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Highly sensitive and rapid LC–ESI-MS/MS method for the simultaneous quantification of uroselective α_1-blocker, alfuzosin and an antimuscarinic agent, solifenacin in human plasma

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ABSTRACT

An accurate, selective and sensitive bioanalytical method has been developed and validated for the simultaneous quantification of alfuzosin and solifenacin in human plasma using propranolol as internal standard (IS). The analytes and IS were extracted in methyl tert-butyl ether, separated on Hypurity C8 column and detected by tandem mass spectrometry with a turbo ion spray interface. The method had a chromatographic run time of 3.0 min and linear calibration curves over the concentration range of 0.25–25 ng/mL for alfuzosin and 0.6–60 ng/mL for solifenacin. The intra- and inter-day accuracy and precision (%CV) evaluated at four quality control levels were within 88.2–106.4% and 0.9–7.7% respectively. The absolute recovery from spiked plasma samples was 71.8% for alfuzosin and 93.1% for solifenacin. Stability of alfuzosin and solifenacin was assessed under different storage conditions. The validated method was successfully employed for bioavailability study after oral administration of 10 mg of alfuzosin hydrochloride and 5 mg of solifenacin succinate tablet formulations in eight healthy volunteers under fed condition.

1. Introduction

Alpha$_1$-adrenergic-receptor antagonists (α_1-blockers) have become the standard of care for the management of benign prostatic hyperplasia (BHP)-related lower urinary tract symptoms (LUTS). However, these agents have the potential to produce orthostatic hypotension and other blood pressure related adverse effects in normotensive patients and those receiving concurrent treatment with other antihypertensive agents. As a result, more uroselective and less vasoactive α_1-blockers are mandatory [1,2]. Clinical uroselectivity refers to the enhanced efficacy in patients with BHP with concomitant reduction in adverse events, for example, a preferential reduction of prostatic urethral obstruction and LUTS that is seen relative to adverse events associated with the cardiovascular system (postural hypotension, syncope, vertigo, dizziness) or central nervous system. (drowsiness, somnolence, fatigue, asthenia) [2,3]. Alfuzosin is an uroselective and competitive alpha$_1$-adrenergic-receptor antagonist, used to treat arterial hypertension and symptomatic benign prostatic hyperplasia, a common disorder in older men [4]. Alfuzosin differs from other α_1-adrenergic-receptor blockers by the absence of a piperidine moiety and the presence of a diaminopropyl spacer, which confers alfuzosin with specific biochemical properties. Alfuzosin acts by inhibiting the postsynaptic alpha$_1$-adrenoceptors on vascular smooth muscle. This inhibits the vasoconstrictor effect of circulating and locally released catecholamines (epinephrine and norepinephrine), resulting in peripheral vasodilation [5–9].

Muscarinic-receptor antagonists are widely prescribed for treatment of the syndrome of overactive bladder and urge urinary incontinence [10,11]. Solifenacin succinate (SOL) is a competitive muscarinic-receptor antagonist approved by the US Food and Drug Administration in 2004 for the treatment of overactive bladder (OAB) with symptoms of urge urinary incontinence, urgency and urinary frequency [11]. Solifenacin is highly selective for muscarinic M3 receptor which is believed to be important in the modulation of bladder function. It reduces smooth muscle tone in the bladder, allowing the bladder to retain larger volumes of urine and reducing the number of micturition, urgency and incontinence episodes [12]. Upon oral administration, solifenacin has a high absolute bioavailability ~88% and is 98% protein bound. Maximum plasma concentrations are reached within 4–6 h. It is extensively metabolized in the liver by the cytochrome P450 3A isoenzymes and approximately 50% of a dose is eliminated renally as parent compound with one active and three inactive metabolites [13–15].

