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Simultaneous quantification of carteolol and dorzolamide in rabbit aqueous humor and ciliary body by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry

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ABSTRACT

A rapid, sensitive and selective method for the simultaneous quantification of carteolol and dorzolamide in rabbit aqueous humor (AH) and ciliary body (CB) has been developed and validated using reversed phase-high performance liquid chromatography (RP-HPLC) with isocratic elution coupled with atmospheric pressure chemical ionization mass spectrometry/mass spectrometry (APCI-MS/MS). The analytes and nadolol (used as internal standard, IS) were purified from AH by protein precipitation. The sample preparation from CB was based on a two steps extraction procedure at different pH, utilizing a liquid–liquid extraction with a mixture of ethyl acetate, toluene and isopropanol 50:40:10 (v/v) at pH 8, followed by a second extraction with ethyl acetate at pH 11. The combined organic extracts were then back extracted into 0.1% aqueous trifluoroacetic acid (TFA). The accuracy and precision values, calculated from three different sets of quality control samples analyzed in sestuplicate on three different days, were within the generally accepted criteria for analytical methods (<15%). The assay proved to be accurate and precise when applied to the *in vivo* study of carteolol and dorzolamide in rabbit AH and CB after single administration of an eye drops containing both drugs.

1. Introduction

β-Blockers or β-adrenoceptor antagonists are a class of therapeutically important drugs widely used for the management of angina pectoris, cardiac arrhythmias, hypertension and myocardial infarction. They are also used in the treatment of glaucoma and ocular hypertension [1,2] due to their property of lowering the intraocular pressure (IOP) by decreasing the production of aqueous humor (AH) at the ciliary process [3,4]. Carteolol (Fig. 1a) is a β-blocking agent with an intrinsic sympathomimetic action. All β-blockers, such as carteolol, with intrinsic sympathomimetic activity, are drugs that exert a partial agonism at the adrenergic receptor while simultaneously block natural endogenous agonist from binding to the receptor. This activity has proved effective in a real reduction of some side effects [5]. Oral carbonic anhydrase inhibitors (CAIs) have been used to lower IOP for the past 40 years. However, the use of these agents have been limited due to associated side effects that include general malaise, fatigue, depression, loss of appetite, gastrointestinal disturbances, weight loss, paresthesias and renal calculi [6,7]. Dorzolamide (Fig. 1b) is a potent and selective inhibitor of human carbonic anhydrase II (CAII), an enzyme that is present in the ciliary epithelium where it plays an important role in the formation of AH [8–10]. The inhibition of CAII by dorzolamide, that presumably takes place by slowing the formation of bicarbonate ions with subsequent reduction in sodium and fluid transport, decreases AH production and lowers IOP in patients with glaucoma or ocular hypertension and who are insufficiently responsive to β-blockers.

Modern fixed combinations pair β-blockers with either prostaglandin analogs [11] or carbonic anhydrase inhibitors [12]. Potential benefits of these combinations include fewer drops per day, fewer bottles of medication (and potentially fewer copayments), reduction in exposure to preservatives, and elimination of the washout effect. For these reasons, a fixed combination for ophthalmic use containing carteolol and dorzolamide at various concentrations has been formulated. Consequently, it is high desirable to have an analytical assay for the simultaneous extraction and determination of carteolol and dorzolamide in the ocular matrices, so as to reduce the time of analysis, with a considerable economic saving, especially when the number of samples to be analyzed is very large.

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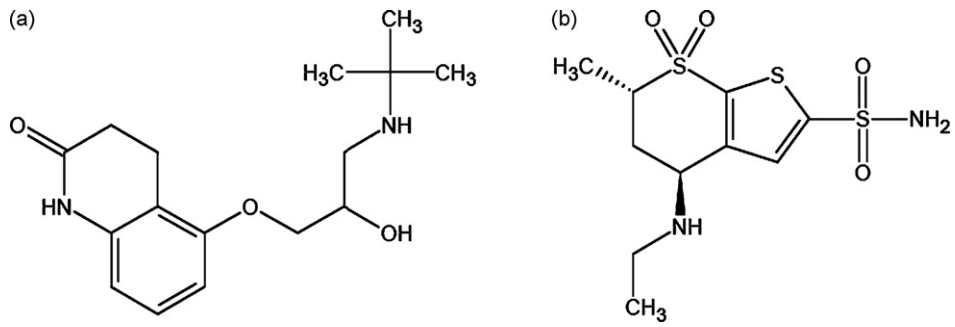

Fig. 1. Chemical structure of carteolol (a) and dorzolamide (b).

In the last years, mass spectrometry (ESI or APCI) coupled with RP-HPLC has become an alternative and powerful technique for quantitative biological analyses. Chromatographic procedures have been reported for the quantification of several β-blockers [13] and dorzolamide [14] in human plasma based on HPLC/APCI-MS. In this paper we describe a rapid, sensitive and selective RP-HPLC/APCI-MS/MS method for the simultaneous determination of carteolol and dorzolamide in rabbit AH and ciliary body (CB) and its application to an *in vivo* study.

