.3	0.8	1.4
	12	100.4	0.7	1.8
	40	99.1	1.2	2.0
Brinzolamide	50	102.6	0.8	1.6
	60	98.3	0.7	1.4

[a] n = 6; [b] n = 5.

precision attained, no replicate analyses are necessary, which decreases time and resources required for samples analysis, as well as waste generation.

3.4.3. Carry-over effect

A sample processed to get a solution containing 20 mg/L brimonidine tartrate and 100 mg/L brinzolamide and a blank were successively injected. The chromatogram does not exhibit any peak or baseline distortion at the window time of the analytes, thus indicating the absence of cross-contamination at these levels.

3.4.4. Robustness

Small changes (those can occur during the common work at the laboratory) were introduced in the selected experimental conditions to evaluate their impact on the main instrumental responses: retention time and peak area, by a one-factor-a-time strategy. A processed sample containing the target concentration of both compounds were analyzed at the maximum, central and minimum value for the studied factor, keeping the other ones at their optimal value, and the RSD% of the obtained retention time and peak area were taken to evaluate variation. The studied parameters and intervals were: SDS concentration, 0.095 – 0.105 M; 1-butanol proportion, 3.9 – 4.1 %; pH, 2.95 – 3.05; low-rate, 0.95 – 1.05 mL/min; injection volume, 18 – 22 μL and detection wavelength, 249 – 259 nm.

The method was considered enough robust to resist random fluctuations for the studied experimental parameters, for all RSD% were < 5.0, which will reduce measurement dispersion in actual practice.

3.4.5. Specificity

The possible interference by excipients or impurities was evaluated by the analysis of a sample processed to get the target concentration. The chromatographic run was extended to 40 min and the UV absorbance spectra were recorded at the same points as described in section 3.1.

In the obtained chromatogram, we observe the front of the chromatogram from 0.9 to 2.0 min, and several baseline distortions between 3.0 and 4.0 min, and a quite stable baseline up to the end of the chromatogram. Peaks corresponding to the analytes were visualized without either any shoulder or overlapping peak. They were also visually compared by overlaying with the chromatogram obtained in the system suitability testing (section 3.1) [37], and were found rather similar.

A peak purity study was performed to check the presence of coeluting compounds. The UV absorption spectra were registered at the retention time, and 50 %- and 5 %- of both fronting and tailing side, and compared with those obtained in section 3.1. No relevant differences were noticed by the naked eye.

Consequently, the eye drop formulation does not contain an interfering compound eluting at or close to the window time of the analytes and there is no risk of overlapping from a strongly retained compound, and then the entire peak area can be assigned to the corresponding drug. The duration of the chromatogram was set to 15 min.

3.5. Analysis of commercial samples

Several commercial eye drop formulations of Simbrinza®, randomly purchased from local chemists, were analyzed to evaluate the suitability of the method for routine analysis. The samples were first processed at the same time and then injected in the same sequence, together with blank and quality control (QC) standard solutions. The results were (brinzolamide/brimonidine tartrate): QC 50/10 mg/L (48.9/9.8 mg/L), sample 1 (label claim 100.8/98.1 %), sample 2 (101.7/102.0 %), sample 3 (97.6/99.5) blank (no signal/no signal), sample 4 (101.9/98.5), sample 5 (97.6/98.3), sample 1 reanalyzed (102.7/99.7), 50/10 mg/L (49.1/10.2 mg/L), sample 2 reanalyzed (99.8/99.3). In all cases, both drugs were plainly detected without overlapping. The chromatogram obtained by the analysis of sample 1 can be seen in Fig. 2B.

Results for QC (relative recovery 97–103 %), blanks (<under LOD) and replicate analysis (difference of < 3 %) were under the acceptance criteria, and then there is not either drift or cross contamination in the measurements. In addition, the studied samples comply with the regulation on the amount of active component (label claim 95 – 105 %).

The steps of the method were short, easy to conduct and semi-automated, and it only requires the preparation of only one solution, and then many samples can be analyzed per day. The procedure only uses available and generic reagents, laboratory material and instrumentation. The chemicals used were harmless and aerially degradable, save a low volume of organic solvent, which makes the procedure safe and environmentally friendly.

3.6. Greenness assessment

The greenness of the method was evaluated using the comprehensive, flexible, and reliable tool Analytical Greenness Metric (AGREE), based on the twelve principles of Green Analytical Chemistry (GAC) [42]. For each principle, specific information preset by the tool is input and transformed into a 0 – 1 score (1 being greener), according to compliance with it, and the global assessment is the average of these twelve scores. Each principle can be assigned different weight by the customer, which introduces a certain degree of flexibility in the measure. The result is visually represented as a clock-like circular pictogram, with the overall score and color representation in the middle, and the color corresponding to each principle in the edges of the circumference. The colors, in order of decreasing greenness are dark green, light green, yellow, orange and red. The procedure is performed as a straightforward and easy way using software AGREE (Universidade de Vigo, Spain and Gdansk University of Technology, Poland) [42].

