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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 606–612
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of tamsulosin in human aqueous humor and serum by liquid chromatography–electrospray ionization tandem mass spectrometry

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Received 10 April 2006; accepted 7 July 2006
Available online 22 August 2006

Abstract

A simple, sensitive and selective LC–MS/MS method was developed for the determination of tamsulosin in human aqueous humor and serum to study the recently reported eye-related adverse effects of this α_1-blocker drug. Aqueous humor samples were analyzed by direct injection, after addition of the internal standard, labetalol. Liquid–liquid extraction with ethyl acetate was used for serum sample preparation. The chromatographic separation was performed on a reversed phase column by gradient elution with acetonitrile −0.1% formic acid at a flow-rate of 0.2 ml/min. Detection and quantification of the analytes were carried out with a linear ion trap mass spectrometer, using positive electrospray ionization (ESI) and multiple reaction monitoring (MRM). The limit of quantification was 0.1 ng/ml for both aqueous humor and serum samples and linearity was obtained over the concentration ranges of 0.1–4.7 ng/ml and 0.1–19.3 ng/ml for aqueous humor and serum samples, respectively. Acceptable accuracy and precision were obtained for concentrations within the standard curve ranges. The method has been used for the determination of tamsulosin in aqueous humor and serum samples from patients that were on tamsulosin medication and underwent cataract surgery.

Keywords: LC–MS/MS; Tamsulosin; Aqueous humor; Serum; Intraoperative floppy iris syndrome

1. Introduction

Tamsulosin (−)-(*R*)-5-[2-[[2-(*O*-ethoxyphenoxy)ethyl]amino]propyl]-2-methoxybenzensulfon-amide (Fig. 1) is an α_{1A}/α_{1D} subtype selective α_1-adrenoceptor antagonist (α_1-blocker) that is the most frequently prescribed medication for the treatment of lower urinary tract symptoms suggestive of benign prostatic hyperplasia (BPH) [1,2]. The α_1-adrenoceptors (α_{1A}, α_{1B}, α_{1D}) take part in the modulation of blood pressure and thus the α_1-blockers were originally developed as antihypertensive agents. Non-selective α_1-receptor blockage has also additional effects, including relaxation of the smooth muscles of bladder neck and prostatic urethra, which are found to relieve the symptoms associated with BPH. Consequently, the first drugs for the treatment of the BPH were non-subtype-selective α_1-blockers. Unfortunately these drugs were commonly associated with cardiovascular adverse effects. These side effects were later avoided by selectively blocking the α_1-adrenoceptor subtypes α_{1A} and α_{1D} with uroselective α_1-blockers like tamsulosin, resulting in the relaxation of the smooth muscles in lower urinary tract with minimal blood pressure-related adverse effects [3].

However, despite the uroselectivity of tamsulosin, recent findings suggest that there is a strong association between systemic use of tamsulosin and iris hypotony [4] or intraoperative floppy iris syndrome (IFIS) [5]. This phenomenon is described to be an inadequate pupil dilation and a sluggish iris during eye operation that compromise cataract surgery and increase the risk for complications. Although the pharmacologic mechanism is not known, it is hypothesized that in addition to blocking the α_{1A}-receptors in the prostate, tamsulosin selectively blocks the iris dilator muscle in which the same receptor subtype dominates [4,5]. After the finding of this new syndrome, it has been suggested that temporarily withholding tamsulosin perioperatively

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doi:10.1016/j.jpba.2006.07.016

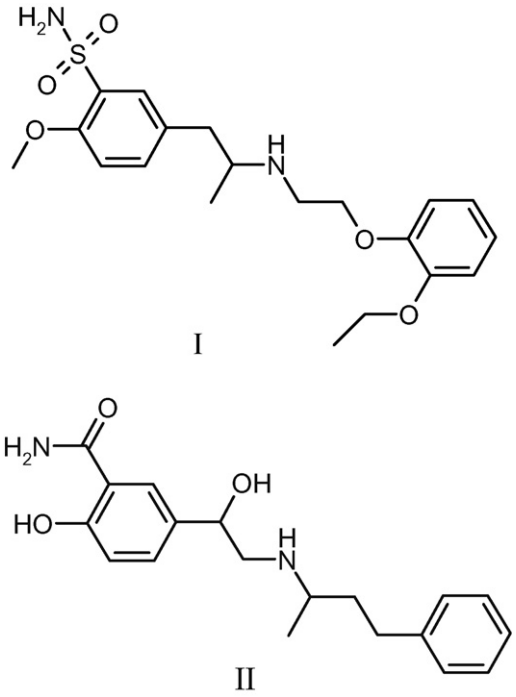

Fig. 1. Chemical structures of tamsulosin (I) and labetalol (II, internal standard).