2. Experimental

2.1. Chemicals and reagents

Carteolol hydrochloride (purity > 99%) was purchased from Otsuka Pharmaceutical (Tokyo, Japan), dorzolamide hydrochloride (purity > 98%) from Neuland Laboratories Limited (Andhra Pradesh, India) and nadolol (purity 99.8%) from Sigma (Milan, Italy). Formic acid (FA), acetic acid (AA) and trifluoroacetic acid (TFA) were obtained from Fluka (Milan, Italy). Sodium hydroxide, mono- and dibasic sodium phosphate, ethyl acetate, toluene, isopropanol and methanol (HPLC grade) were purchased from Merck (Milan, Italy). Distilled water was prepared with a Milli-Q Synthesis A10 System (Millipore, Milan, Italy), HPLC grade water and acetonitrile were provided by Carlo Erba (Milan, Italy). Blank, drug-free AH and CB samples were collected from adult, healthy male pigmented rabbits in the SIFI's laboratory. All chemicals and solvents were of the highest purity commercially available and were used without further purification.

2.2. In vivo study

Male pigmented rabbits, weighing 1.8–2.2 kg, used throughout the study were purchased from Harlan (Italy). Prior to experiments the rabbits were housed in standard cages and allowed free access to food and water. The experiments using rabbits were carried out according to the Guiding Principles in the Care and Use of Animals (DHEW Publication NIH 80-23) and the ARVO Resolution on the Use of Animals in Research.

In vivo studies were carried out treating rabbits with a single administration of 50 μL of an eye drops containing carteolol hydrochloride and dorzolamide hydrochloride at various concentrations. The vehicle was phosphate buffer isotonic and at different pH.

After euthanasia, AH and CB were collected from the rabbits in polypropylene tubes after administration of the eye drops in a time course of 15–360 min. All AH and CB samples were sealed and stored at −80 °C until analysis. The samples were extracted and processed as described in Sections 2.4.1 and 2.4.2.

2.3. Preparation of standard and QC solutions

Stock solutions of carteolol hydrochloride, dorzolamide hydrochloride and nadolol were prepared at a concentration of 1 mg/mL dissolving 10 mg of each compound in 10 mL of methanol and stored at −20 °C.

For the validation of AH analytical procedure, the stock solutions of carteolol, dorzolamide and nadolol were diluted daily with methanol to obtain working solutions of appropriate concentration. 50 μL of each working solution were added to 150 μL of AH blank to prepare six calibration standards at the following concentrations: 50, 125, 250, 500, 1000 and 2500 ng/mL for carteolol and 125, 250, 500, 1000, 2500 and 5000 ng/mL for dorzolamide. The IS concentration was 500 ng/mL. The QC samples for carteolol were prepared in AH blank at the following three different concentrations: 100 ng/mL (the low QC, LQC), 400 ng/mL (the medium QC, MQC) and 2000 ng/mL (the high QC, HQC). The QC samples for dorzolamide were prepared in AH blank at the following three different concentrations: 200 ng/mL (LQC), 1250 ng/mL (MQC) and 4000 ng/mL (HQC).

For the validation of the CB analytical procedure, the stock solutions of carteolol, dorzolamide and nadolol were diluted daily with methanol to obtain working solutions of appropriate concentration. Aliquots of the working solutions were then diluted with CB blank in phosphate buffer to a final volume of 2 mL to achieve six calibration standards containing carteolol and dorzolamide at the following concentrations: 2, 5, 10, 20, 40 and 80 μg/g of CB for both analytes. The IS concentration was 20 μg/g. QC samples were prepared independently in CB blank at the following three different concentrations for both analytes: 6 μg/g (LQC), 32 μg/g (MQC) and 64 μg/g (HQC).

2.4. Extraction procedures

2.4.1. Aqueous humor

For the preparation of AH samples for quality control (QC) and calibration curve determination, 150 μL of rabbit AH blank were spiked with 50 μL of the appropriate working solution of each analyte and 50 μL of IS solution at a concentration of 3 μg/mL. For the *in vivo* study, 100 μL of methanol and 50 μL of IS at a concentration of 3 μg/mL were added to 150 μL of AH obtained from treated rabbits. All samples were vortexed for 60 s and cooled for 30 min in ice. After centrifugation at 12,000 rpm for 10 min at 4 °C, 250 μL of the supernatant were evaporated to dryness under vacuum in a concentrator (Eppendorf 5301). The residue was reconstituted in 250 μL of the HPLC mobile phase and 20 μL were injected into the HPLC/MS system.

2.4.2. Ciliary body

For the preparation of CB samples for QC and calibration curve determination, 25 mg of rabbit CB blank in 2 mL of phosphate buffer

(pH 8.0) were spiked with an adequate aliquot of working solution of each analyte and 25 μL of IS solution at a concentration of 20 μg/mL. For the *in vivo* study, 25 mg of CB from treated rabbits were added to 2 mL of phosphate buffer (pH 8.0) and spiked with 25 μL of IS solution at a concentration of 20 μg/mL. All samples were vortexed for 60 s and a liquid–liquid extraction was performed by shaking the aqueous layer for 20 min with 5 mL of ethyl acetate, toluene and isopropanol 50:40:10 (v/v). After centrifugation at 5000 rpm for 5 min at 25 °C, the organic layer was transferred into a flask. Then, 100 μL of 4% aqueous NaOH (pH 11.0) was added to the remaining aqueous phase and a second liquid–liquid extraction was performed for 20 min with 5 mL of ethyl acetate. After centrifugation, the organic layer was combined with the first organic extract and 200 μL of 0.1% aqueous TFA were added to the resulting organic phase. The two layers were vortexed for 15 min and centrifuged for 5 min. The aqueous phase was separated and evaporated to dryness under vacuum in a concentrator. The residue was reconstituted with 1 mL of the HPLC mobile phase and 20 μL were utilized for the RP-HPLC/APCI-MS/MS analysis.