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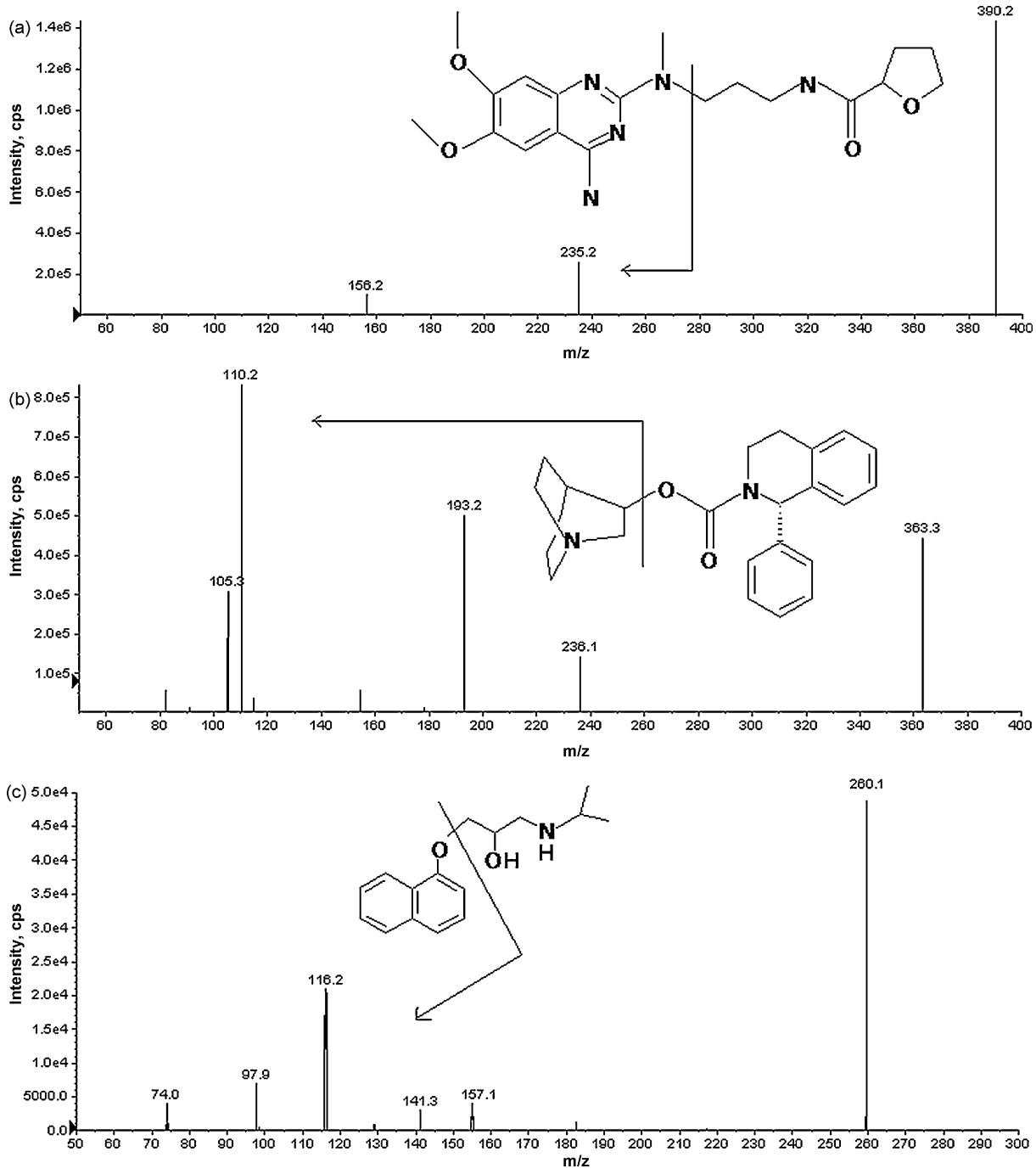

Fig. 1. Product ion mass spectra of (a) alfuzosin, (b) solifenacin and (c) propranolol (IS).

Few assays are reported in literature for the determination of alfuzosin in biological matrices. Rouchouse et al. [16] have determined enantiomers of alfuzosin in human plasma on a second-generation alpha$_1$-acid glycoprotein chiral stationary by high-performance liquid chromatography. The limit of quantification for each isomer was 1 ng/mL. Another HPLC method with fluorescence detection has been described by Guinebault et al. [17] using liquid–liquid extraction and large injection volume technique. The calibration graphs were linear between 1 and 200 ng/mL in blood plasma and 0.05–10 μg/mL in urine for alfuzosin. Carlucci et al. [18] have given an HPLC method using a column switching procedure without extraction to isolate the drug from biological matrix with an LLOQ of 2 ng/mL. A sensitive, selective and rapid LC–MS/MS method for alfuzosin is proposed by Wiesner et al. [19] in human plasma. Following liquid–liquid extraction in tert butyl methyl ether, the samples were chromatographed on Supelco Discovery C18 column in a run time of 4 min. The mean recovery was 82.9% with a lower limit of quantification of 0.3 ng/mL. Recently, alfuzosin was analyzed in human plasma by a selective and automated sample extraction on a molecularly imprinted polymer [20]. The high selectivity of the support coupled to the chromatographic system permitted an easy and fast analysis of the drug with a limit of quantification of 15 μg/mL by HPLC-UV.

Table 1
Intra-batch and inter-batch precision and accuracy.

Level	Conc. added (ng/mL)	Intra-batch				Inter-batch			
		n	Mean Conc. found (ng/mL)[a]	%RE[b]	%CV[c]	*n*	Mean Conc. found (ng/mL)[d]	%RE[b]	%CV[c]
		Alfuzosin							
LLOQ	0.25	6	0.23	−9.4	7.7	18	0.22	−10.5	7.1
LQC	0.75	6	0.66	−11.8	2.7	18	0.68	−9.4	4.1
MQC	9.0	6	8.93	0.7	4.1	18	8.89	−1.3	4.7
HQC	18.0	6	17.06	−5.2	5.1	18	16.49	−8.4	4.7
		Solifenacin							
LLOQ	0.60	6	0.59	−0.8	2.9	18	0.61	1.9	6.8
LQC	1.80	6	1.71	−5.0	0.9	18	1.74	−3.6	3.8
MQC	21.6	6	22.75	5.3	3.6	18	22.98	6.4	3.0
HQC	43.2	6	42.51	−1.6	2.5	18	42.68	−1.2	3.0

[a] Mean of six replicates observations at each concentration.
[b] Relative error.
[c] Coefficient of variance; *n*, total number of observation.
[d] Mean of eighteen replicates observations over three different analytical runs.