might be a solution to avoid the IFIS [6,7]. However, this has not always been effective and it seems that receptor binding of tamsulosin might continue for some time beyond the disappearance of a plasma level of tamsulosin [8,9].

In order to study this phenomenon and the proposed ophthalmic penetration of tamsulosin, a method was needed to estimate tamsulosin levels in the aqueous humor of patients that were on tamsulosin medication and underwent cataract surgery. Also the serum concentration of tamsulosin at the time of the eye operation was needed to compare the state of medication to the aqueous humor concentrations and to the clinical observations. A single instrumental method that would enable the analysis of both aqueous humor and serum samples in one analytical sequence was preferred over two separate methods. However, to the author's knowledge, no methods have been reported for the measurement of tamsulosin in aqueous humor or serum. Moreover, bioanalytical methods available for the quantification of tamsulosin in body fluids are few, being mostly developed for plasma concentration measurements relating to pharmacokinetic studies [10–15]. Some papers also describe methods for plasma dialysate, urine or tissue analysis [9,16]. These methods were considered unsuitable for the analysis of small volumes of aqueous humor samples and also, with few exceptions, the internal standards used in these methods are not commercially available.

In this study, a simple and sensitive method for the analysis of tamsulosin in aqueous humor and serum samples was developed. The method enables both types of samples to be analyzed within the same analytical sequence using identical instrument conditions and an easily available internal standard. The method is based on a HPLC with gradient elution and MS/MS detection by multiple reaction monitoring with highly selective and sensitive linear ion trap mass spectrometer. The method was validated in terms of selectivity, linearity, accuracy, precision and stability for both serum and aqueous humor sample analysis and successfully applied to study the IFIS and the effect of withholding tamsulosin before cataract surgery of patients on tamsulosin medication. A detailed report of these studies is under preparation.

2. Experimental

2.1. Chemicals

Tamsulosin hydrochloride was obtained from Fermion Oy (Espoo, Finland). Labetalol hydrochloride (internal standard, IS) was purchased from Sigma (St. Louis, MO, USA), HPLC-grade acetonitrile was from Rathburn (Walkerburn, UK). Formic acid and sodium bicarbonate were from Riedel-de Haen (Seelze, Germany) and ethyl acetate from LabScan (Dublin, Ireland). Water was purified using a Milli-Q Gradient system (Millipore, Milford, MA, USA). All the reagents used were of analytical grade.

2.2. Chromatographic equipment and conditions

The HPLC system comprised of a Finnigan Surveyor MS Pump and a Finnigan Surveyor Autosampler (Thermo Electron, San Jose, CA, USA) with a Waters XTerra C8 column (50 mm × 2.1 mm, 3.5 μm, Waters, Milford, MA, USA) and a Zorbax XDB-C8 Narrow-Bore Guard Column (Agilent Technologies, Palo Alto, CA, USA). Column temperature was maintained at 30 °C. Separations of both serum and aqueous humor samples were performed by gradient elution at a flow rate of 0.2 ml/min. The mobile phase consisted of water–formic acid (A; 100:0.1, v/v) and water–acetonitrile–formic acid (B; 50:50:0.1, v/v/v). The gradient program was as follows: 0–5 min: 10% B → 100% B; 5–6 min: 100% B → 10% B; 6–8 min: 10% B → 10% B.