2.5. HPLC/APCI-MS/MS conditions

The quantitative analyses were performed using a Surveyor MS pump and a Surveyor autosampler maintained at 25 °C. 20 μL of each sample were loaded onto a reversed phase Waters Simmetry C_{18} column (150 mm × 2.1 mm i.d., 100 Å, 3.5 μm) equipped with a precolumn. The column was eluted at 25 °C with H_2O + 0.5% (v/v) FA/CH_3CN + 0.5% (v/v) FA 90:10 (v/v) (mobile phase) for 15 min. The flow rate was 0.180 mL/min. The HPLC system was interfaced with a ThermoFinnigan LCQ-DECA ion trap mass spectrometer equipped with an APCI ion source operating in MS/MS positive ion mode under the following conditions: vaporizer temperature 420 °C; capillary temperature 180 °C, sheat gas 80 a.u.; corona discharge needle +5 μA. MS/MS spectra were scanned in the *m*/*z* range 190–290 for carteolol, 230–330 for dorzolamide and 200–300 for IS, consecutively. The collision energy was 28% a.u. for carteolol and IS, and 25% a.u. for dorzolamide. The isolation width, activation Q and activation time were 3 mass units, 0.250 and 30.00 ms, respectively. Quantitative data was processed using the "Excalibur LCquan" software.

2.6. Method validation

Validation was carried out on three different days for each matrix, following the guidelines for Bioanalytical Method Validation published by the FDA [15].

2.6.1. Selectivity, calibration linearity, detection and quantification limits

AH and CB blank samples from six different rabbits were analyzed with the described procedure for peaks interfering with the detection of the analytes or the IS.

The AH and CB calibrators at six concentration levels obtained as described in Section 2.3 and subjected to the respective extraction procedures (see Sections 2.4.1 and 2.4.2) were used to verify the linearity. The ratio of the peak areas of the analyte to the peak areas of IS was calculated. Calibration curves for carteolol and dorzolamide were constructed by unweighted (Equal) least-squares linear regression analysis of the peak area ratios of analyte/IS versus the concentration of analyte, were used to quantify carteolol and dorzolamide in AH and CB, and were employed to obtain QC values from their peak area ratios. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value [15].

The limit of detection (LOD), and limit of quantification (LOQ) were determined on the basis of the standard deviation of the response and the slope using the equation reported [16].

2.6.2. Accuracy and precision

QC samples (LQC, MQC and HQC), run in sestuplicate at each concentration level on three different days, were used to assess the accuracy and intra-day and inter-day precision of the method. The accuracy and precision were calculated using the formulas published elsewhere [17] and the criteria for acceptability of the data included accuracy within ±15% standard deviation (SD) from the nominal values and precision within ±15% relative standard deviation (RSD) [15].

2.6.3. Stability experiments

All stability studies were conducted at two concentration levels, LQC and HQC, using three replicates at each concentration level. Freeze-thaw was evaluated to the third cycle. In each cycle samples were frozen at −20 °C for 20 h, thawed and kept at room temperature (25 °C) for 4 h.

To estimate short-term stability QC samples were left at room temperature and analyzed after 8 h. Analyte stability for long-term storage was tested by analyzing QC samples after storage at −20 °C for 35 days. For estimation of stability of the processed samples under the conditions of HPLC–MS analysis QC, samples were left in the autosampler at 25 °C and injected, under the routine analytical run conditions, after 20 h.

Samples are considered to be stable if assay values are within the acceptable limits of accuracy (±15% SD) and precision (±15% RSD) [15].

2.6.4. Recovery, matrix effect and process efficiency

In order to determine the recovery, matrix effect and process efficiency, two groups (pre-extraction and post-extraction spiked) of LQC, MQC and HQC samples were prepared in quadruplicate. The pre-extraction QC samples were obtained as described in Sections 2.4.1 and 2.4.2 for AH and CB, respectively. The post-extraction spiked QC samples were prepared applying the same procedures on AH and CB blank. The extracts were then spiked with the appropriate amount of the working solutions of carteolol and dorzolamide before drying the samples.

The recovery was calculated as the response ratio (carteolol or dorzolamide peak area/IS peak area) measured in pre-extraction spiked samples, as a percentage of that measured from post-extraction spiked samples.

The matrix effect was determined by comparison of response ratio in post-extraction spiked QC samples (value *A*) with that of response ratio observed in HPLC mobile phase (value *B*). The matrix effect was calculated as $(1 - A/B) \times 100$.

The process efficiency was estimated as the analyte response observed after the extraction versus the analyte response observed in the HPLC mobile phase.