Like alfuzosin, there are limited number of sensitive and selective methods for the determination of solifenacin in biological fluids. Nanagihara et al. [21] have developed and validated a sensitive and specific method for the simultaneous determination of solifenacin and its active metabolite M1 (4S-hydroxy solifenacin) in rat plasma. The procedure involves a two-step liquid–liquid extraction followed by detection on a semi-micro-HPLC-UV to achieve an LLOQ of 2 ng/mL. However, the major drawback of the method is the use of large plasma volume (1.0 mL) for processing and very long chromatographic run time (25 min).

An excellent review is presented by Novara et al. [22] regarding the use of anticholinergic drugs, alone or in combination with α-blockers, in patients with lower urinary tract symptoms due to benign prostatic hyperplasia and concomitant overactive bladder syndrome, to assess the role of antimuscarinic drugs in patients with BPH. Based on the available data a combination therapy of α-blockers and antimuscarinic agents can be considered promising in terms of safety and efficacy. However, well-designed placebo-controlled, long-term randomized-controlled trials (RCTs) are needed to assess their long-term safety and efficacy.

Thus, the objective of the present study was to develop and validate a sensitive, and a high-throughput LC–ESI-MS/MS method for simultaneous quantitation of alfuzosin and solifenacin for routine sample analysis. To the best of our knowledge, this is a first report on their simultaneous estimation in human plasma by any analytical technique. The extraction procedure employed is highly efficient and gives quantitative and reproducible recoveries for both the analytes and the internal standard. The method presented is simple, selective and rugged to support a pharmacokinetic study of alfuzosin and solifenacin for the recommended dose of 10 mg of alfuzosin hydrochloride and 5 mg of solifenacin succinate in human subjects.

2. Experimental

2.1. Chemicals and materials

Reference standards of alfuzosin hydrochloride and solifenacin succinate were provided by Ranbaxy Lab. (New Delhi, India). Propranolol hydrochloride (IS) was obtained from Ipca Lab. (Mumbai, India). All these standards had purity ≥98%. HPLC grade methanol

Table 2
Relative matrix effect results for alfuzosin and solifenacin.

Sr. No.	Plasma	Peak area response ratio			
		Alfuzosin		Solifenacin	
		LQC	HQC	LQC	HQC
1	Lot-1	0.101	2.492	0.102	2.700
2		0.100	2.504	0.111	2.611
3	Lot-2	0.098	2.501	0.104	2.644
4		0.096	2.479	0.103	2.674
5	Lot-3	0.106	2.602	0.110	2.775
6		0.103	2.476	0.101	2.674
7	Lot-4	0.100	2.476	0.110	2.756
8		0.101	2.481	0.104	2.649
9	Lot-5	0.102	2.647	0.107	2.886
10		0.108	2.550	0.111	2.700
11	Lot-6	0.106	2.452	0.109	2.784
12		0.112	2.444	0.115	2.769
Mean peak area response ratio		0.103	2.509	0.107	2.719
S.D.[a]		0.005	0.061	0.004	0.078
%CV[b]		4.4	2.4	4.1	2.9

[a] Standard deviation.
[b] Coefficient of variance.

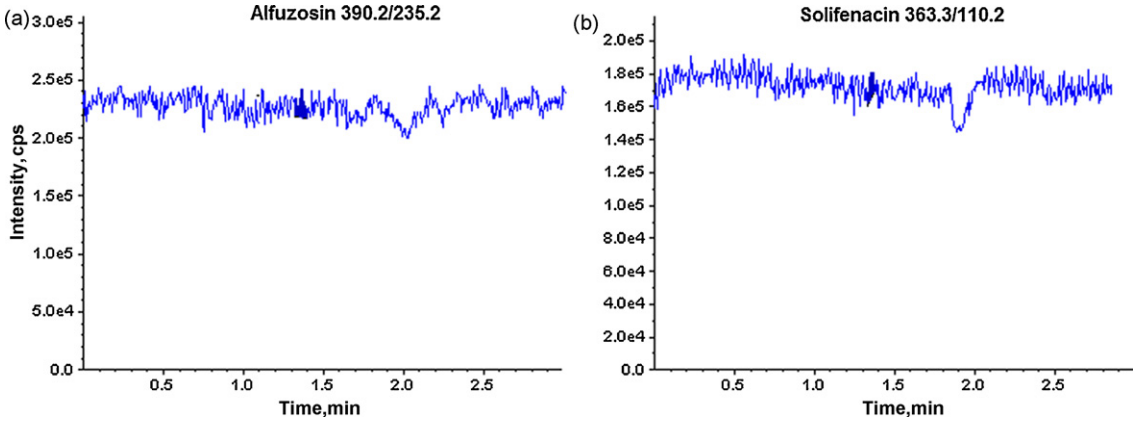

Fig. 2. Post-column infusion MRM LC–MS/MS ion current chromatograms of (a) alfuzosin (390.2 → 235.2) and (b) solifenacin (363.3 → 110.2).

and acetonitrile were purchased from J.T. Baker INC (Phillipsburg, NJ, USA). AR grade ammonium formate and formic acid were procured from Qualigens Ltd. (Mumbai, India). Methyl tert butyl ether used for extraction was procured from Merck Pvt. Ltd. (Mumbai, India). Purified water was generated from Milli-Q A10 gradient water purification system purchased from Millipore (Bangalore, India). Blank human blood was collected with K_3 EDTA from healthy and drug free volunteers. After centrifugation at 2061 × *g* at 10 °C, plasma was collected and stored at −20 °C.