2.3. Mass spectrometric equipment and conditions

The mass analysis was carried out with a Finnigan LTQ linear ion trap mass spectrometer equipped with Finnigan Ion Max electrospray ionization source operating in the positive ion mode (Thermo Electron, San Jose, CA, USA). The following instrument conditions were used: nitrogen sheath and auxiliary gas flow rates 25 and 5 arbitrary (instrument) units, respectively, spray voltage 4.5 kV, capillary temperature 350 °C, capillary voltage 18 V and tube lens offset 75 V. Collection time for the ion trap was set at 100 ms and the following transitions were monitored: m/z 409.3 → 228.0 and m/z 409.3 → 271.0 for tamsulosin and m/z 329.0 → 311.0 for labetalol. Divert valve was programmed to allow eluent flow into the mass spectrometer from 2 to 5 min of each run. Data acquisition was conducted using Xcalibur 1.4 SR1 software. For quantification, peak area ratios of the analyte to the internal standard were calculated as a function of the concentration of the analyte using LCquan 2.0 software.

2.4. Preparation of standards

Stock solutions of tamsulosin (0.20 mg/ml) and labetalol (0.25 mg/ml) were prepared in methanol and stored at −20 °C.

Working solutions were prepared daily by diluting stock solutions with water. Calibration standards used for the analysis of serum samples were prepared by adding 50 μl of the tamsulosin working solutions and 30 μl of the IS working solution (0.2 μg/ml) in 1 ml of drug free human serum. The spiked samples were then treated following the sample preparation procedure as described in Section 2.5 to give a series of standards with tamsulosin concentration of 0.1, 0.2, 0.4, 0.8, 3.9, 9.7 and 19.3 ng/ml.

Calibration standards for aqueous humor samples were prepared by diluting tamsulosin stock solution with water and adding IS working solution. Tamsulosin concentrations in these calibration standards were 0.05, 0.09, 0.46, 0.93, 1.39 and 2.78 ng/ml and IS concentration was 25 ng/ml.

2.5. Sample preparation

Before cataract surgery, a venous blood sample was taken for the determination of serum tamsulosin concentration. An aqueous humor sample was taken at the beginning of the operation from the freshly incised corneal side port. All samples were frozen immediately and thawed unassisted at room temperature prior to analysis.

To 1 ml of serum in a glass tube, 30 μl of IS working solution (0.2 μg/ml) was added and the tube was briefly mixed by vortexing. After that, 1 ml of saturated sodium bicarbonate and 5 ml of ethyl acetate were added and the sample was vortex-mixed for 2 min and centrifuged for 10 min at 1500 × *g*. The organic phase was removed and evaporated to dryness under a stream of nitrogen at 40 °C and reconstituted with 200 μl of mobile phase. Thirty-five microliters of this solution was injected for analysis. For quality control (QC) samples, 1 ml of drug free human serum was spiked with 50 μl of appropriate tamsulosin working solutions and treated as mentioned above in order to contain 0.4, 3.7 and 9.2 ng/ml of tamsulosin.

Aqueous humor samples were prepared by adding 20 μl of IS working solution (63 ng/ml) to 30 μl of aqueous humor in a borosilicate glass HPLC vial insert. This solution was then carefully mixed using a micropipette and a 35 μl aliquot was injected for analysis. Quality control samples at three different concentrations (0.1, 0.5 and 2.8 ng/ml) were prepared following the same procedure as above using tamsulosin stock solution diluted with water.

The study adhered to the tenets of the Declaration of Helsinki and was approved by ethics committee of the Central Hospital of Central Finland. The measurements were only taken after the subjects had given their informed consent.

2.6. Assay validation

Calibration standards were analyzed before the samples within each analytical run. Calibration curve included a blank sample (matrix sample processed without internal standard) and a zero sample (matrix sample processed with internal standard). The goodness of fit of the linear calibration curves was determined with correlation coefficients and the deviation of standards from their nominal concentration was calculated. The lower limit of quantification (LLOQ) for the measurement of tamsulosin in both serum and aqueous humor was determined by calculating precision and accuracy for five LLOQ samples that were independent of the calibration curves.

Precision and accuracy of the method were determined by analyzing the QC samples at three concentrations ($n = 3$ at each concentration level) on three separate days. Accuracy was calculated by comparing the mean experimental concentrations of assayed QC samples with their nominal values, and percentage values were used as the index. Relative standard deviations (R.S.D.%) of the concentrations were used as an index of precision. Additional study on accuracy of aqueous humor assay was performed by using human aqueous humor-based QC sample that was prepared as described in Section 2.5 at the concentration level of 0.5 ng/ml.

