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DEVELOPMENT AND VALIDATION OF SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF BRIMONIDINE INTO OCULAR IMPLANTS

Júlio Fernandes de Souza[1]*, Kamila Nunes Maia[1], Sílvia Ligório Fialho[2], Frank Pereira de Andrade[1], Gisele Rodrigues da Silva[1]

[1]School of Pharmacy, Federal University of São João del-Rei, Divinópolis, Minas Gerais, Brazil.

[2]Pharmaceutical and Biotechnological Development, Ezequiel Dias Foundation, Belo Horizonte, Minas Gerais, Brazil.

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***Correspondence for Author:**
Dr. JF De Souza.
Federal University of São João del-Rei (UFSJ). Rua Sebastião Gonçalves Coelho, 400, Chanadour, Divinópolis, Minas Gerais, Brazil.

ABSTRACT

A spectrophotometric method was developed and validated to perform the determination of brimonidine incorporated into chitosan implants and released from them. Brimonidine was detected at 258 nm. The analytical method was linear ($R^2 > 0.99$) over the concentration range of 0.5 to 15 μg mL^{-1} of brimonidine, precise (RSD < 5.0%), accurate (with average recoveries ranging of 96 to 102%) and robust. The theoretical quantitation limit was 0.015 μg mL^{-1}. The validated method was applied to quantify the brimonidine incorporated into chitosan implants; and it demonstrated the uniform distribution of the drug in these implantable devices. Finally, the spectrophotometric method showed the controlled and prolonged *in vitro* release of brimonidine that was incorporated into the chitosan.

Keywords: method validation; brimonidine; *in vitro* drug release; brimonidine-loaded chitosan implants; spectrophotometry.

1. INTRODUCTION

Glaucoma is an optic neuropathy with chronic and progressive evolution, in which there are typical changes in the optic nerve head, in the layers of the retinal nervous fibers and increased intraocular pressure above 21 mmHg [1-2]. The elevation of intraocular pressure

may cause progressive reduction of visual acuity and visual field loss, with possible progression to irreversible blindness [3].

Currently the treatment of glaucoma is based on cholinergic drugs, beta blockers, adrenergic agonists, carbonic anhydrase inhibitors, prostaglandin analogues and hyperosmotic agents [2]. Many patients need more than one drug to achieve the ideal intraocular pressure [4-5]. Brimonidine, as shown in Figure 1, is an alpha-adrenergic agonist drug that reduces secretion of aqueous humor and increases uveo escleral outflow [6]. It is useful as monotherapy and adjunctive therapy in treatment of glaucoma. A combination of brimonidine and timolol in eye drops effectively reduces intraocular pressure and offers the advantage of both drugs in only one eye drop. The neuroprotective capacity of brimonidine is an additional advantage to its use in glaucoma and ocular hypertension [7].

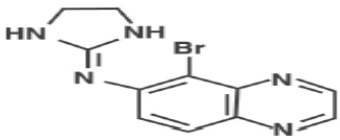


Fig. 1 Chemical structure of the brimonidine.

These drugs are administrated using eye drops of multiple doses, which contain preservatives. However, the prolonged administration of these adjunvants could promote a severe inflammatory response in the tissues of the anterior segment of the eye [8-9]. To overcome the drawbacks of these conventional pharmaceutical dosage forms, biodegradable ocular implants could be developed [10], providing controlled and prolonged release of the drugs in the anterior segment of the eye to treat the glaucoma, excluding the use of preservatives.

Several reports have described methods for quantifying brimonidine in different samples and using various analytical techniques such as high performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrophotometry (LC/MS), capillary electrophoretic analysis (CEA) and gas chromatography coupled with MS (GC/MS) [11-21]. A HPLC method and two LC/MS/MS methods have been reported for the quantitation of brimonidine in eye drops [11-13]. A LC/MS/MS method has been reported for the identification of synthetic impurities of brimonidine [14] and few HPLC methods have been reported for quantifying brimonidine in plasma, serum, aqueous humor [15,16] and in ocular tissues [17,18]. A CEA of brimonidine in biological fluid and a GC/MS assay for analysis of brimonidine in human plasma were also reported [19,20]. An extensive survey of literature did not reveal any

validated UV-spectrophotometric method for quantifying of brimonidine in ophthalmic formulations [21,22].