2.2. Liquid chromatography and mass spectrometric conditions

The liquid chromatography system (Shimadzu, Kyoto, Japan) consisted of binary LC-20AD prominence pump, autosampler (SIL-HTc), solvent degasser (DGU-20A_3 prominence) and temperature-controlled compartment for column (CTO 10AVP). The analytical column, Hypurity C8 (50 mm × 4.6 mm internal diameter, 5 μm particle size) from Thermo (India) Pvt. Ltd. (Bangalore, India) was used for separation of analytes and IS. The flow rate of the mobile phase under isocratic condition was kept at 0.4 mL/min. The auto sampler temperature was set at 10 °C and the injection volume was 5 μL. The mobile phase consisted of 2 mM ammonium formate (pH 3.0, adjusted with formic acid) in water: acetonitrile (15:85, v/v). The column oven temperature was maintained at 45 °C and the total LC run time was 3.0 min.

An API-3000 LC–MS/MS triple quadrupole mass spectrometer equipped with a Turbo Ion Spray™ ionization source (Applied Biosystems/MDS Sciex, Toronto, Canada) was used for tandem mass spectrometry. MS/MS analysis was performed in multiple reaction monitoring (MRM) and positive ionization mode, using mass transition *m*/*z* 390.2 → 235.2 for alfuzosin; *m*/*z* 363.3 → 110.2 for solifenacin; and *m*/*z* 260.1 → 116.2 for propranolol (IS). Fig. 1 shows the mass spectra of product ions for analytes and IS respectively. The source dependant parameters optimized were nebuliser gas: 4.14 × 10^4 Pa, curtain gas: 3.72 × 10^5 Pa, source temperature: 400 °C. Collision activated dissociation (CAD) gas was set at 4.14 × 10^4 Pa. Compound dependant parameters set for alfuzosin, solifenacin and IS were declustering potential (DP): 40, 38, and 37 V; collision energy (CE): 39, 38, 26 eV and cell exit potential (CXP): 15, 19, 10 V; entrance potential (EP): 12, 10, 10 V, focusing potential (FP): 280, 260, 250 V respectively. Ion spray voltage (ISV) was set at 5500 V. Q1 and Q3 were maintained at low and unit resolution respectively. Dwell time was kept at 200 ms and no cross talk was found between transitions. Peak areas were integrated using analyst software version 1.4.2.

2.3. Standard and quality control preparation

The standard stock solutions of alfuzosin, solifenacin and propranolol (IS) were prepared by dissolving their accurately weighted compounds in methanol to give a final concentration of 1000 μg/mL for alfuzosin and solifenacin and 500 μg/mL for propranolol. The standard stock solutions of analytes were than serially diluted with methanol: water (70:30, v/v) to obtain working solutions of required concentration range. All the solutions were stored at 2–8 °C and were brought to room temperature before use.

The calibration standards and quality control (QC) samples were prepared by spiking (5% of the total plasma volume) with working solutions. Calibration samples were prepared at concentration of 0.25, 0.5, 1, 2.5, 5, 10, 15, 20 and 25 ng mL^{-1} for alfuzosin and 0.6, 1.2, 2.4, 6, 12, 24, 36, 48 and 60 ng/mL for solifenacin. Quality control samples were prepared at 0.75 ng/mL (low quality control, LQC), 9 ng/mL (medium quality control, MQC) and 18 ng/mL (high quality control, HQC) for alfuzosin and 1.8 ng/mL (LQC), 21.6 ng/mL (MQC) and 43.2 ng/mL (HQC) for solifenacin. Aliquots of spiked plasma samples were taken in micro-centrifuge tubes and stored at −20 °C.

2.4. Sample preparation

All frozen subject samples, calibration standards and quality control samples were thawed at room temperature prior to analysis. The samples were adequately vortexed and centrifuged at 1811 × *g* for 2 min at 10 °C to settle any solid present. An aliquot of 0.5 mL plasma sample was mixed with 50 μL of internal standard (0.3 μg/mL) and 3.0 mL of methyl tert butyl ether was added. The mixture was vortexed for 2 min, followed by centrifugation at 1811 × *g* for 5 min at 10 °C. The organic layer was separated and then evaporated under a gentle stream of nitrogen at 50 °C. The sample was reconstituted with 0.5 mL mobile phase and 5 μL was used for injection in the LC–MS/MS system.

2.5. Methodology for validation

The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, stability and dilution integrity according to USFDA guidelines [23].

Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, 2 haemolysed and 2 lipemic. They were processed by the proposed extraction protocol and their response was assessed at the retention time of analytes and the internal standard. Six LLOQ samples for alfuzosin and solifenacin were prepared from the screened blank plasma samples which had the least interference.