Extraction recovery of the serum assay was determined by comparing the detector responses (tamsulosin peak areas) of serum QC samples to that of reference samples (prepared by diluting the stock solutions with mobile phase to the QC sample concentrations).

Analyte stability was evaluated by determining the stock solution stability, freeze and thaw stability and short-term temperature stability. Stock solutions of tamsulosin and labetalol (IS) were stored at −20 °C and measured against fresh stock solutions. Freeze and thaw stability of serum samples was determined by spiking blank human serum with tamsulosin to low and high QC sample concentrations (0.4 and 9.2 ng/ml; $n = 3$) and subjecting the samples to three freeze and thaw cycles (−80 °C to room temperature) before analysis. Short-term temperature stability was determined by storing the spiked QC samples at room temperature before sample preparation and analysis. Freeze and thaw stability of aqueous humor samples was determined by analyzing three replicate samples from spiked aqueous humor before and after subjecting the samples to three freeze and thaw cycles. Short-term temperature stability was determined by storing the spiked samples at room temperature before sample preparation.

3. Results and discussion

3.1. Chromatography

Concentration of tamsulosin in the aqueous humor samples was presumed to be minimal because of the very high plasma protein binding of tamsulosin [9]. In addition, the small volume of aqueous humor recovered during the eye operation made sample concentration by means of evaporation unfeasible. For these reasons, the focus of the chromatographic method development was to find suitable instrument conditions to allow direct injection of large volumes of aqueous humor without causing excessive peak distortion, while maintaining high sensitivity and the ability to analyze serum samples with the same method.

Reversed phase column was selected with dimensions of 2.1 mm × 50 mm for added sensitivity due to small peak volumes. As the injection volume of 35 μl was about 35% of the column volume, gradient elution was employed to achieve good chromatographic peak shape and reasonably short reten-