Therefore, in this study, the spectrophotometric method with UV detection was developed and validated to determine brimonidine incorporated into chitosan implants and verify the controlled and sustained release of the drug from the implants. The proposed method is advantageous since it is extremely simple and fast, providing unequivocal quantification of brimonidine in the implants.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

Chitosan (average molecular weight 190-310) was purchased from Sigma Aldrich® (USA). Brimonidine Tartrate (molecular weight 442.24) was purchased from Iffect Chemphar Company® (China). Acetic acid was purchased from Cromato Chemicals® (Brazil). Purified water was supplied by a water purification system Milli-Q®(USA).

2.2 Instrumentation and analytical conditions

A spectrophotometer Nova Instruments®Model Nova 1600UV (Brazil) with double beam UV-visible, spectral width of 2 nm, wavelength accuracy of 0.5 nm and a 10mm quartz cuvette was used to measure the absorbance of all the solutions. The spectra were displayed automatically by the system software UV-Probe. The detection of drug was performed at 258nm. The placebo solution was used as blank.The analytical balance Marte® AY220 (Brazil), the digital ultrasonic bath Sanders Medical Soniclean 2PS (Brazil) and the incubator Tecnal TE420 were also used.

2.3 Preparation of Chitosan films and incorporation of the Brimonidine

Chitosan drug-loaded films were produced by a casting/solvent evaporation technique [23]. Solutions with approximately 500 mg of chitosan were prepared separately in 25 mL volumetric flasks. Approximately 15 mL of 2% v/v acetic acid aqueous solution was added in each flask. These solutions were sonicated for 10 minutes and the volume were adjusted to 25 mL with the same solution [24]. Approximately 56.25 mg of brimonidine was dissolved by stirring in each solution to make the solution completely homogeneous. Next, these solutions were poured on Petri glass dishes, which were transported to a greenhouse at 37 °C and maintained for 24 hours. Next, these films were dried at room temperature for 1 to 3 days [25]. The final aspect of the implants was a transparent yellow plastic.The dried films were cut into

circles of 4 mm of diameter and 0.3 mm of thick, each with a dose of 1.0 mg of brimonidine for tests [24]. The blank matrix film, without the drug,was made analogously to the process described above [25].

2.4 Preparation of the Solutions

2.4.1 Brimonidine standard solution

Approximately 56.25 mg of brimonidine was dissolved in a 25 mL volumetric flask. Approximately 15 mL of 2% v/v acetic acid aqueous solution was added. The solution was sonicated for 10 minutes and the volume was adjusted to 25 mL with the same solution.

2.4.2 Brimonidine sample solution

One implant was transferred to a volumetric flask of 25 mL and 5mL of 2% v/v acetic acid aqueous solution was added. The solution was sonicated for 10 minutes and the volume was adjusted to 25 mL with the same solution.

2.4.3 Placebo solution

One implant containing chitosan (without drug) was transferred to a 25 mL volumetric flask and 15 mL of 2% v/v acetic acid aqueous solution was added. The solution was sonicated for 10 minutes and the volume was adjusted to 25 mL with the same solution. The solution was filtered.

2.5 Method validation

The spectrophotometric method was validated by determining the parameters of linearity, selectivity (matrix effect), precision, accuracy, robustness and quantification limit [26,27].

2.5.1 Linearity and Matrix Effect

The linearity was performed according to procedures reported by Souza and Junqueira (2005) [28]. The calibration curve was constructed using six brimonidine standard concentrations (0.025; 0.05; 0.075; 0.1; 0.125 and 0.150 µg mL^{-1}) in 3 independent replicates run in random order. To verify the matrix effect, a second calibration curve was obtained using six brimonidine standard concentrations (0.025; 0.05; 0.075; 0.1; 0.125 and 0.150 µg mL^{-1}), in the presence of the chitosan solution, in 3 independent replicates run in random order. The linear regression analysis was done by the ordinal least squares method (OLSM). The residue analysis was performed [29] and outliers were deleted by using the Jacknife standardized residual test [26].

The test of Jacknife was applied successively and the maximum exclusion of 22.2% of the original points was considered [27].The validation of the use of the OLSM through the corresponding assumptions was achieved using the following tests for the residual assumptions: normality (Ryan–Joiner test) [28], homoscedasticity (Brown–Forsythe test) [30,31] and independency (Durbin–Watson test) [32]. For the model assumption, the lack-of-fit test (ANOVA) ($p > 0.05$) and the significance of the regression ($p > 0.05$) were considered [33]. Finally, considering that the linear model was adequate, the slope and intercept were obtained from the constructed calibration curves (with and without matrix), and these parameters were compared. The homogeneity of the residual variances of the calibration curves (with and without matrix) was evaluated using the lack-of-fit test (ANOVA) ($p > 0.05$) in order to verify if the slopes and intercepts could be compared by t-Student test assuming combined or distinct variances [28,34].