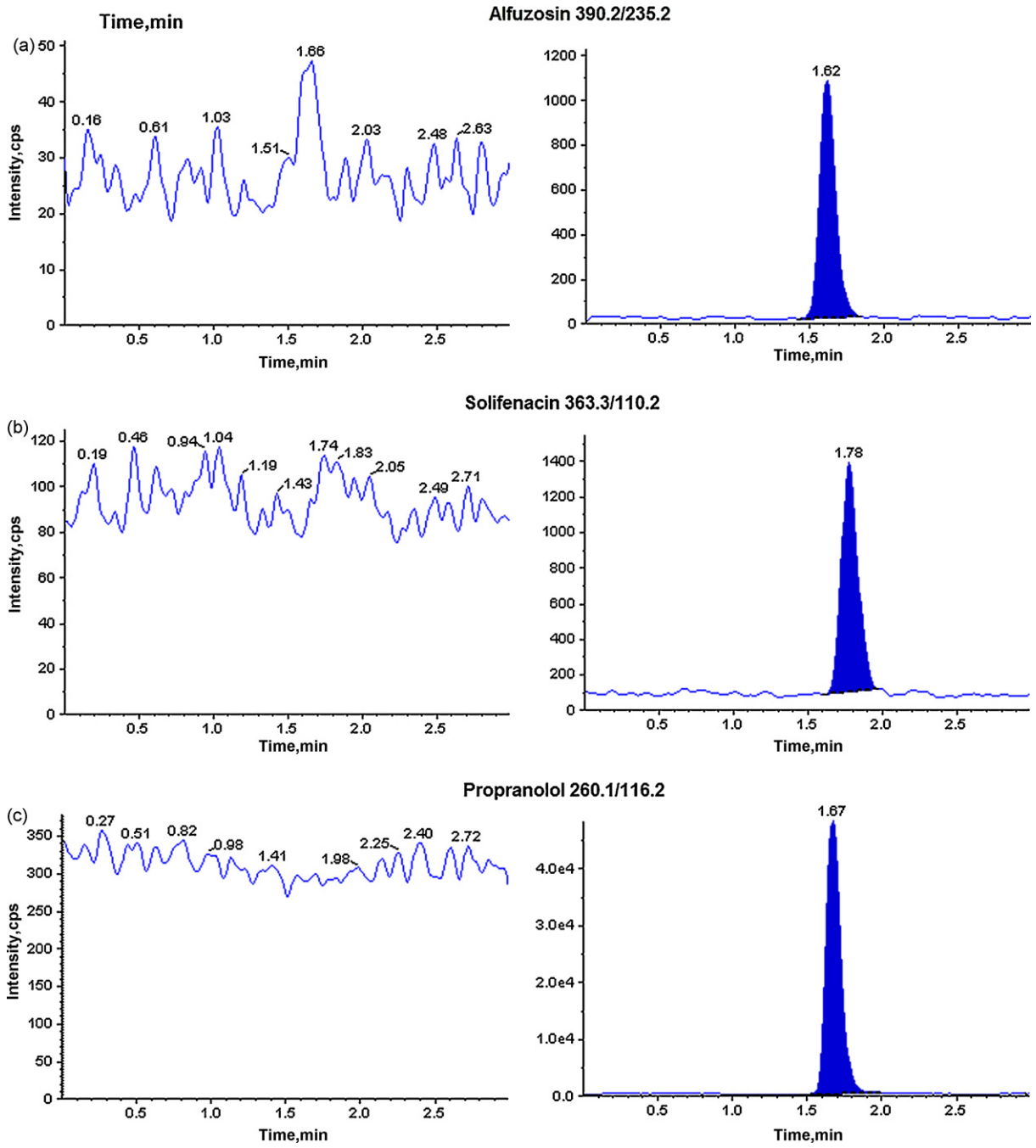

Fig. 3. MRM chromatograms of (a) alfuzosin (390.2 → 235.2), (b) solifenacin (363.3 → 110.2) and (c) propranolol (260.1 → 116.2, IS) in blank plasma and at LLOQ respectively.

Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle is able to avoid any carry forward of injected sample in subsequent runs. The design of the study comprised of the following sequence of injections: blank plasma → LLOQ → ULOQ → blank plasma to check for any possible interference due to carryover.

The linearity of the method was determined by analysis of standard plots associated with a nine-point standard calibration curve. Three linearity curves containing nine non-zero concentrations were analyzed. The calculation was based on the peak area ratio of analyte versus the area of internal standard. The concentration of the analytes were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x$) for alfuzosin and solifenacin. The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the range tested.

Intra-day and inter-day accuracy and precision of the method were assessed at four different concentrations levels (LLOQ, LQC, MQC and HQC) for each analyte. Mean and standard deviation (S.D.) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (%CV) respectively.

Recovery represents the extraction efficiency of a method and was performed at LQC, MQC and HQC levels. Absolute recovery was evaluated by comparing peak area of extracted samples to the peak area of unextracted samples (quality control working solutions spiked in mobile phase).

Table 3
Stability results for alfuzosin and solifenacin.