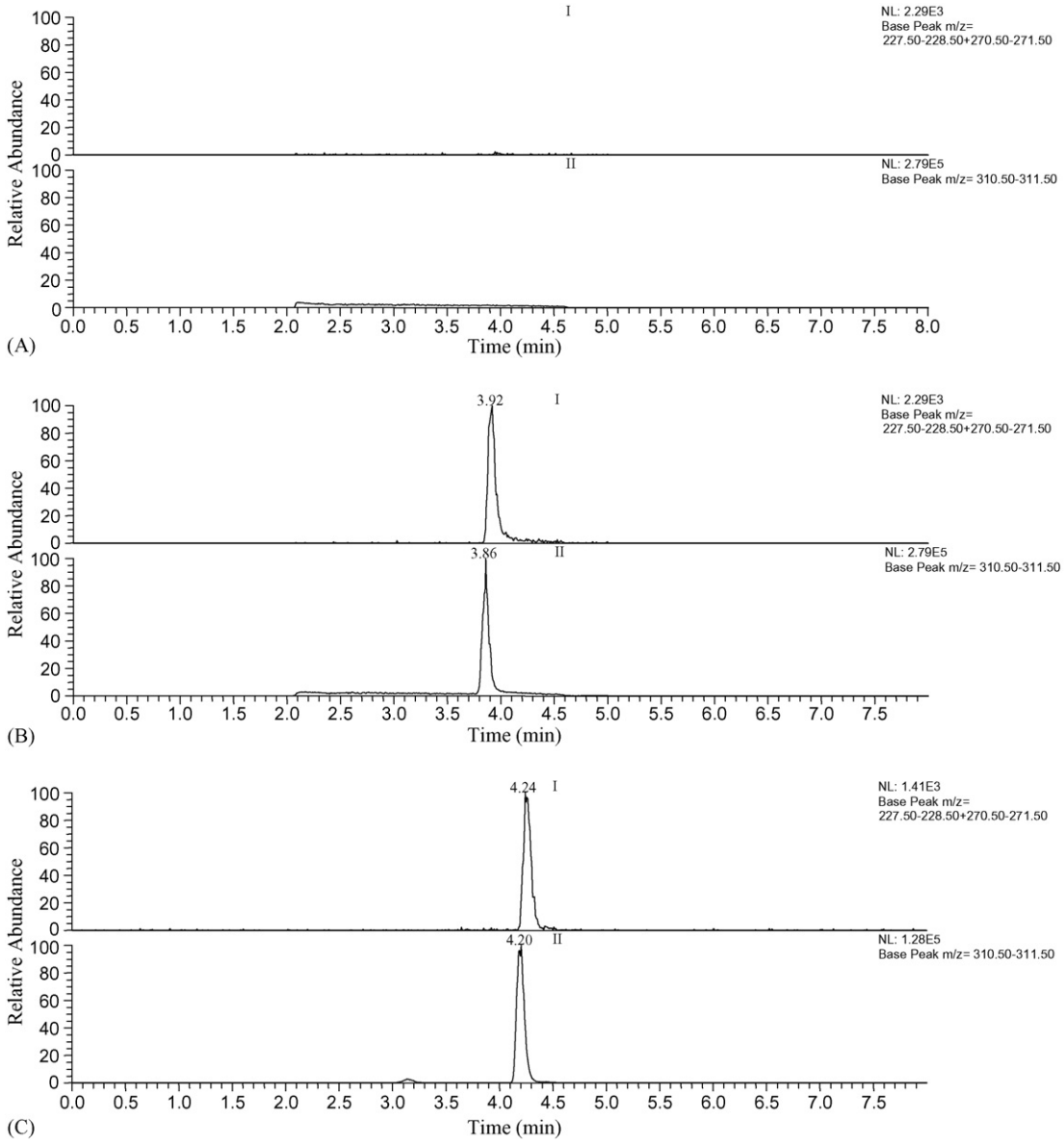

Fig. 2. Representative base peak chromatograms of: (A) blank aqueous humor (AH) sample; (B) blank AH sample spiked with tamsulosin (0.5 ng/ml) and IS (41.7 ng/ml); (C) AH sample from a patient on tamsulosin medication (0.4 mg daily) at the time of cataract surgery. Peak I, tamsulosin; peak II, labetalol (IS).

tion times. The highly selective MRM detection permitted close elution of tamsulosin and IS, which can be utilized to ensure uniform ionization environment for both the analytes. Acetonitrile and formic acid were selected as the mobile phase components because of their good MS compatibility. Representative chromatograms for the analysis of aqueous humor and serum are shown in Figs. 2 and 3, respectively.

3.2. Mass spectrometry

The protonated molecular ions for tamsulosin and IS were found to be *m/z* 409.3 and *m/z* 329.0, respectively. The most intensive product ions were *m/z* 228.0 and *m/z* 271.0 for tamsulosin and *m/z* 311.0 for the IS (Fig. 4). By monitoring the transitions *m/z* 409.3 → 228.0 + 271.0 and *m/z* 329.0 → 311.0, a highly selective and sensitive assay for tamsulosin was developed. Labetalol was found to be a suitable internal standard for the analysis of tamsulosin in serum with comparable extraction and ionization properties. The IS was also added to the aqueous humor samples to compensate the variability in ionization.

3.3. Calibration curve and sensitivity

Calibration curves were linear over the investigated concentration ranges with mean correlation coefficients of 0.994 and 0.998 for serum and aqueous humor assays, respectively. Deviation of calibration standards from their nominal concentrations at the LLOQ was less than 20%. The LLOQ for the determination of tamsulosin in both aqueous humor and serum, defined as the lowest concentration analyzed with a precision less than 20% and accuracy of 80–120%, was 0.1 ng/ml.

