

## Simple and Rapid HPLC Method for the Determination of Alfuzosin in Human Plasma

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### ABSTRACT

A simple, sensitive and precise HPLC method for the quantitation of alfuzosin in human plasma has been developed and validated. Commercially available atenolol was used as an internal standard. After liquid-liquid extraction, alfuzosin and atenolol (I.S.) in human plasma were analyzed using mobile phase containing 25 % v/v acetonitrile and 75 % v/v water (containing 1ml/L triethylamine as peak modifier, pH adjusted to 2.5 with orthophosphoric acid). Chromatographic separation was achieved on a BDS Hypersil-C18 column (50 mm × 4.6 mm i.d., particle size 5µm) using isocratic elution at (flow rate 0.5 mL / min). The peak was detected using a fluorescence detector programmed for 0-2 min at Ex 222 nm and Em 300 nm for atenolol and 2-4 min at Ex 265 nm and Em 380 nm for alfuzosin, and the total time for a chromatographic separation was ~ 4 min. The validated quantitation ranges of this method were 0.1–25 ng/ml with coefficients of variation between 1.6 - 4.8%. Mean recoveries were 98.0±0.9 %. The within and between batch precision was 2.74-3.28% and 2.65 - 2.77 %, respectively. The within and between batch relative error (bias) were -7.1– (-0.3) % and -1.60 - 2.46 %, respectively. Stability of alfuzosin in plasma was ≥ 94.9%, with no evidence of degradation during sample processing and 30 days storage in a deep freezer at -70 ± 5°C. The absolute extraction recoveries of drug from plasma was ≥ 98%. This validated method is sensitive and simple with between-batch precision of ≤ 3%. The method can be used for bioequivalence and pharmacokinetic studies of alfuzosin formulations.

**Keywords:** Alfuzosin, Assay; HPLC; Plasma.

### INTRODUCTION

Alfuzosin, *N*-{3-[(4-amino-6,7-dimethoxy-2-quinazoliny) methylamino] propyl} tetrahydro-2-furancarboxamide hydrochloride (Fig.1), is an antagonist of  $\alpha_1$  post-synaptic adrenergic receptors, showing some myorelaxant effects<sup>(1,2)</sup>. Alfuzosin is a basic compound with a  $pK_a$  value of 8.13 and is stable under normal conditions of temperature and light<sup>(3)</sup>. Alfuzosin, a quinazoline derivative, is a selective and competitive antagonist of  $\alpha_1$ -adrenoceptor-mediated contraction of

prostatic smooth muscle. As a result it decreases sympathetically controlled muscle tone and improves the rate of urinary flow. Alfuzosin differs from other quinazoline derivatives (prazosin, terazosin, doxazosin) by the presence of a diaminoethyl spacer and the absence of a piperidine moiety<sup>(4)</sup>.

Several methods have been described for the determination of alfuzosin in plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with fluorimetric and luminescence detection, achieving lower limits of quantification (LLOQ) of around 1 ng/ml<sup>(3, 5, 6, 7, 8, 9, 10)</sup>. A highly sensitive and rapid tandem mass spectrometry method for alfuzosin in plasma<sup>(11)</sup> and LC-ESI-MS/MS

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method for simultaneous quantification of alfuzosin and solifenacin in human plasma<sup>(12)</sup> have been reported. The calibration range for alfuzosin was 0.391-32.3 and 0.25-25 ng/ml.

For routine clinical analysis a high throughput analysis is required. A simple, sensitive and rapid analytical method is necessary for quantitation of the concentrations of alfuzosin in human plasma in order to support pharmacokinetic and bioequivalence studies.

The aim of this study was to develop and validate a more selective and sensitive assay method than the previously described with minimal sample preparation and short chromatography time. This new method makes use of a fluorometric detector in conjunction with liquid chromatography to increase the selectivity, which allows

for more sensitive and rapid chromatography and is well suited for bioequivalence studies involving large numbers of samples. To achieve this we used short column (50 mm × 4.6 mm), and suitable commercial internal standard.

The method reported in this paper is a simple, rapid, sensitive and accurate HPLC–RF method to determine alfuzosin in plasma utilizing commercially available internal standard. The present method has the following advantages over the other reported methods: (1) sensitivity is increased to 0.1ng/ml, (2) rapid as the run time is only 4 minutes, (3) only 500µl of plasma sample is required for sample preparation and finally (4) organic waste is less as it utilizes less mobile phase.