2.5.2 Precision

The intra-assay precision (repeatability) was evaluated by assaying solutions at concentrations of 0.05, 0.1 and 0.15 µg mL^{-1} on the same day. The solutions were prepared by incorporating the brimonidine in chitosan solution (n = 3 for each concentration). Similarly, the inter-assay precision or intermediate precision was evaluated on three consecutive days (n = 9 for each concentration). The precision was expressed as relative standard deviation (RSD) amongst responses.

2.5.3 Accuracy

Brimonidine standard solutions at three different levels of concentrations (0.05; 0.10 and 0.15 µg mL^{-1}) were added to chitosan solution. At each level, solutions were prepared and the recovery percentage was calculated (n = 3 for each concentration). The accuracy was evaluated on three different and consecutive days.

2.5.4 Robustness

Robustness was determined by analyzing the same brimonidine standard solutions of 0.1 µg mL^{-1} at the pre-established operating condition (wavelength - 258 nm) and also by changing this operating analytical condition (wavelengths - 256 and 260 nm). The brimonidine content was determined for each condition and the obtained data were submitted to statistical analysis (analysis of variance test).

2.5.5 Quantitation limit

The quantitation limit was determined with base on the peaks obtained in the spectrophotometric reading. Solutions brimonidine 0.01 mg/ml were prepared, evaluated and analyzed separately in triplicate in a spectrophotometer [35]. The value of the limit of quantification was defined as the lowest concentration which can be determined quantitatively with appropriate precision and accuracy. It was calculated from the calibration curve and could be expressed as: $\text{Quantitation limit} = 10\ \sigma/b$ where σ is the standard deviation of the response and b is the slope of the calibration curve [26,27].

2.6 Determination of brimonidine content into chitosan implants

The standard and sample solutions of brimonidine were prepared at a concentration of 0.01 mg mL^{-1}. The solutions samples (n = 10) were analyzed by the validated spectrophotometric method, while the drug content in each implant was expressed as the percentage of pre-specified value.

2.7 *In vitro* Release of brimonidine from chitosan implants

The *in vitro* release of brimonidine from chitosan implants was analyzed for 30 consecutive days. The procedure was performed in an incubator at 70 rpm and 37 °C. The implants were placed in different tubes containing 3 mL phosphate buffer solution (PBS pH 7.4) (n = 5) [24]. At predetermined intervals, 3 mL of the PBS was sampled and the same volume of fresh PBS was added to each tube. The amount of brimonidine released from the each implant was quantified by the validated spectrophotometric method and expressed as a cumulative percentage of drug released into the medium.

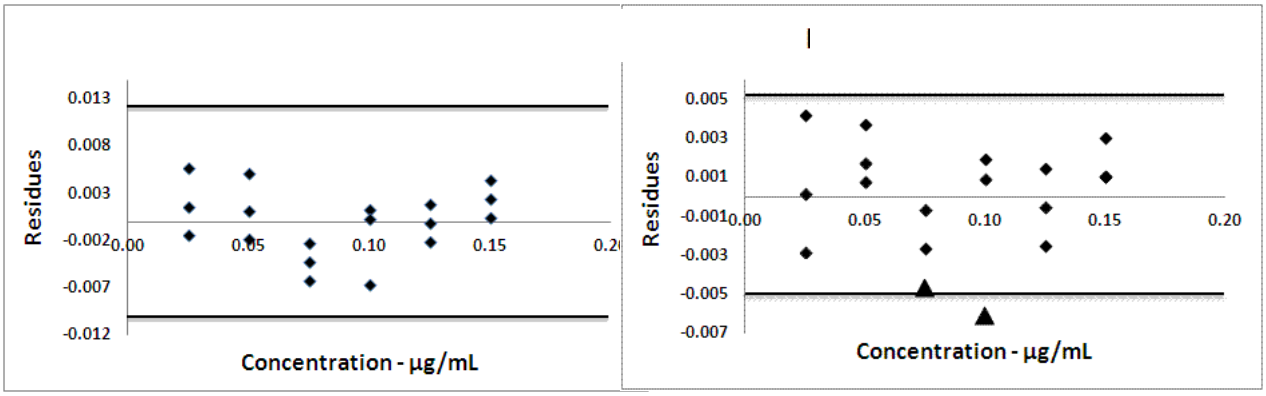
3. RESULTS AND DISCUSSION

3.1 Development of the spectrophotometric method

In this study, an ultraviolet spectrophotometric method was developed and validated for determination of brimonidine incorporated into chitosan implants and released from them. Initially, an ultraviolet spectroscopic scanning run of brimonidine standard solution was performed and an intense absorption band at 258 nm was visualized. The ultraviolet spectra of the placebo solution was also recorded and it was identified the existence of interferences or overlaps with the brimonidine response at 258 nm, indicating that all samples should be analyzed using a placebo solution as blank in order to eliminate the matrix effect [36].