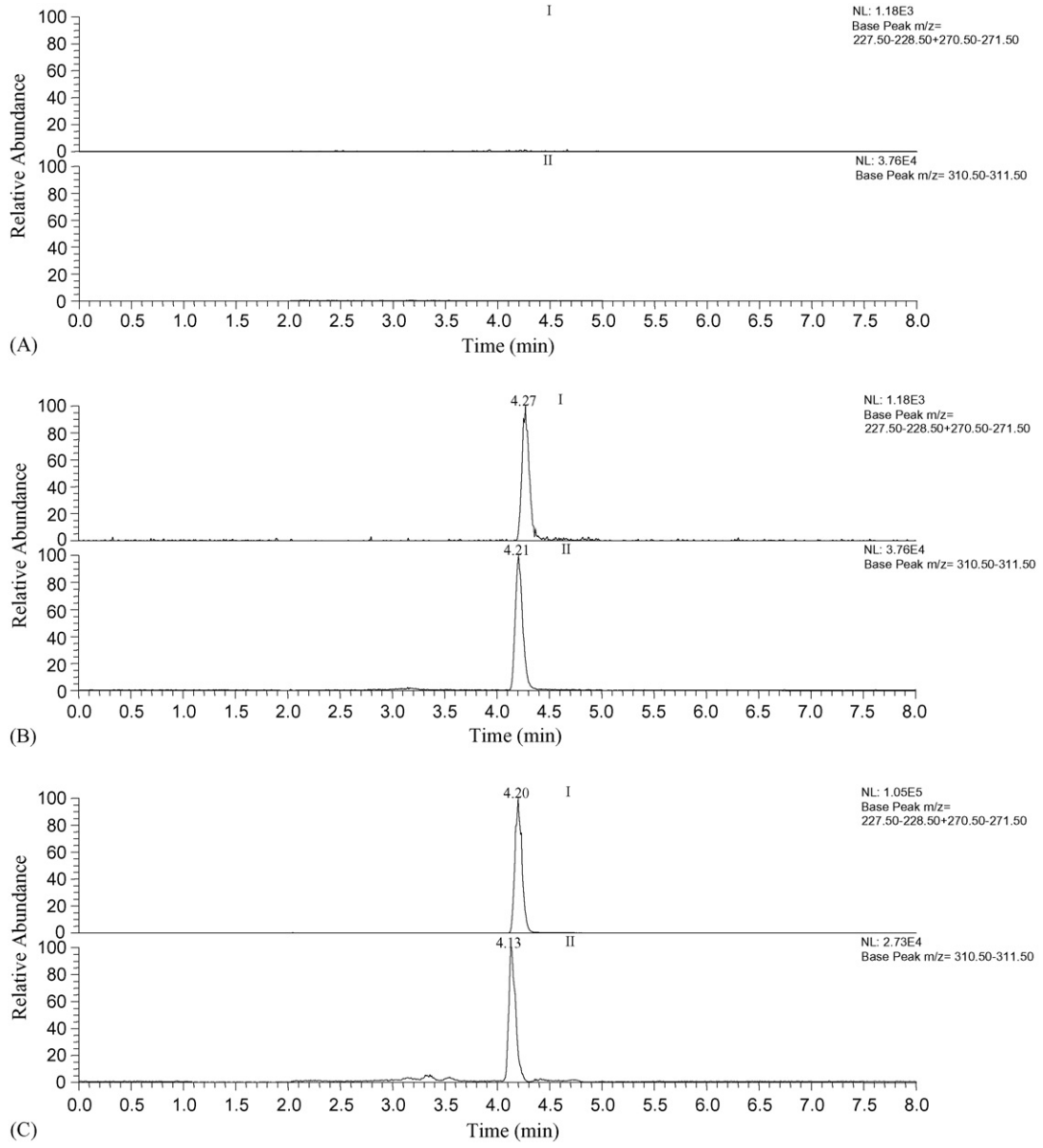

Fig. 3. Representative base peak chromatograms of: (A) blank serum sample; (B) blank serum sample spiked with tamsulosin (3.7 ng/ml) and labetalol (IS) (5.8 ng/ml); (C) serum sample from a patient on tamsulosin medication (0.4 mg daily) at the time of cataract surgery. Peak I, tamsulosin; peak II, labetalol (IS).

The calibration curve equation is $y = bx + c$, where y represents the tamsulosin to IS peak area ratio and x represents the concentration of tamsulosin. The mean equation of the calibration curves ($n = 3$) for serum samples with standard deviations shown in parenthesis was $y = 0.384(0.136)x - 0.002(0.004)$. Best curve fit was achieved using a weighed ($1/x^2$) linear regression. The mean equation of the calibration curves ($n = 3$) for aqueous humor samples with standard deviations shown in parenthesis was $y = 0.0713(0.0379)x + 0.00010(0.00014)$. Calibration curves for the aqueous humor assay were obtained by unweighted linear regression. The concentrations of the calibration standards for aqueous humor samples (0.05–2.78 ng/ml) equated to the tamsulosin concentrations of 0.08–4.67 ng/ml in aqueous humor before addition of the internal standard. The limited amount of human aqueous humor available prevented the construction of a matrix based calibration curve. However, accuracy of the method was confirmed by spiking blank aqueous humor with tamsulosin. As aqueous humor is water-like, clear solution with low protein content, several published methods for the analysis of human aqueous humor report the use of water or mobile phase-based calibration curve with a proof of accuracy, as blank human aqueous humor is generally not available [17–21].