3. Results and discussion

3.1. Extraction procedures

Sample preparation is a basilar step for determination of analytes in biological samples. Protein precipitation using methanol was found appropriate for the quantification of carteolol and dorzolamide in rabbit AH. In fact, this ocular fluid contains smaller amounts of proteins compared to other samples. Therefore, satisfying results were obtained adopting a simpler preparation procedure. On the contrary, the quantitative extraction of the two analytes together with the IS from CB required the development of

a much more laborious procedure. Liquid–liquid extraction with various organic solvents (methyl-tert-butyl ether, diethyl ether, dichloromethane, hexane, ethyl acetate, toluene and isopropanol) was evaluated. Finally, it was found that two extractive steps at different pH values were necessary. In the first extraction step at pH 8.0, only dorzolamide (pK_a of the protonated secondary amino group 6.35; pK_a of the sulphonamide group 8.5) is in the neutral form and therefore is extracted by the organic phase (ethyl acetate, toluene and isopropanol 50:40:10, v/v) while carteolol and nadolol (pK_a of the protonated secondary amino group 9.75 and 9.67, respectively), being mostly in the protonated form, remained prevalently in the aqueous layer. Increasing the pH to 11.0, these two compounds deprotonate and are consequently extracted by ethyl acetate. The analytes and IS were then back extracted from the combined organic phase with 0.1% TFA and the acidic aqueous phase, containing all the three compounds, was separated, evaporated and redissolved in the mobile phase for the HPLC/MS analysis.

3.2. Mass spectrometry

The possibility of using electrospray ionization (ESI) or APCI in the positive and negative ion mode was investigated. APCI in the positive ion mode was found to be the most sensitive ionization technique for the two analytes and IS. In a direct infusion experiment, the mass spectra for carteolol, dorzolamide and IS showed peaks at *m*/*z* 293.2, 325.0, 310.2, corresponding to the respective protonated molecular ions $[M+H]^+$.

To eliminate possible isobaric ions that can interfere in the quantitative analysis and improve sensitivity and selectivity, quantification of the studied analytes was performed in MS/MS mode rather than in MS full scan. The $[M+H]^+$of carteolol at *m*/*z* 293.2 was then isolated and fragmented in the ion trap. The MS/MS spectrum is characterized by an abundant signal at *m*/*z* 237.1, due to a neutral loss of isobuthene from the $[M+H]^+$ (Fig. 2a). This fragment ion was chosen for the quantification of carteolol. Similarly, the following fragment ions were used for quantification purposes: for the dorzolamide fragment ion at *m*/*z* 280.0 (Fig. 2b), arising from the precursor ion at *m*/*z* 325.0 by the elimination of ethylamine, and for the IS fragment ion at *m*/*z* 254.1 (Fig. 2c), corresponding to a loss of isobuthene from the *m*/*z* 310.2 precursor ion.

3.3. Liquid chromatography

In order to detect the two analytes together with the IS in a single chromatographic run, several procedures were tested. In particular, different stationary reversed phases, eluents and composition of ionization agents (FA, AA and TFA) in the mobile phase were assayed. A Waters Simmetry C_{18} column, eluted at 25 °C with H_2O + 0.5% (v/v) FA/CH_3CN + 0.5% (v/v) FA 90:10 (v/v) at a flow rate of 0.180 mL/min, was selected because it provided the best conditions for the elution of the compounds. In these conditions, symmetric peaks and suitable short retention times were obtained for all the molecules. Dorzolamide, carteolol and IS were eluted at 3.70, 7.57 and 7.93 min, respectively, as reported in Fig. 3a. Fig. 3

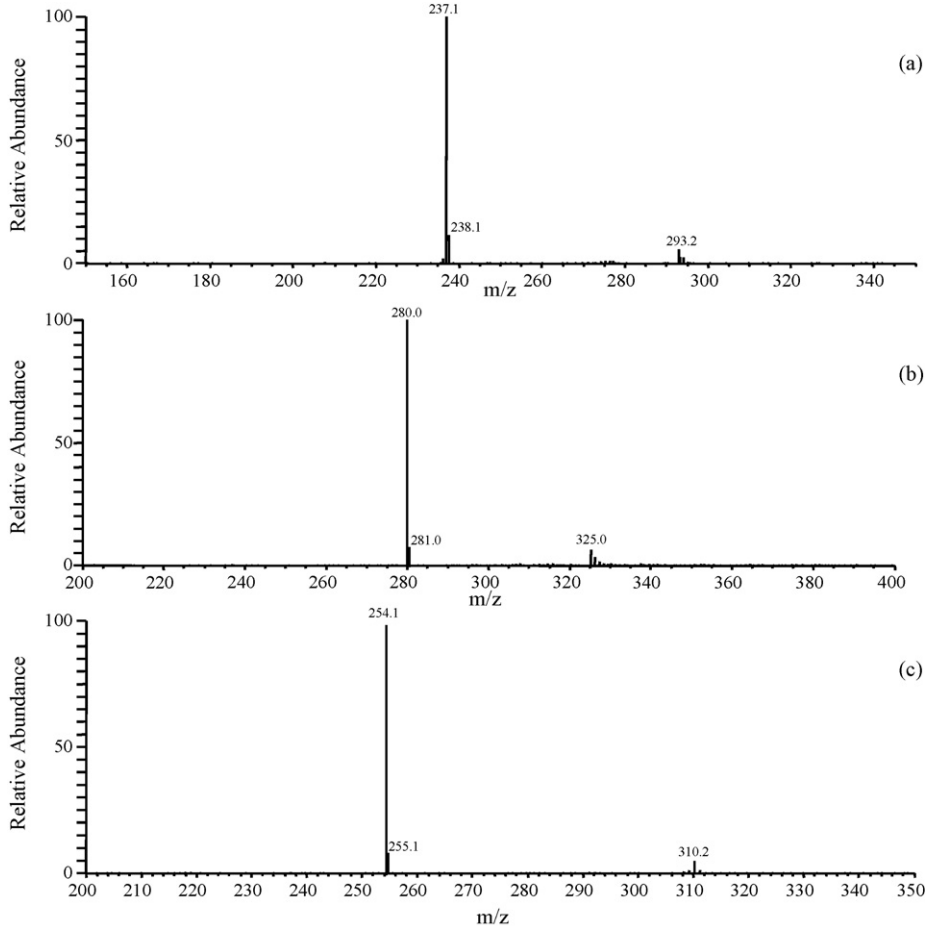

Fig. 2. Full scan product ion mass spectra of the [M+H]+ at *m*/*z* 293.2 (carteolol) in the HPLC mobile phase (a), *m*/*z* 325.0 (dorzolamide) (b) and *m*/*z* 310.2 (nadolol) in the HPLC mobile phase (c).