3.2 Validation of the method

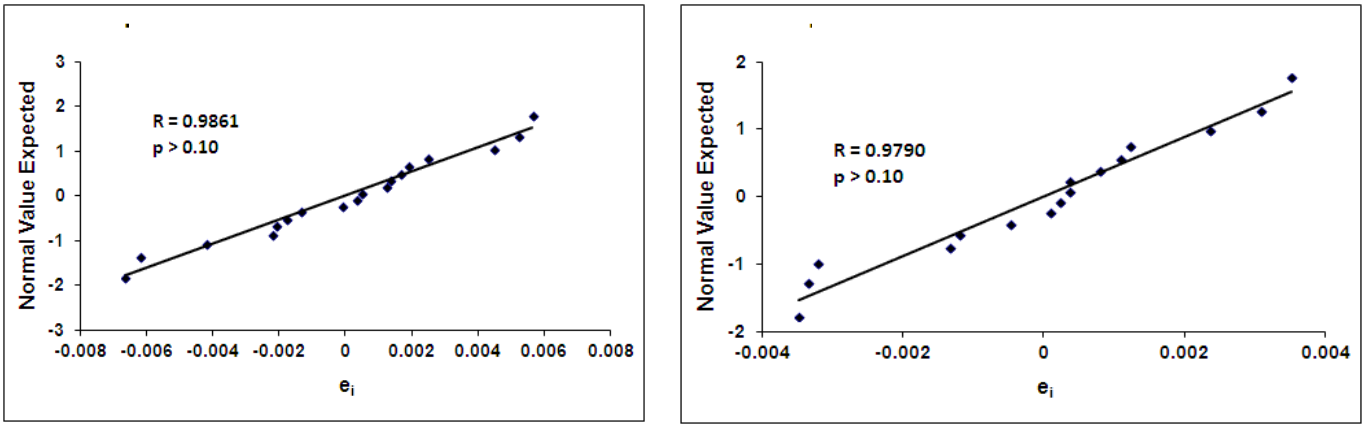
The calibration curves of pure brimonidine and brimonidine in the placebo solution were plotted using five standard concentrations of brimonidine (0.025; 0.05; 0.075; 0.1; 0.125 and 0.150 µg mL^{-1}) in 3 independent replicates run in random order. Examinations of the residual plots indicated no outliers for the calibration curve from pure brimonidine and indicated two outliers for the calibration curve from brimonidine in chitosan solution in the levels 0.075 and 0.1 µg mL^{-1} (Figure 2).



A) **B)**

Fig. 2: Residual plots for outlier diagnose by Jacknife standardised residuals test for pure brimonidine (A) and brimonidine in placebo solution (B).

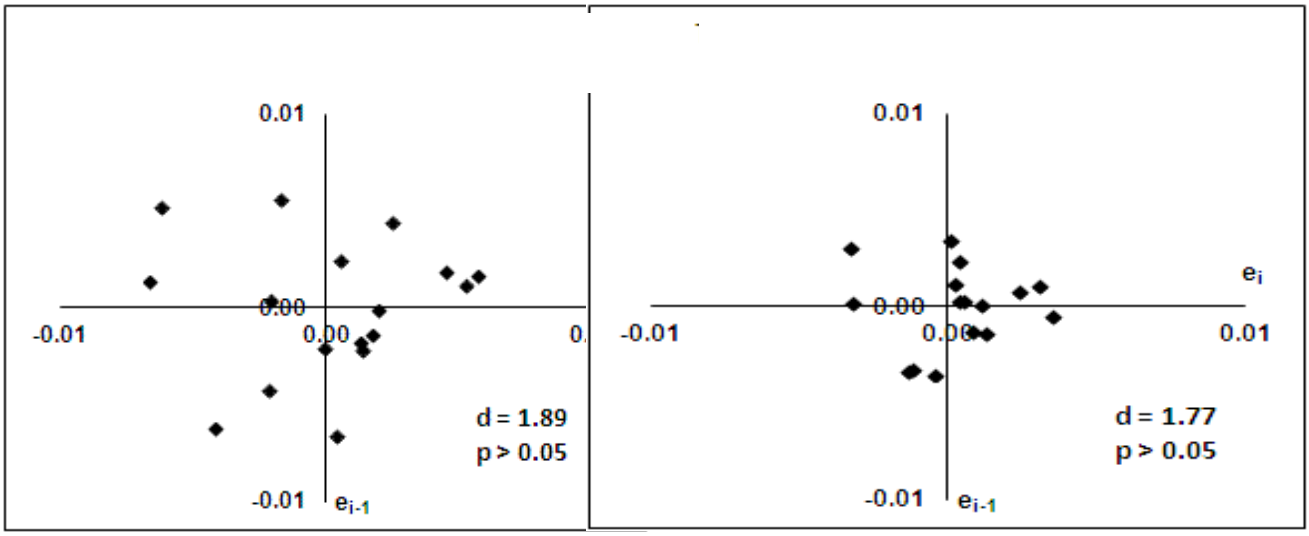
The assumption that residuals are normally distributed was confirmed. The QQ plots and the respective Ryan–Joiner correlation coefficients are illustrated in Figure 3.