3.4. Accuracy and precision

Accuracy and precision of the method were satisfactory for our purpose. As shown in Table 1, for each QC level of tamsulosin in serum, the inter-day precision was less than 17.8% and accuracy was within $100.0 \pm 6.9\%$. For each QC level of tamsulosin in aqueous humor, the inter-day precision was less than 9.6% and accuracy was within $100.0 \pm 7.8\%$. Because of the

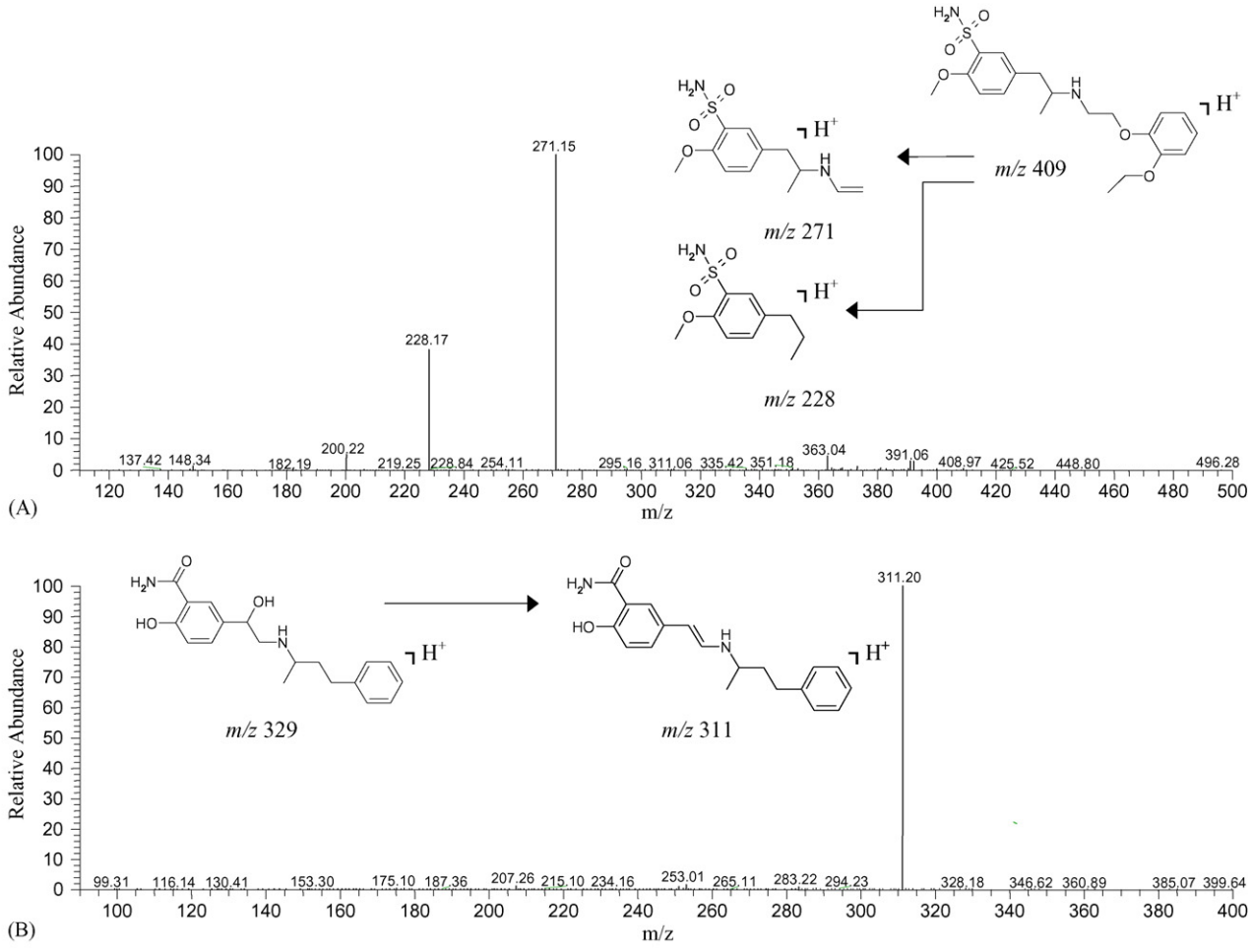

Fig. 4. Positive product ion spectra $[M+H]^+$ of: (A) tamsulosin and (B) labetalol (IS) with proposed product ion structures.