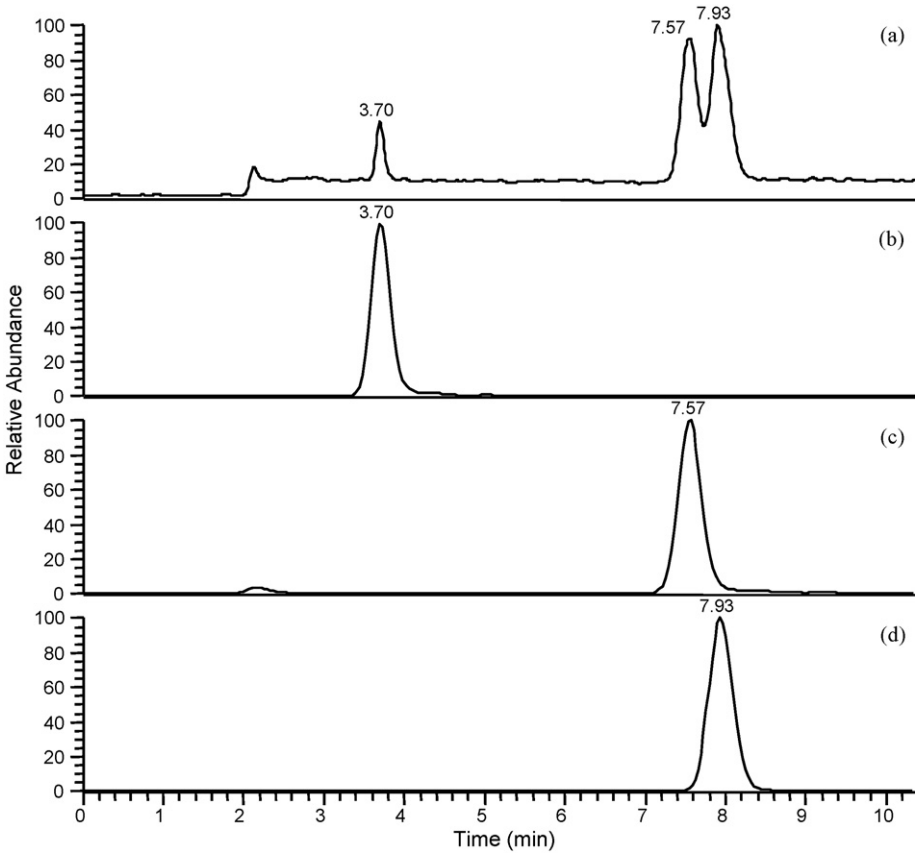

Fig. 3. (a) Total ion current (TIC) of dorzolamide, carteolol and nadolol, (b) single ion current (SIC) of the fragment ion at *m*/*z* 280.0 for dorzolamide, (c) SIC of the fragment ion at *m*/*z* 237.1 for carteolol, and (d) SIC of the fragment ion at *m*/*z* 254.1 for nadolol.

also shows the single ion current (SIC) of the fragment ion at *m*/*z* 280.0 for dorzolamide (Fig. 3b), the SIC of the fragment ion at *m*/*z* 237.1 for carteolol (Fig. 3c) and the SIC of the fragment ion at *m*/*z* 254.1 for IS (Fig. 3d). Even if carteolol and IS eluted unresolved, no attempts were made to resolve the two components because the quantification of each analyte is determined monitoring the area of its specific fragment ion, and therefore complete separation of the compounds is not required.

3.4. Assay validation

The assay was found to be selective for all tested compounds. No interfering peaks were observed in the extract of AH and CB samples. The six-point calibration curve, obtained by unweighted (Equal) linear regression, showed good linearity over the whole concentration range (50–2500 ng/mL for carteolol and 125–5000 ng/mL for dorzolamide in AH; 2–80 μg/g for both analytes in CB), which covered the concentrations typically found in AH and CB, respectively, in treated rabbits. The correlation coefficients for regression equations, generated in three different days, were 0.9998 ± 0.0001 (mean ± standard deviation) in AH and 0.9999 ± 0.0001 in CB for carteolol. For dorzolamide, they were 1.0000 ± 0.0001 in AH and 0.9998 ± 0.0003 in CB.

The slope and the intercept were calculated for each calibration curve. A slope of 0.00202 ± 0.00006 (mean ± standard deviation) and an intercept of 0.04418 ± 0.00602 for carteolol and a slope of 0.00040 ± 0.00002 and an intercept of 0.00519 ± 0.00572 for dorzolamide were determined in AH. A slope of 0.10488 ± 0.01204 and an intercept of −0.02914 ± 0.04682 for carteolol and a slope of 0.01286 ± 0.00085 and an intercept of 0.00338 ± 0.00249 for dorzolamide were calculated in CB.