A) **B)**

Fig.3: Normal QQ plots of residuals for A) pure brimonidine and B) brimonidine in chitosan solution: ei, residual and R, correlation coefficient of Ryan–Joiner test.

The quantitation limit was calculated and was found the value of 0.015 µg mL^{-1}. Residuals were statistically independent and the Durbin–Watson statistic was 1.89 (pure brimonidine) and 1.77 (brimonidine in placebo solution) ($p > 0.05$), demonstrating that the autocorrelation was not observed (Figure 4).



A) **B)**

Fig.4: Plots of residuals autocorrelation for A) pure brimonidine and B) brimonidine in chitosan solution: ei, residual and d, Durbin–Watson statistic.

Homoscedasticity was accessed by the modification proposed by Brown and Forsythe for the Levene test [37,38]. Table 1 shows that the residual variability across all concentration levels was not significantly different ($p > 0.05$), indicating homoscedasticity [28].

Table 1.Residual homoscedasticity evaluation by modified Levene Test.

Statistic	Pure Brimonidine	Brimonidine with Chitosan
t_L	0.924	1.23
P	0.369	0.240

The results of normality and homoscedasticity tests and independency of residuals indicated that the use of OLSM was appropriate. Additionally, a high significance ($p > 0.05$) of the regression was observed, while the lack-of-fit was not significant ($p > 0.05$) for the brimonidine pure and brimonidine in placebo solution curves (Table 2).

Table 2. ANOVA statistic for regression including lack-of-fit test.

Source	degrees of freedom	sum of squares	mean square	variance ratio (*F*)	significance (*p*)
Pure brimonidine				18686.33	5.64×10^{-26}
Due to regression	1	3.01×10^{-1}	3.01×10^{-1}		
Residual	16	2.58×10^{-4}	1.61×10^{-5}		
Lack-of-fit	4	4.02×10^{-5}	1.01×10^{-5}	0.555	6.99×10^{-1}
Pure error	12	2.17×10^{-4}	1.81×10^{-5}		
Total	17	3.01×10^{-1}			
Brimonidine in placebo solution				37071.46	7.54×10^{-24}
Due to regression	1	4.60×10^{-1}	4.60×10^{-1}		
Residual	13	1.61×10^{-4}	1.24×10^{-5}		
Lack-of-fit	4	9.08×10^{-5}	2.27×10^{-5}	2.899	8.52×10^{-2}
Pure error	9	7.05×10^{-5}	7.83×10^{-6}		
Total	14	4.60×10^{-1}			

Therefore, the relationship between the absorbances and the brimonidine concentrations was linear. The x-y plots and the respective OLSM statistics are presented in Figure 5.

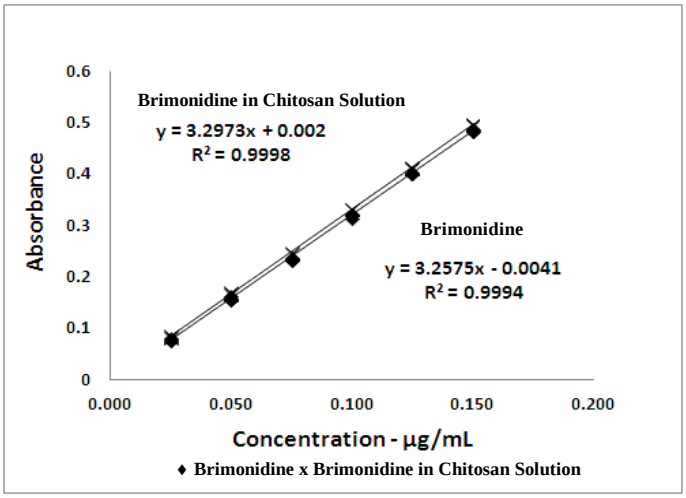


Fig.5: Analytical curves of pure brimonidine and brimonidine in placebo solution. The regression equations and determination coefficients (R^2) were expressed.