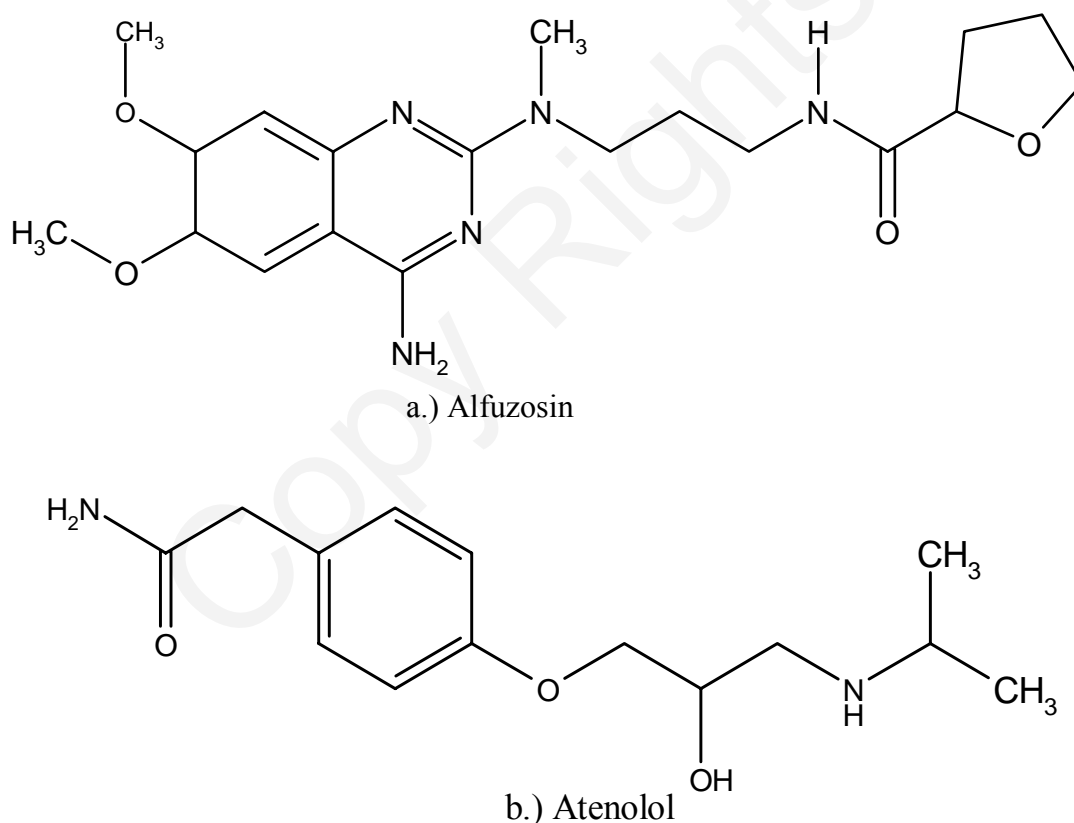


Fig. (1): Structure of a) Alfuzosin b) Atenolol

## EXPERIMENTAL

### Chemicals and reagents

Alfuzosin (99.2%) and atenolol (99%) were obtained from Tabuk Pharmaceutical Manufacturing Company (KSA). Chemical structures are presented in figure 1. HPLC grade Lichrosolv acetonitrile, methanol and water were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

### Drug Solutions Stock solutions

The stock solution of alfuzosin (0.5 mg/ml) and atenolol (1 mg/ml) for generating a standard curve prepared by dissolving an appropriate amount of each were compound in methanol respectively. Working solution of alfuzosin was obtained by further diluting the stock standard solution with methanol. Working solution of atenolol (15 µg/ml) was prepared by diluting stock solution with HPLC grade water.

### Preparation of Standard and quality control samples

Working solution of alfuzosin for the calibration (2.0, 10.0, 20.0, 50.0, 100.0, 200.0, 300.0 and 500 ng/ml) and quality control sample (6.0, 250.0 and 400.0 ng/ml) were prepared separately. Working solution for calibration and quality control were prepared by diluting the suitable concentration of stock solution to 20 ml in volumetric flask using methanol. The internal standard solution (15 µg/ml) was prepared by diluting the stock solution with HPLC grade water.