Stability	Storage condition	Level	Alfuzosin					Solifenacin				
			A[a]	%CV	B[b]	%CV	%Mean change	A[a]	%CV	B[b]	%CV	%Mean change
Bench top	Room temperature (6 h)	LQC	0.70	4.5	0.66	3.6	−5.5	1.66	3.6	1.63	2.4	−1.3
		HQC	16.48	2.0	16.38	1.6	−0.6	42.46	2.0	41.98	2.4	−1.1
Process	Autosampler (10 °C, 50 h)	LQC	0.69	3.9	0.67	6.7	−3.2	1.79	3.3	1.68	2.0	−6.1
		HQC	16.88	2.3	16.79	3.0	−0.6	39.76	2.0	40.56	2.7	2.0
Freeze and thaw	After 5th cycle at −20 °C	LQC	0.70	2.2	0.73	8.0	4.4	1.79	2.5	1.86	3.3	4.1
		HQC	16.78	2.6	16.98	3.1	1.2	40.89	4.1	41.84	3.6	2.3
Long-term stability	72 days at −20 °C	LQC	0.66	2.7	0.69	4.7	4.7	1.71	0.9	1.79	2.4	4.4
		HQC	17.06	5.1	17.35	1.2	1.7	42.51	2.5	42.20	1.8	−0.7
Dry extract stability	13 h, 2–8 °C	LQC	0.69	3.9	0.68	3.0	−1.1	1.79	3.3	1.73	2.2	−3.3
		HQC	16.88	2.3	16.82	2.7	−0.4	39.76	2.0	40.58	3.6	2.0

[a] Mean comparison concentration (ng/mL).
[b] Mean stability concentration found (ng/mL).

To evaluate the magnitude of matrix ion suppression/enhancement effect at the retention time and MRM of the analytes, post-column analytes infusion experiment was conducted. A standard solution of alfuzosin and solifenacin at MQC concentration level in mobile phase was infused post-column via a 'T' connector at 5 μL/min employing Harvard infusion pump. Aliquots of 5 μL of extracted control plasma were then injected into the column and MRM LC–MS/MS chromatograms were acquired for both the analytes. Any dip in the baseline upon injection of extracted control plasma would indicate ion suppression, while a peak at the retention time of alfuzosin and solifenacin indicates ion enhancement. To study the effect of matrix on analyte quantitation with respect to consistency in signal, relative matrix effect was checked in six different lots of K3 EDTA plasma. Two replicates, each at LQC and HQC levels were prepared from these lots of plasma and checked for the accuracy in terms of %bias in all the QC samples. Cross selectivity experiment was conducted for alfuzosin and solifenacin at ULOQ level by comparing the peak area at their respective retention times.

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed by comparing area response of stability samples of analytes and the internal standard with the area response of sample prepared from fresh stock solutions. The results should be within the acceptable limit of ±10% change for stock solution stability experiment. Bench top stability, extracted sample stability (process stability), freeze–thaw stability, dry extract stability and long-term stability were performed at LQC and HQC level using six replicates at each level. To meet the acceptance criteria, the difference between the stability and fresh samples should be within ±15%.

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was carried out at 1.5 times the ULOQ concentration. Six replicates samples of 1/2 and 1/4th concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

3. Results and discussion

3.1. Method development

To develop a rapid, selective and sensitive method, mass acquisition parameters, chromatographic conditions and an efficient extraction procedure are mandatory. Mass spectrometry parameters can play an important role in selectivity and sensitivity while chromatography has an impact on selectivity and run time of the method. Simple and efficient extraction procedure can help to obtain clearer samples for quantitative recoveries. Selection of internal standard is equally important as it constantly takes care of the analytes for quantification.

Initially, solution (100 ng/mL) of both the analytes and IS in methanol was infused to acquire optimum mass spectrometer parameters. Using ESI as ionization source, tuning was done in positive mode as both the compounds have the ability to accept protons and give protonated species $[M+H]^+$ ions in Q1 mode. Protonated parent ions for alfuzosin, solifenacin and propranolol were observed at m/z 390.3, 363.4 and 260.1 respectively. Higher and consistent response was achieved after optimum setting of the compound dependent parameters like DP, EP and FP. Addition of formic acid in infusion solution further enhanced the ionization (protonation), resulting in higher response. Fragmentation was car-

ried out using nitrogen as CAD gas in the collision cell. Most stable and intense product ions were observed at m/z 235.2, 110.2 and 116.2 for alfuzosin, solifenacin and propranolol respectively. For alfuzosin, it was difficult to get a consistent and intense product ion even at higher collision energy, thus the ion at m/z 235.2 was selected for further study.

To set the chromatographic conditions, different buffers like ammonium acetate, ammonium formate with methanol/acetonitrile were tried. Acidic mobile phase was preferred as the compounds are basic ($pK_a > 8.0$). Use of long column was deliberately avoided in order to have a short chromatographic runtime, thus hypurity C8 column (50 mm × 4.6 mm) was used. 2 mM ammonium formate (pH 3.0, adjusted with formic acid): acetonitrile (15:85, v/v) was finalized as mobile phase as it gave good peak shapes with desired response. Low pH buffer enhanced protonation and helped in eluting the analytes completely without tailing, which assisted in proper quantification of analyte peaks. Further, high content of acetonitrile in the mobile phase assisted in eluting the analytes and IS within 3 min. Fine tuning was performed with mobile phase to optimize the source dependent parameters in mass spectrometer. Nebuliser gas (gas1), heater gas (gas2) and source temperature (400 °C) had a major impact on signal intensity. The noise level decreased significantly at higher curtain gas, which resulted in increased signal to noise ratio. However, ion spray voltage did not have any major impact on the intensity.