For this method, the twelve principles, input provided, and the corresponding scores were (all with the same weight):

1. Sample Pretreatment Activities: external sample pre- and treatment and batch analysis (reduced number of steps) (0.3).
2. Minimal Sample Size and Minimal Number of Samples Are Goals: 0.25 mL (0.85).
3. In Situ Measurements Should Be Performed: off-line (0.0).
4. Integration of Analytical Processes and Operations Saves Energy and Reduces the Use of Reagents: 3 steps or less (1.0).
5. Automated and Miniaturized Methods Should Be Selected: semi-automatic, not miniaturized (0.25).
6. Derivatization Should Be Avoided: No derivatization (1.0).
7. Generation of a Large Volume of Analytical Waste Should Be Avoided and Proper Management of Analytical Waste Should Be Provided: 65 mL (0.14).
8. Multianalyte or Multiparameter Methods Are Preferred versus Methods Using One Analyte at a Time: 2 analytes, 4 samples per hour (0.45).
9. The Use of Energy Should Be Minimized: LC (1.0).
10. Reagents Obtained from Renewable Source Should Be Preferred: None of the reagents was bio-based (0.0).
11. Toxic Reagents Should Be Eliminated or Replaced: 2.6 mL (0.38).
12. The Safety of the Operator Should Be Increased: threats not avoided: toxic to aquatic life (0.8).

The procedure obtained a final score of 0.51 (yellow), which indicates that it is fairly green (Fig. 3). The main issues were the off-line sample preparation and the use of HPLC, which were unavoidable. The program also considers the volume of solutions used as “high”, although it is low for HPLC-based methods.

4. Conclusions

In the current study, a method based on HMLC coupled to UV absorbance detector has been successfully developed and validated for the quantification of brinzolamide and brimonidine tartrate in eye drop

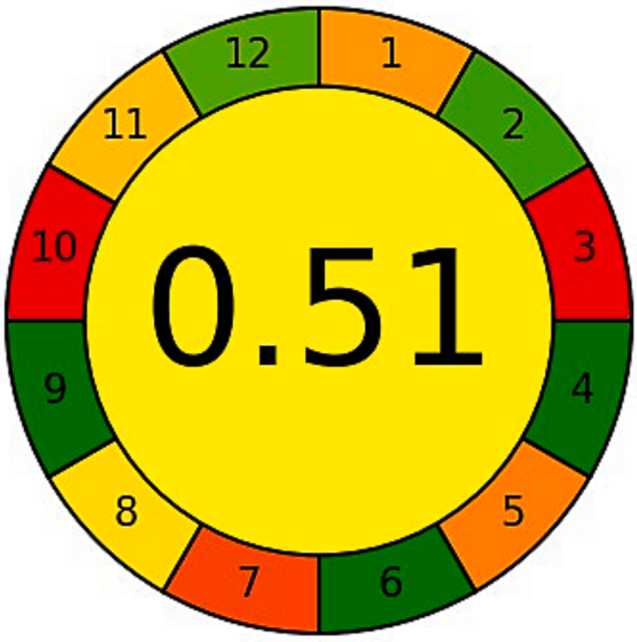

Fig. 3. AGREE scores of the method.

formulation. The main features are the low volume of organic solvent manipulated and wasted, and the simplicity of sample treatment, which does not require any purification step, achieved because of the strong solubilizing power of micellar solutions. The effect of SDS and organic modifier on elution strength and the partition constants of the different equilibria occurring in the column were studied using an experimental design, what provided a better understanding of the retention mechanism. We found out this was essentially governed by electrostatic attraction. The method was demonstrated to be reliable, short, easy-to-handle, available, safe, ecofriendly and with a high sample throughput, which makes it a valuable alternative for routine analysis in pharmaceutical control.

CRediT authorship contribution statement

Daniel García-Ferrer: Writing – original draft, Validation, Software, Investigation, Formal analysis. **Juan Peris-Vicente:** Writing – original draft, Validation, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization, Project administration. **Devasish Bose:** Visualization, Supervision, Resources, Project administration, Funding acquisition. **Abhilasha Durgbanshi:** Writing – review & editing, Software, Investigation, Data curation, Conceptualization. **Samuel Carda-Broch:** Writing – review & editing, Visualization, Supervision, Methodology, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement/Funding

The authors are thankful to DST-FIST and DST-PURSE (Department of Science and Technology, Ministry of Science and Technology, India) for supporting with FIST and PURSE scheme (Sl.No.64 Dated 31-05-2016) and to the “Generalitat Valenciana” (Valencian Regional Government, Spain) for support by project CIAICO/2023/010. We thank the Universitat de València for paying the APC for open access publishing.

Data availability

Data will be made available on request.

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