Fifty micro-litre of working standard solution was added to 950 µl of drug free plasma to obtain alfuzosin concentration of 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 25.0 ng/ml. Similarly, the quality control sample of alfuzosin as a single batch [of concentration 0.3 (low), 12.5 (medium) and 20.0 ng/ml (high)] were prepared by spiking the 5% appropriate working solution to 95% of pooled blank drug free plasma. The quality control samples were divided into aliquot in test tube and were stored at  $-70 \pm 5$  °C until analysis. All stock solutions were stored between 4-8 °C.

### Chromatographic conditions

The HPLC system (Agilent Technologies, Inc., Santa Clara, CA) consisted of a constant solvent delivery system

(G1311A), a spectrofluorometric detector (G1321A) equipped with an auto-sample injector (ALS-G1329) fitted with a 100 µl sample loop. The analytical column employed was BDS Hypersil C18 (50 mm × 4.6 mm i.d., 5 µm particle size, Thermo Electron Corporation, USA). The data were captured using Microsoft Windows-XP based Chem. Station for LC system Rev. B.03.01 [317] 2001-2007 chromatographic software. The mobile phase was comprised of containing 25 % v/v acetonitrile and 75 % v/v water (containing 1ml/L triethylamine as peak modifier, pH adjusted to  $2.5 \pm 0.1$  with orthophosphoric acid using Orion Research Model 611 pH Meter). The mobile phase was filtered through 0.45 µm Millipore filter before used and degassed in an ultrasonic bath. All separations were performed isocratically at a flow rate of 0.5 ml/min. The detector was programmed for 0-2 min at an excitation wavelength of 222 nm and an emission wavelength of 300 nm for atenolol and from 2-4 min at an excitation wavelength of 265 nm and an emission wavelength of 380 nm for alfuzosin.

### Sample processing

All plasma samples were thawed at room temperature. Thawed plasma (500 µl) was transferred to a 15ml glass test tube and the 50 µl of internal standard (15 µg/ml) was added. Mixture was vortexed and 75 µl of 2M-sodium hydroxide was added. Then 6 ml aliquot of extraction solvent diethylether : dichloromethane (7:3, v/v) was added using Ceramus® Classic Bottle top Dispenser (Hirsch Mann Laborgerate GmbH, Eberstadt, Germany). The sample was vortexed for 10 min using Vibrax Vortexer (Model VX-Z VXR Basic, IKA Werke GmbH & Co. Staufen, Germany) and centrifuged for 2 min at  $1000 \times g$ . The organic layer was transferred to a 10 ml test tube. The organic layer was evaporated and the residue was reconstituted in 200 µl of mobile phase, vortexed and an aliquot of 100 µl was injected into HPLC.

### Bioanalytical method validation

The method was validated for selectivity, specificity, linearity, precision and accuracy, recovery, stability and dilution integrity according to USFDA guidelines<sup>(13)</sup>.

Calibration curves were made from blank (a plasma sample processed without IS), a zero sample (a plasma

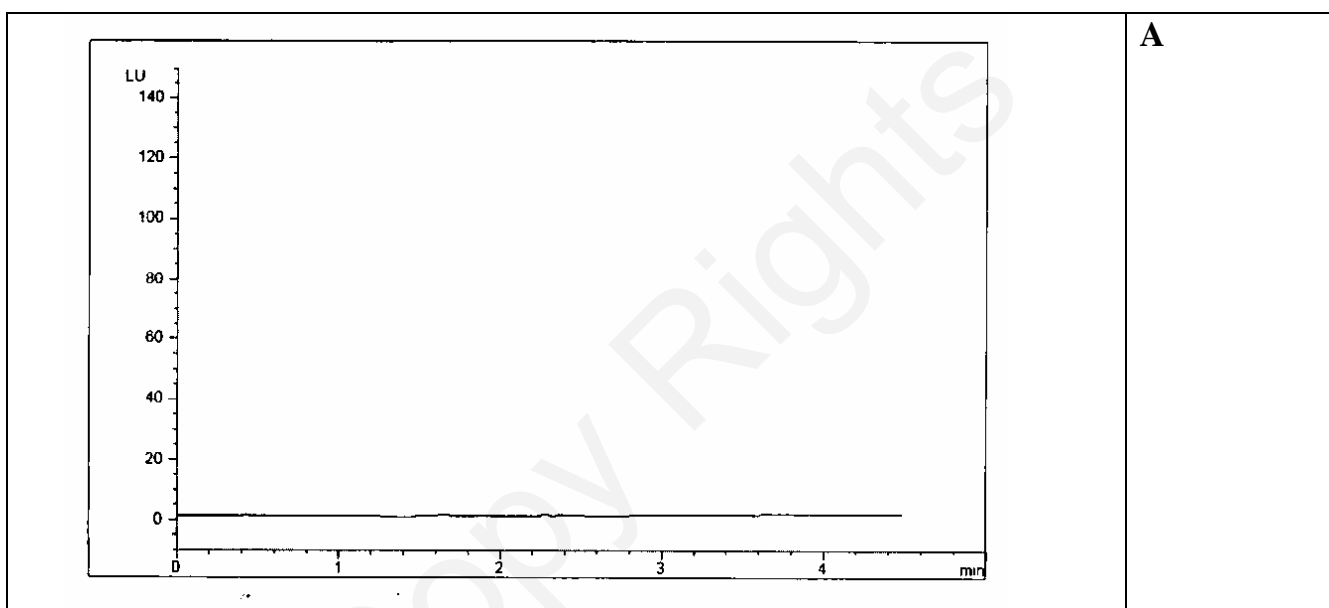
processed with IS) and eight non-zero samples covering the total range (0.1-25.0 ng/ml), including lower limit of quantitation (LLOQ). Each validation run consisted of system suitability sample, blank sample, a zero sample, eight non-zero sample (n=5) covering the total range (0.1-25.0 ng/ml) and quality control sample at three concentration (n=10 at each concentration). Samples were analysed from lower to high concentration at the beginning of each validation run. Other samples were distributed randomly through the run.