For plasma extraction, liquid–liquid extraction was preferred as this method is cost effective and has ability to remove interferences from sample matrix. Initially, protein precipitation was tried using acidic acetonitrile and methanol as precipitating agents. However, the recovery was very poor (<40%) due to the presence of co-eluting compounds which suppress the ionization. Moreover, this method was unable to remove interferences from sample matrix. The other drawbacks of this method were column clogging and frequent cleaning of MS source. Medium polar solvents like diethyl ether, methyl tert butyl ether, ethyl acetate were used to extract the compounds as alfuzosin and solifenacin have the log P values of 1.6 and 3.1 respectively. Methyl tert butyl ether was the best solvent that gave consistent recoveries (≥70%) for both the analytes and IS. Sample preparation is simple and quick as back extraction was not required. No interference was found at the retention time of analytes and IS.

No major suppression/enhancement was found at the retention time of alfuzosin, however a minor suppression was observed at the retention time of solifenacin as shown in Fig. 2, which did not have a significant impact on analyte quantification. Absolute matrix effect was checked by injecting unextracted (the solution prepared in mobile phase) and extracted sample according to the method outlined by Matuszewski et al. [24]. The response observed for extracted sample was around 5–6% less compared to unextracted sample for both the analytes.

Propranolol, used as an internal standard in the present study was adequate to control any analytical variation due to solvent evaporation, integrity of the column and ionization efficiency. There was no effect of IS on analyte recovery, sensitivity or ion suppression.

3.2. Selectivity and sensitivity

Chromatograms for blank plasma and at LLOQ in Fig. 3 demonstrate the sensitivity and selectivity of the method with retention times of 1.82, 1.78 and 1.67 min for alfuzosin, solifenacin and IS respectively. The interference observed at the retention time of analytes and IS was less than 2% in all blank plasma samples. %CV for six LLOQs was less than 8% and %accuracy was within 95–105% for both the analytes. There was no response found in blank plasma after highest calibration standard (ULOQ) injection, which indicates no carryover of the analyte in subsequent runs.

3.3. Linearity, accuracy and precision

All three calibration curves were linear from 0.25 to 25 ng/mL with correlation coefficient $r \geq 0.9984$ for alfuzosin and 0.6–60 ng/mL with $r \geq 0.9996$ for solifenacin. A straight line fit was made through the data points to give the linear equation $y = 0.083x + 0.001$ for alfuzosin and $y = 0.061x - 0.004$ for solifenacin, where y is the peak area ratio of the analyte to IS and x is the concentration of the analyte. The standard deviation values for slope, intercept and correlation coefficient (r) found was 0.006, 0.001 and 0.001 for alfuzosin and 0.005, 0.003 and 0.0002 for solifenacin respectively.

For inter-day, three runs and for intra-day, a single run was assayed. Each run contains six replicates at four concentration levels (LLOQ, LQC, MQC and HQC). Intra-day and inter-day precision was less than 8% while the accuracy (%RE) was within −11.8 to 6.4% for both analytes with respect to their nominal concentration as given in Table 1.

3.4. Relative matrix effect and recovery

Consistency of matrix effect was checked by injecting unextracted LQC and HQC samples prepared by spiking externally with working solution in extracted plasma. This was checked in different six plasma lots including a haemolysed and lipemic lot. The %CV for all six lots was less than 4.5% (Table 2). Cross selectivity experiment indicated no interference of alfuzosin on solifenacin and vice versa.

The recovery found at LQC, MQC and HQC level was 69.7, 73.6 and 72.1% for alfuzosin; 93.2, 95.1 and 90.9% for solifenacin respectively. The precision (%CV) among three QC levels found was 7.4, 4.8 and 3.5 for alfuzosin and 6.3, 9.5 and 5.2 for solifenacin. Recovery of IS was 89.9% with a %CV of 4.5.

3.5. Stability results and dilution integrity

Stock solutions of both analytes and IS were stable at room temperature for 24 h and at 2–8 °C for 17 days. Alfuzosin and Solifenacin in control human plasma at room temperature were stable at least for 6 h and for minimum of five freeze and thaw cycles. Process stability was of 50 h at 10 °C. Spiked plasma samples stored at −20 °C for long-term stability experiment were stable for at least 72 days. Dry extract stability was of 13 h at 2–8 °C. Different stability experiments in plasma and the values for the precision and percent change are shown in Table 3.

The mean back calculated concentrations for 1/2 and 1/4th dilution samples were within 85–115% of their nominal values. The coefficient of variation (%CV) for 1/2 and 1/4th dilution samples of analytes was less than 3.8%.

3.6. Application of the method on human volunteers

The proposed method was successfully applied to a bioavailability study in eight healthy human male subject samples for a formulation containing 10 mg of alfuzosin hydrochloride extended release and 5 mg of solifenacin succinate under fed condition. All subjects were informed of the aim and risk involved in the study and their consent were obtained. The work was approved and subject to review by Institutional Ethics Committee, an independent body comprising of lawyers, medical doctors, social workers, pharmacologists and an academician. The procedures followed while dealing with human subjects were based on International Conference on Harmonization, E6 Good Clinical Practice (ICH, E6 GCP)