Table 1
Accuracy and precision of the method for the determination of tamsulosin in human serum and aqueous humor ($n = 3$ days, three replicates per day)

Matrix	Nominal concentration (ng/ml)	Precision (R.S.D.%)	Accuracy (%)
Serum	Intra-day		
	0.4	5.9	110.8
	3.7	3.3	111.4
	9.2	6.8	111.2
	Inter-day		
	0.4	8.3	102.1
	3.7	17.8	93.1
	9.2	11.0	100.7
Aqueous humor	Intra-day		
	0.1[a]	10.3	99.3
	0.5	8.2	101.6
	2.8	5.6	101.6
	Inter-day		
	0.1[a]	6.6	104.4
	0.5	5.9	104.6
	2.8	9.6	107.8

[a] Concentration of the lower limit of quantification (LLOQ).

small amounts of tamsulosin in aqueous humor, the lowest QC level was set at the LLOQ of the method. Five replicate samples at this concentration level resulted in a precision of 10.4% and accuracy of 104.6%. The results indicate acceptable accuracy and precision of the method.

An additional study for the accuracy of the aqueous humor assay was made using drug free human aqueous humor for constructing a QC sample at the concentration of 0.5 ng/ml. Result of this experiment indicated that the water-based calibration curve was suitable for our purpose, showing an accuracy of 78% at the concentration level studied.

3.5. Extraction recovery

Extraction recovery was determined for serum samples and results showed that the recovery of tamsulosin was 66–77% for tamsulosin and 49–60% for the IS at the QC concentrations of 0.4, 3.7 and 9.2 ng/ml.

3.6. Stability

Stock solutions of tamsulosin and labetalol (IS) were stable for at least 6 and 12 months, respectively. No significant degra-

dation of tamsulosin in serum samples (0.4 and 9.2 ng/ml) was observed after three freeze and thaw cycles (accuracy 113.9 and 104.5%, respectively) or after 4 h at room temperature (deviation from the initial concentration −11.9 and −2.0%, respectively). Tamsulosin was also found to be stable in aqueous humor after three freeze and thaw cycles and after 4 h storage at room temperature (deviation from the initial concentration −2.8 and 0.7%, respectively).

3.7. Application

The analytical method described in this paper yielded satisfactory results for the determination of tamsulosin in human serum and aqueous humor. The method has been successfully applied to study the interconnections between tamsulosin concentration in serum and the anterior chamber of the eye and their effect on the characteristics of the iris during cataract surgery of patients on tamsulosin medication. The mean serum and aqueous humor concentrations of 11 patients receiving tamsulosin were 12.7 ± 4.1 and 0.4 ± 0.3 ng/ml, respectively (mean ± S.D.). After a pause of 7–28 days in the use of tamsulosin, in four out of five cases, measurable amounts of tamsulosin still remained in the aqueous humor but not in serum. The results indicate the ophthalmic penetration of tamsulosin and its prolonged presence in the anterior chamber of the eye.

4. Conclusions

A simple and sensitive method using liquid chromatography with electrospray ionization ion trap mass spectrometry (LC–ESI-MS/MS) was developed for the determination of tamsulosin in human serum and aqueous humor. The method enables the analysis of both serum and aqueous humor samples within the same analytical sequence using easily available internal standard, offers high selectivity and sensitivity and enables direct injection of aqueous humor samples. The method is considered adequate for its purpose and it was capable of demonstrating that small amount of tamsulosin is present in the anterior chamber of human eye during normal tamsulosin medication and even after temporarily withholding tamsulosin. To the authors' knowledge, this is the first published method to show the presence of tamsulosin in the human aqueous humor.

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