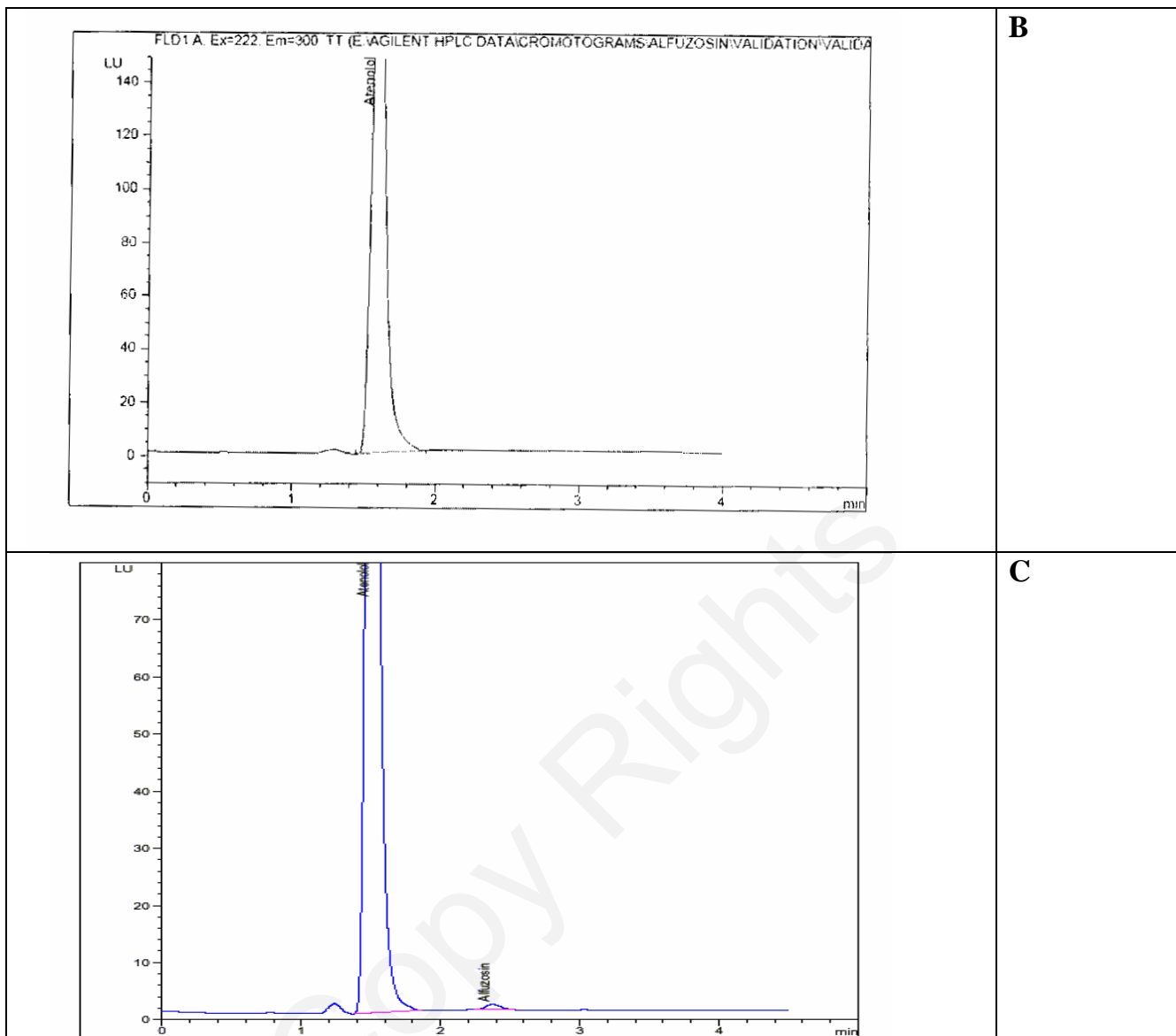
Such calibration curves were generated on 3 consecutive days. Linearity was assessed by weighted (x) least square regression analysis. The acceptance criterion for each back-