The accuracy observed for the mean back-calculated concentrations, calculated as the percentage deviations from nominal concentration (DEV%), were less than ±3% for each analyte in each matrix; while the precision values, calculated as the relative standard deviation (RSD%), were less than 4% for each analyte in each matrix (Table 1). This indicates that the data of the unweighted (Equal) linear regression equation is in accordance with the acceptable criteria. The LOD was determined as 0.25 ng/mL for carteolol and 0.58 ng/mL for dorzolamide in AH, and 2.53 ng/g for carteolol and 0.35 μg/g for dorzolamide in CB. The LOQ values were 0.75 and 1.77 ng/mL for carteolol and dorzolamide, respectively, in AH, and 7.68 ng/g and 1.06 μg/g for carteolol and dorzolamide, respectively, in CB.

The accuracy and precision of the assay were determined as described in Section 2 and the data is shown in Table 2. The within-run and between-run precision values were less than 1% for both analytes in both matrices. Likewise, the accuracy was less than ±0.5% of the nominal value for both analytes in both matrices. The accuracy and precision values were found to be within the accepted limits.

The mean observed concentrations deviated less than ±2% at LQC and HQC concentrations for carteolol and dorzolamide in both matrices for all stability tests (Tables 3 and 4). The results were found to be within the assay variability limits during the entire process.

Recovery, matrix effect and process efficiency were determined as described in Section 2 and the data is shown in Table 5. The

Table 1
Back-calculated concentrations from calibrators run in duplicate on three different days in AH and CB ($N = 6$).

	Carteolol concentration in AH (ng/mL)					
	50	125	250	500	1000	2500
Mean (ng/mL)	48.64	126.76	254.47	508.47	1001.13	2498.16
SD (ng/mL)	1.78	1.39	4.35	6.25	5.13	6.59
RSD (%)	3.65	1.10	1.71	1.23	0.51	0.26
DEV (%)	2.71	−1.41	−1.79	−1.69	−0.11	0.07
	Dorzolamide concentration in AH (ng/mL)					
	125	250	500	1000	2500	5000
Mean (ng/mL)	124.50	249.84	501.37	1004.45	2504.60	4997.02
SD (ng/mL)	0.80	1.95	3.60	7.42	6.78	8.53
RSD (%)	0.64	0.78	0.72	0.74	0.27	0.17
DEV (%)	0.40	0.07	−0.27	−0.45	−0.18	0.06
	Carteolol concentration in CB (μg/g)					
	2	5	10	20	40	80
Mean (μg/g)	2.03	5.02	10.02	19.80	40.02	80.04
SD (μg/g)	0.06	0.05	0.13	0.35	0.27	0.19
RSD (%)	3.18	0.90	1.29	1.74	0.69	0.24
DEV (%)	−1.41	−0.37	−0.19	1.02	−0.06	−0.05
	Dorzolamide concentration in CB (μg/g)					
	2	5	10	20	40	80
Mean (μg/g)	2.00	5.02	10.12	19.99	40.23	80.06
SD (μg/g)	0.05	0.05	0.19	0.15	0.58	0.20
RSD (%)	2.38	0.96	1.92	0.77	1.44	0.25
DEV (%)	−0.06	−0.37	−1.19	0.07	−0.56	−0.08

Table 2
Back-calculated concentrations of carteolol and dorzolamide resulting from 18 QC samples in three analytical runs in AH and CB ($N = 18$).

	Carteolol concentration in AH (ng/mL)		
	100	400	2000
Mean (ng/mL)	100.00	401.36	2002.15
SD (ng/mL)	0.64	2.09	3.45
RSD (%)	0.64	0.52	0.17
DEV (%)	0.00	−0.34	−0.11
Within-run precision (%)	0.66	0.54	0.17
Between-run precision (%)	0.20	0.18	0.02
	Dorzolamide concentration in AH (ng/mL)		
	200	1250	4000
Mean (ng/mL)	199.07	1252.04	4003.86
SD (ng/mL)	0.95	2.06	5.78
RSD (%)	0.48	0.16	0.14
DEV (%)	0.46	−0.16	−0.10
Within-run precision (%)	0.44	0.15	0.15
Between-run precision (%)	0.23	0.07	0.05
	Carteolol concentration in CB (μg/g)		
	6	32	64
Mean (μg/g)	6.01	32.03	64.02
SD (μg/g)	0.02	0.04	0.04
RSD (%)	0.36	0.12	0.07
DEV (%)	−0.25	−0.08	−0.02
Within-run precision (%)	0.35	0.13	0.07
Between-run precision (%)	0.13	0.05	0.02
	Dorzolamide concentration in CB (μg/g)		
	6	32	64
Mean (μg/g)	6.03	32.02	64.03
SD (μg/g)	0.02	0.04	0.05
RSD (%)	0.38	0.12	0.08
DEV (%)	−0.45	−0.05	−0.05
Within-run precision (%)	0.41	0.12	0.07
Between-run precision (%)	0.16	0.01	0.02

Table 3
Stability of carteolol in AH and CB ($N = 3$).