These plots demonstrated linearity in the range from 0.025 to 0.150 µg mL^{-1} for brimonidine pure and brimonidine in direct contact with the chitosan matrix [28]. To demonstrate the selectivity of the spectrophotometric method, the slopes and intercepts of the calibration curves were compared using the t-Student test. Accordingly, there was no significant difference in the slopes (t´b = 1.70 and t critic = 2.68). There was significant difference in the intercepts (t´a = 2.06 and t critic = 2.68) of the calibration curves of the pure brimonidine and brimonidine in chitosan solution, indicating existence of the matrix effect.

The repeatability or intra-assay precision and intermediate precision or inter-assays precision were expressed as the relative standard deviation (RSD) of a series of measures of different concentrations of brimonidine in chitosan solutions. The data obtained for precision were summarized in Table 3.

Table 3. Mean content of brimonidine in the intra-assay and inter-assay precision.

Intra-assay precision (Day 1)			**Intra-assay precision (Day 2)**		
Addition (µg / mL)	**Recovery (%)**	**Relative Error**	**Addition (µg / mL)**	**Recovery (%)**	**Relative Error**
0.05	96.0 ± 4.3 (RSD = 4.3)	4.1	0.05	97.1 ± 3.2 (RSD = 3.3)	2.9
0.1	99.2 ± 3.6 (RSD = 3.7)	0.8	0.1	98.0 ± 1.4 (RSD = 1.5)	2
0.5	99.1 ± 2.3 (RSD = 2.4)	0.9	0.5	102.0 ± 1.0 (RSD = 0.9)	-2
Intra-assay precision (Day 3)			**Inter-assay precision**		
Addition (µg / mL)	**Recovery (%)**	**Relative Error**	**Addition (µg / mL)**	**Recovery (%)**	**Relative Error**
0.05	97.5 ± 2.3 (RSD = 2.4)	2.6	0.05	96.9 ± 3.3 (RSD = 3.4)	3.2
0.1	102.0 ± 2.0 (RSD = 1.6)	-1.6	0.1	99.6 ± 2.8 (RSD = 2.8)	0.4
0.5	102.0 ± 0.5 (RSD = 0.5)	-2.1	0.5	101.0 ± 2.0 (RSD = 2)	-1

The RSD values were lower than 5% for all levels of concentrations, indicating appropriate intra-assay and inter-assays precision of the spectrophotometric method [30]. Additionally, it was observed that recoveries ranged from 96 to 102%, indicating accuracy of the developed method [40], which showed relative errors less than 5%.

In the robustness (Table 4), the recoveries ranged from 97 to 101% at wavelengths evaluated and statistical analysis showed no significant difference between results obtained. Thus, the

brimonidine content remained unaffected by deliberated modification of a specific spectrophotometric parameter, demonstrating the robustness of the method.

Table 4: Recovery of brimonidine to assess the robustness.

Wavelength (nm)	Recovery (%)
256	99.2 ± 0.7 (RSD = 0.7)
258	100.0 ± 1.0 (RSD = 1)
260	98.4 ± 0.7 (RSD = 0.7)

3.3 Determination of the content of brimonidine into chitosan implants

Brimonidine incorporated into polymeric implants was determined by applying validated spectrophotometric method. The results showed that the content of brimonidine was uniformly distributed in implants of chitosan and no unit was outside of the interval of 85 to 115% of the pre-indicated amount of brimonidine (1.0 mg of drug per implant), as shown in Table 5.

Table 5. Brimonidine content into chitosan implants (n = 10).

Implants	Content of Brimonidine (%)
1	104.85
2	99.39
3	100.00
4	105.76
5	100.91
6	104.85
7	99.39
8	100.00
9	106.36
10	103.94
Mean Content	102.55
RSD	2.78%

3.4 *In vitro* release of brimonidine incorporated into chitosan implants

Brimonidine leached from the chitosan implant was measured using the validated spectrophotometric method. Figure 6 shows the cumulative release of brimonidine from implants of chitosan over a period of 30 consecutive days. The implants promoted a controlled and sustained release of the drug, once the entire content of brimonidine was leached from the implant during the analyzed period.