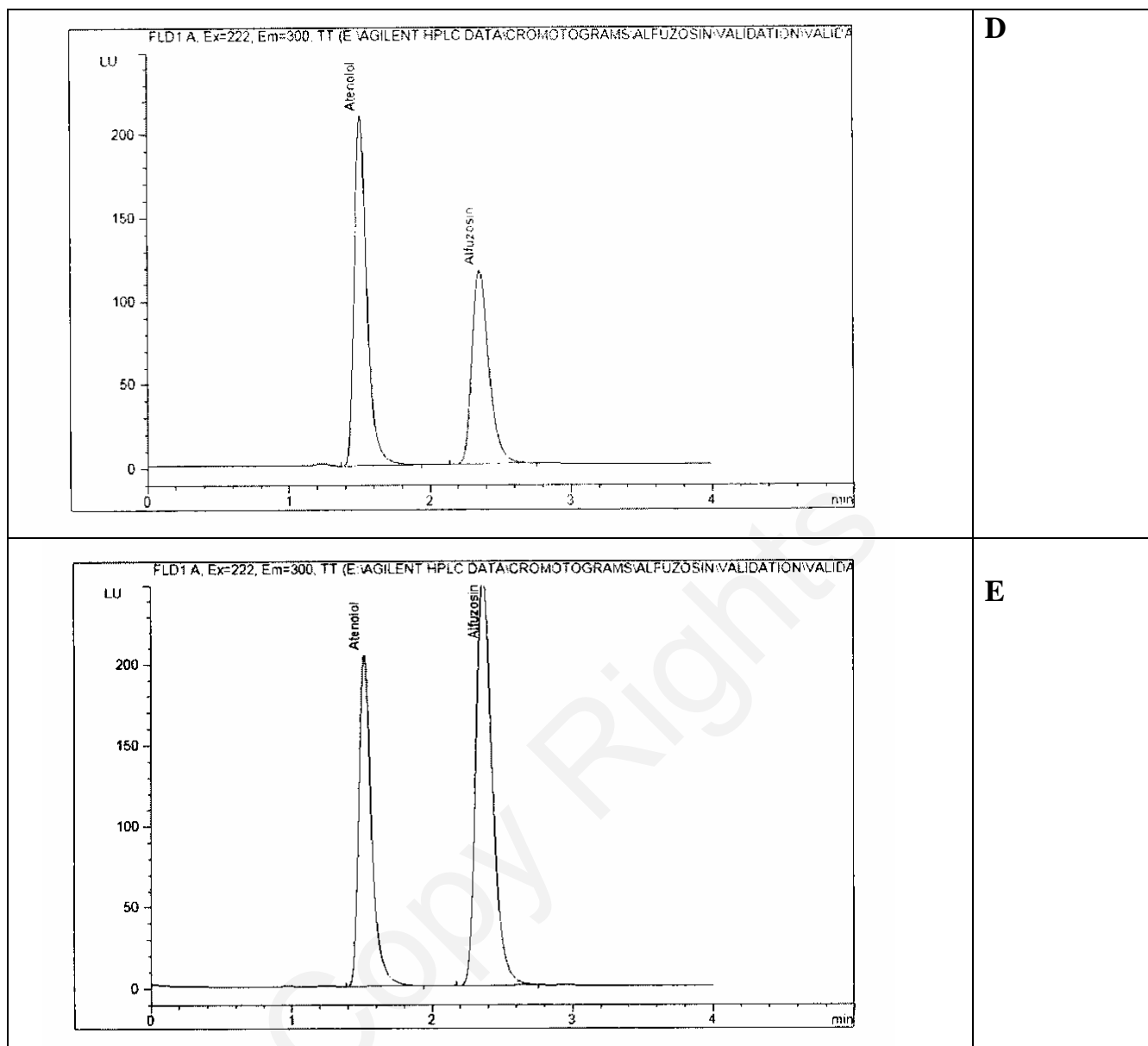
calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