Nominal concentration (ng/mL)	Matrix	Stability	Mean (ng/mL)	RSD (%)	DEV (%)
100	AH	0 h[a]	100.00	0.64	0.00
		3rd freeze–thaw	99.76	0.06	0.24
		8 h (short term)	100.07	0.06	−0.07
		35 days at −20 °C	100.08	0.11	−0.07
		20 h (autosampler)	100.03	0.02	−0.03
2000	AH	0 h[a]	2002.15	0.17	−0.11
		3rd freeze–thaw	2003.54	0.27	−0.07
		8 h (short term)	2000.89	0.01	0.06
		35 days at −20 °C	2000.35	0.04	0.09
		20 h (autosampler)	2003.56	0.27	−0.07
Nominal concentration (μg/g)	**Matrix**	**Stability**	**Mean (μg/g)**	**RSD (%)**	**DEV (%)**
6	CB	0 h[a]	6.01	0.36	−0.25
		3rd freeze–thaw	6.00	0.15	0.21
		8 h (short term)	6.00	0.14	0.29
		35 days at −20 °C	6.03	0.14	−0.26
		20 h (autosampler)	6.00	0.07	0.28
64	CB	0 h[a]	64.02	0.07	−0.02
		3rd freeze–thaw	63.99	0.12	0.03
		8 h (short term)	63.99	0.06	0.03
		35 days at −20 °C	63.95	0.04	0.10
		20 h (autosampler)	63.98	0.15	0.05

[a] 0 h indicates the mean back-calculated concentrations reported in Table 2.

recovery of the compounds from both matrices was greater than 93% at LQC, MQC and HQC concentrations. The matrix effect of the analytes from both matrices was less than 9.5% at LQC, MQC and HQC concentrations. The process efficiency of the molecules from both matrices was greater than 88% at LQC, MQC and HQC concentrations.

The procedure has proven to be applicable in the analysis of authentic AH and CB samples from rabbits treated with eye drops containing carteolol and dorzolamide. For example, Fig. 4 shows the concentration–time profiles for carteolol (a) and dorzolamide (b) in rabbit AH at different times (15, 30, 60, 120, 240, 360 min) after a single administration of 50 μL of an eye drops containing carteolol hydrochloride (2%, w/v) and dorzolamide hydrochloride (2%, w/v) at pH 6. Pharmacokinetic parameters are as follows: for carteolol T_{max} is 60 min, C_{max} is 715.40 ± 152.23 ng/mL and AUC is 92.69 ± 17.61 (min μg)/mL; for dorzolamide T_{max} is 60 min, C_{max} is 1224.20 ± 214.48 ng/mL and AUC is 155.89 ± 26.51 (min μg)/mL. It is worth to observe that the pharmacokinetic profile for carteolol and dorzolamide in AH is comparable to that reported when these drugs were administered alone [18,19], thus suggesting that no drug–drug interaction occurs when carteolol and dorzolamide are administered together.

The presented assay is the first validated procedure for the simultaneous determination of carteolol and dorzolamide in rabbit AH and CB.

Table 4
Stability of dorzolamide in AH and CB ($N = 3$).

Nominal concentration (ng/mL)	Matrix	Stability	Mean (ng/mL)	RSD (%)	DEV (%)
200	AH	0 h[a]	199.07	0.48	0.46
		3rd freeze–thaw	202.87	1.60	−1.91
		8 h (short term)	200.25	0.17	−0.59
		35 days at −20 °C	200.58	0.12	−0.76
		20 h (autosampler)	200.10	0.05	−0.51
4000	AH	0 h[a]	4003.86	0.14	−0.10
		3rd freeze–thaw	4002.94	0.06	0.02
		8 h (short term)	4000.81	0.02	0.08
		35 days at −20 °C	4000.10	0.02	0.09
		20 h (autosampler)	4002.11	0.04	0.04
Nominal concentration (μg/g)	**Matrix**	**Stability**	**Mean (μg/g)**	**RSD (%)**	**DEV (%)**
6	CB	0 h[a]	6.03	0.38	−0.45
		3rd freeze–thaw	5.98	0.26	0.70
		8 h (short term)	6.00	0.43	0.47
		35 days at −20 °C	6.01	0.19	0.32
		20 h (autosampler)	5.99	0.39	0.54
64	CB	0 h[a]	64.03	0.08	−0.05
		3rd freeze–thaw	64.05	0.02	−0.03
		8 h (short term)	63.96	0.09	0.11
		35 days at −20 °C	64.03	0.04	0.00
		20 h (autosampler)	64.01	0.09	0.03

[a] 0 h indicates the mean back-calculated concentrations reported in Table 2.

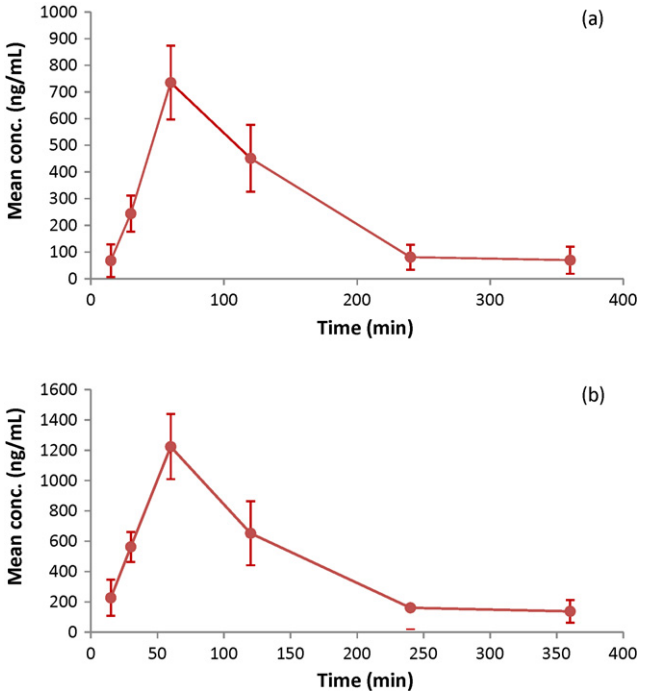

Fig. 4. AH concentration–time profiles for (a) carteolol and (b) dorzolamide after a single administration of an eye drops containing carteolol hydrochloride (2%, w/v) and dorzolamide hydrochloride (2%, w/v) at pH 6.