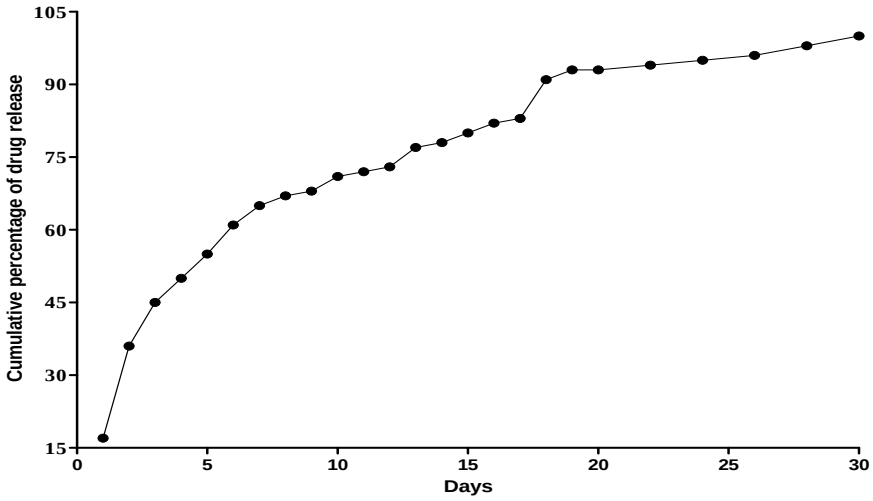


Fig. 6 *In vitro* brimonidine release from the implants (n = 5). RSD was lower than 5% for all samples.

4. CONCLUSION

A spectrophotometric method was developed and validated in terms of linearity, matrix effect, quantitation limit, precision, accuracy and robustness to determine the brimonidine-loaded chitosan implants. The validated method quantified unequivocally the brimonidine content into chitosan implants. Additionally, the analytical method demonstrated the controlled and sustained *in vitro* release of brimonidine from the polymeric implantable devices.

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REFERENCES

1. Mello P, Mandía-Júnior C. II Brazilian Consensus on Primary Open-Angle Glaucoma. Planmark, 2005.
2. Paranhos-Júnior A, Omi C, Prata-Júnior J. Brazilian Society of Brazilian Consensus on Glaucoma. Best Point, 2009.
3. Foster A, Gilbert C, Johnson G. Changing patterns in global blindness: 1988-2008. Community Eye Health, 2008; 21 (1): 37-39.
4. Bell NP, Ramos JL, Feldman RM. Safety, tolerability, and efficacy of fixed combination therapy with dorzolamide hydrochloride 2% and timolol maleate 0.5% in glaucoma and ocular hypertension. Clin Ophthalmol, 2010; 4 (1): 1331-1346.
5. Claxton AJ, Cramer J, Pierce C. A systematic review of the associations between dose regimens and medication compliance. Clin Ther, 2001; 23 (1): 1296-1310.
6. Schuman JS. Short and long-term safety of glaucoma drugs. Expert Opin Drug Saf, 2002;1 (1): 181-194.
7. Cantor LB. Brimonidine in the treatment of glaucoma and ocular hypertension. Ther Clin Risk Manag, 2006;2 (1): 337–346.
8. Boyle JE, Ghosh K, Gieser DK, Adamsons IA. A randomized trial comparing the dorzolamide-timolol combination given twice daily to monotherapy with timolol and dorzolamide: Dorzolamide-Timolol Study Group. Ophthalmol, 1998; 105 (1): 1945-1951.
9. Razeghinejad MR, Sawchyn AK, Katz LJ. Fixed combinations of dorzolamide-timolol and brimonidine-timolol in the management of glaucoma. Expert Opin Pharmacother, 2010; 11 (1): 959-968.
10. Russ HH. Glaucoma and Ocular Surface: Evidence Based Medicine. http://www.universovisual.com.br/publisher/preview.php?id_mat=3098.
11. Shirke RR, Pai N. RP-HPLC determination of brimonidine tartrate in brimonidine tartarate eye drops. Ind Drugs, 2002; 39 (9): 484-486.
12. Ni J, Rowe R, Heidelbaugh T, Sinha S, Acheampong A. Characterization of benzimidazole and other oxidative and conjugative metabolites of brimonidine *in vitro* and in rats *in vivo* using on-line H/D exchange LC-MS/MS and stable-isotope tracer techniques. Xenobiotica, 2007; 37 (2): 205-220.
13. Jiang S, Chappa AK, Proksch JW. A rapid and sensitive LC/MS/MS assay for the quantitation of brimonidine in ocular fluids and tissues. J of Chromat B, 2009; 877 (3): 107-114.