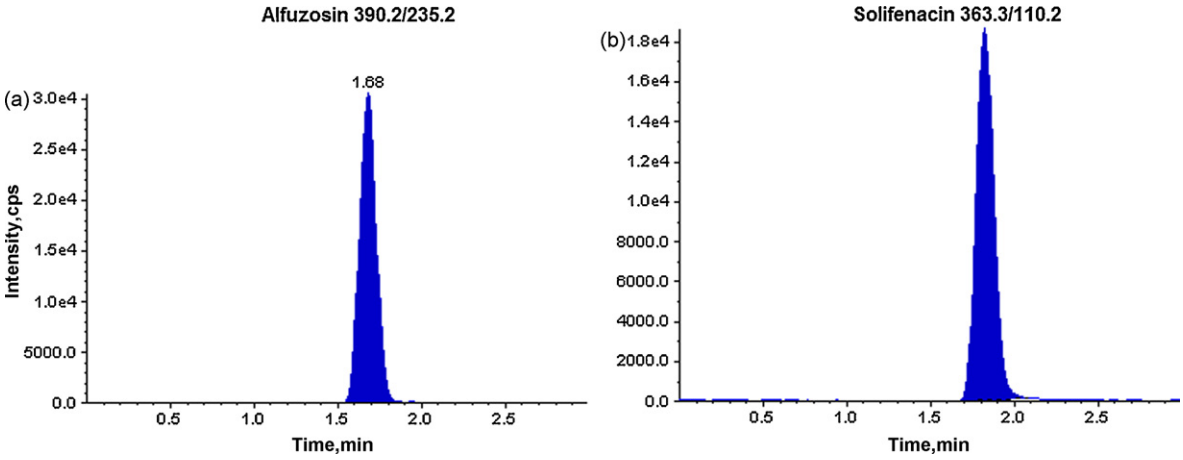

Fig. 4. Real subject chromatograms of (a) alfuzosin and (b) solifenacin at 3.5 and 4.0 h respectively.

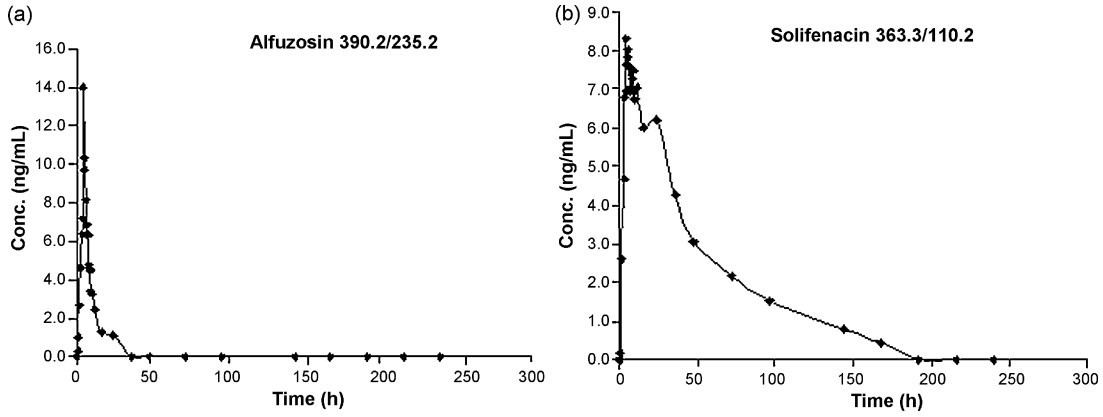

Fig. 5. Mean pharmacokinetic profile of (a) alfuzosin and (b) solifenacin after oral administration of 10 mg of alfuzosin hydrochloride and 5 mg of solifenacin succinate tablet formulation to eight healthy human subjects under fed condition.

guidelines [25]. Health check up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. All subjects were negative for HIV, HBSAg and HCV tests. Each subject was orally administered a single dose of test formulation after recommended wash out period of 28 days with 240 mL of water. Drinking water was not allowed and supine position was restricted 2 h post-dose. Standardized meals were provided as per schedule. Blood samples were collected in tubes containing K3 EDTA before and after 0.5, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 12.0, 16.0, 24.0, 36.0, 48.0, 72.0, 96.0, 144.0, 168.0, 192.0, 216.0 and 240.0 h of administration of drug. Blood samples were centrifuged at 1811 × *g* for 10 min and plasma was separated, stored at −20 °C until use. All 248 samples including the calibration, QC and subject samples were run and analyzed in only 3 days and precision and accuracy for calibration and QC samples were within acceptable limits. Fig. 4 gives the MRM chromatograms of real subjects for alfuzosin and solifenacin. $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ and C_{max} (AUC: area under curve, C_{max}: peak plasma concentration) were calculated. Mean plasma concentration versus time profiles for the treatment, under fed condition for alfuzosin and solifenacin are presented in Fig. 5.

4. Conclusion

This LC–ESI-MS/MS method is the first report for the simultaneous determination of alfuzosin and solifenacin in human plasma. It is highly sensitive and selective with a short turnaround time. The validated method presents a simple and cost effective extraction procedure with quantitative and reproducible recoveries for alfuzosin and solifenacin. No interferences from endogenous plasma components or other sources were found and no "cross talk" effect was observed in plasma samples. The on-column loading of alfuzosin (125 pg) and solifenacin (300 pg) per injection volume was very less compared to all other reported procedures [16–21]. The validation data demonstrates good precision and accuracy of the method. Also, the established LLOQ is sufficiently low to conduct a pharmacokinetic study of alfuzosin and solifenacin.

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