Table 5
Recovery, matrix effect and process efficiency for carteolol and dorzolamide in AH and CB ($N = 4$).

	Carteolol concentration in AH (ng/mL)		
	100	400	2000
Recovery (%)	99.82	99.72	99.99
Matrix effect (%)	1.76	3.30	1.10
Process efficiency (%)	98.06	96.42	98.89
	Dorzolamide concentration in AH (ng/mL)		
	200	1250	4000
Recovery (%)	98.87	98.99	98.68
Matrix effect (%)	8.94	7.35	9.32
Process efficiency (%)	90.94	92.63	90.68
	Carteolol concentration in CB (μg/g)		
	6	32	64
Recovery (%)	94.66	97.98	97.39
Matrix effect (%)	5.14	7.58	4.90
Process efficiency (%)	89.79	90.56	92.62
	Dorzolamide concentration in CB (μg/g)		
	6	32	64
Recovery (%)	95.82	93.24	97.73
Matrix effect (%)	5.47	5.95	7.58
Process efficiency (%)	90.58	87.70	90.33

4. Conclusions

A sensitive, selective and rapid method was developed for the simultaneous quantification of carteolol and dorzolamide in rabbit AH and CB by HPLC–MS/MS. Because no gradient elution and no chromatographic separation are necessary, the need of "fine tuning" of the HPLC is eliminated. After the extraction procedures, the recovery of two analytes is greater than 98% and 93% in AH and CB, respectively. The results obtained for selectivity, accuracy and precision, short-term and long-term stability and stability following freeze–thaw cycles were found to be within the acceptable limits. The analytical method here presented proved to be linear, accurate and precise for the simultaneous determination of carteolol and dorzolamide concentrations in rabbit ocular matrices and was used for the *in vivo* studies of these drugs in rabbit AH and CB.

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References

[1] T.J. Zimmerman, J. Ocul. Pharmacol. 9 (1993) 373.
[2] G.D. Novack, M.J. O'Donnell, D.W. Molloy, J. Am. Geriatr. Soc. 50 (2002) 956.
[3] J.A. Nathanson, PNAS, USA 77 (12) (1980) 7420.
[4] S.E. Moroi, P.R. Lichter, in: J.G. Hardman, L.E. Limbird, A.G. Gilman (Eds.), Ocular Pharmacology, in Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill, New York, 2001, p. 1821.
[5] C.D. Black, H.J. Mann, Drug Intell. Clin. Pharm. 18 (1984) 554.
[6] N. Pfeiffer, Surv. Ophthalmol. 42 (1997) 137.
[7] M.F. Sugrue, Prog. Retin. Eye Res. 19 (2000) 87.
[8] J.J. Baldwin, G.S. Ponticello, P.S. Anderson, M.A. Mercko, W.C. Randall, H. Schwan, M.F. Sugrue, P.S. Gautheron, J. Grove, P. Mallorga, M.P. Viader, B.M. McKever, M.A. Navia, J. Med. Chem. 32 (1989) 2513.
[9] L.M. Hurvitz, P.L. Kaufman, A.L. Robin, R.N. Weinreb, K. Crawford, B. Shaw, Drugs 41 (1991) 514.
[10] T.J. Blacklock, P. Sohar, J.W. Buthcher, T. Lamanec, E.J.J. Grabowski, J. Org. Chem. 58 (1993) 1672.
[11] T. Sugiyama, S. Kojima, O. Ishida, T. Ikeda, Acta Ophthalmol. 87 (2008) 797.
[12] D. Hartenbaum, Clin. Ther. 18 (1996) 460.
[13] H.H. Maurer, O. Tenberken, C. Kratzsch, A.A. Weber, F.T. Peters, J. Chromatogr. A 1058 (2004) 169.
[14] M.L. Constanzer, C.M. Chavez, B.K. Matuszewski, J. Pharm. Biomed. Anal. 15 (1997) 1001.
[15] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CV), 2001, available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.
[16] Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005, available at: http://www.ich.org/LOB/media/MEDIA417.pdf.
[17] N.J. Lakhani, E.R. Lepper, A. Sparreboom, W.L. Dahul, J. Venitz, W.D. Figg, Rapid Commun. Mass Spectrom. 19 (2005) 1176.
[18] M. Higashiyama, T. Tajika, K. Inada, A. Ohtori, J. Ocul. Pharmacol. Ther. 22 (2006) 333.
[19] H.H. Sigurdsson, E. Stefánsson, E. Gudmundsdóttir, T. Eysteinsson, M. Thorsteinsdóttir, T. Loftsson, J. Control. Release 102 (2005) 255.