14. Kvapil L, Grepl M, Hradil P. ACTA Universitatis Palackianae Olomucensis Facultatis Medicae, 2003; 42: 19-26.
15. Acheampong A, Tang-Liu DDS. J Pharma Biomed Anal, 1995; 13: 995-1002.
16. Karamanos NK, Lamari F, Katsimpris J, Gartaganis S. Bio Chromat, 1999; 13: 86-88.
17. Acheampong AA, Shackleton M, John B, Burke J, Wheeler L, Tang-Liu DDS. Drug Metab Dispos, 2002; 30: 421-429
18. Chien DS, Homsy JJ, Gluchowskil C, Tang-Liu DDS. Curr Eye Res, 1990; 9: 1051-1059.
19. Tzovolou DN, Lamari L, Mela MK, Gartaganis SP, Karamanos SK. Capillary electrophoretic analysis of brimonidine in aqueous humor of the eye and blood sera and relation of its levels with intraocular pressure. Biomed Chromat, 2000; 14 (5): 301-305.
20. Acheampong A, Diane DN. Measurement of brimonidine concentrations in human plasma by a highly sensitive gas chromatography/mass spectrometric assay. J Pharma Biomed Anal, 1995; 3:995-1002.
21. Chandran S, Pandey S, Deshpande P, Bhagav P. Development and validation of stability indicating UV spectrophotometric method for the estimation of brimonidine tartrate in pure form, formulations and preformulation studies. Der Pharmacia Lettre, 2010; 2 (3): 106-122.
22. Aburahma MH, Mahmoud AA. Biodegradable ocular inserts for sustained delivery of brimonidine tartarate: preparation and *In Vitro/In Vivo* evaluation. AAPS PharmSciTech, 2011; 12 (4): 1335-1347.
23. Rodrigues LB, Leite, HF, Yoshida, MI. *In vitro* release and characterization of chitosan films as dexamethasone carrier. Int J Pharm, 2009; 23:1–6.
24. Ribeiro CRV. Effect of slow-release implants of Bevacizumab intrabeculectomy experimental rabbits. Master's dissertation. RibeirãoPreto, Brazil, 2011.
25. Fulgêncio, GO, Viana, FAB, Ribeiro, RR, Yoshida, MI, Faraco, AG, Cunha-Júnior, AS. New Mucoadhesive Chitosan Film for Ophthalmic Drug Delivery of Timolol Maleate: *In Vivo* Evaluation. J Ocular Pharm&Ther, 2012; 28 (4): 350-358.
26. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005. Validation of Analytical Procedures: Text and Methodology.
27. Brazil, National Agency for Sanitary Vigilance, Resolution RE No. 899, 29/5/2003. Guide for validation of analytical and bioanalytical methods, Official Gazette, Executive, Brasilia, 2003.

28. Souza SVC, Junqueira RG. A procedure to assess linearity by ordinary least squares method. Anal Chim Acta, 2005; 552: 25-35.
29. United States Pharmacopoeia; 34th ed, The United States Pharmacopoeial Convention: Rockville, 2011.
30. Meyer PC, Zund RE. Statistical methods in analytical chemistry. John Wiley & Sons, New York, 1993.
31. Belsley DA, Kuh E, Welsch RE. Regression diagnostics: identifying influential data and sources of collinearity. Wiley, New York, 1980.
32. Horwitz W. Protocol for the design, conduct and interpretation of method-performance studies: Revised 1994 (Technical Report), Pure Appl Chem, 1995; 67: 331-343.
33. Ryan TA, Joiner BL. Normal probability plots and tests for normality. The State College, Pennsylvania State University, 1976.
34. Snedecor GW, Cochran WG. Statistical methods. 8th ed, Jowa State University, Ames, 1996.
35. Saliba JB, Júnior ASC, Gomes ECL, Mansur HS, Silva GR. Development and Validation of a High Performance Liquid Chromatographic Method for Determination of Cyclosporine-A from Biodegradable Intraocular Implants. Quim Nova, 2011; 34 (1): 140-144.
36. Silva GR, Rodrigues FF, Perasoli FB, Pereira AF, Andrade FP. Development and Validation of Spectrophotometric Method for the Determination of Digoxin Controlled Released from Polymeric Implants. World J Pharm Res, 2013; 2 (6): 3255-3269.
37. González AG, Herrador MA. A pratical guide to analytical method validation, including measurement uncertainty and accuracy profiles. Trends Anal Chem, 2007; 26(3): 227-238.
38. Santos MCM, Costa VM, Pereira AF, Silva-Cunha A, Filho SL, Gomes AJPS, Silva GR. Development and validation of spectrophotometric method for determination of methotrexate incorporated into PLGA implants. Int J Drug Dev & Res, 2013; 5 (1): 154-160.39.