#### Specificity and selectivity

To evaluate the specificity of the method, drug free plasma sample was carried through the assay procedure and the retention times of the endogenous compounds in the plasma were compared with those of alfuzosin or atenolol (internal standard). Specificity of the method was assessed to test the matrix influence between different plasma samples. Interference from OTC (over the counter) medication was also investigated. The tested compounds were salicylic acid, paracetamol, ibuprofen, mefenamic acid, caffeine and nicotinamide.







**Figure 2: Chromatograph resulting from (A) Analysis of blank human plasma, (B) human plasma spiked with 15 $\mu$ g/ml Atenolol (IS) (C) human plasma spiked with 0.1ng/ml of Alfuzosin and IS, (D) human plasma spiked with 12.5 ng/ml of alfuzosin and IS (E) human plasma spiked with 25 ng/ml of alfuzosin and IS.**

### Sensitivity

The limit of detection was defined, as analyte responses are at least five times the response compared to blank response. The lowest standard on the calibration

curve was defined as the limit of quantification as an analyte peak was identifiable, discrete, and reproducible with a precision of less than or equal to 20% and accuracy of 80–120%.

**Table 2: Statistical evaluation of the analysis results for alfuzosin in standard curves**

Concentration added (ng/mL)	Concentration found (mean ± S.D., n=15) (ng/mL)	Precision (%)	Bias (%)
0.1	0.11±0.004	3.5	6.5
0.5	0.52±0.02	3.9	4.2
1.0	1.09±0.02	1.6	8.6
2.5	2.78±0.06	2.4	11.0
5.0	5.26±0.16	3.1	5.2
10.0	10.46±0.50	4.8	4.6
15.0	15.21±0.46	3.0	1.3
25.0	25.00±0.73	3.9	-0.02

### Accuracy and precision

Within-batch accuracy and precision evaluation were performed by repeated analysis of alfuzosin in human plasma. The batch consist of calibration standards (n=5), ten replicates of LLOQ, low, medium and high quality control samples, while between batch accuracy and precision were assessed by analysis of similar sequence of samples on three separate occasions. The overall precision of the method expressed as relative standard deviation and accuracy of the method expressed in term of relative error (bias).

### Recovery

The efficiency of alfuzosin extraction from human plasma was measured analyzing three levels of QC samples. The drug recovery was determined by comparing peak area obtained from the spiked QC plasma samples after extraction and reconstitution to the standard solution at the same concentration of the spiked QC plasma samples.

### Stability

The bench top stability was examined by keeping replicates of the low, medium and high plasma quality control samples at room temperature for approximately 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Auto-sampler stability of alfuzosin was tested by analysis of processed and reconstituted low, medium and high plasma QC samples, which are stored in the auto-sampler

tray for 24 h at 5±1°C. Stability of alfuzosin in human plasma was tested after storage at approximately –70±5 °C for 30 days. For each concentration and each storage condition, three replicates were analyzed in one analytical batch. The concentration of alfuzosin after each storage period was related to the initial concentration as determined for the samples.

### Stock solution stability

The working solution (1.875 µg/ml) of alfuzosin was repeatedly (n=3) injected into the chromatograph immediately after preparation (time 0) and at 3, 6, and 9 hours after bench top storage at room temperature and 4°C. This injection protocol was repeated after 1, 8, 15 and 30 days storage of this solution between 4-8°C.

## RESULTS AND DISCUSSION

### Separation and specificity

Figure 2 shows the representative chromatograms of (A) blank plasma, (B) blank plasma with internal standard, (C) human plasma spiked with 0.1 ng/ml (LLOQ) alfuzosin and with internal standard, (D) human plasma spiked with 12.5 ng/ml alfuzosin and with internal standard (E) human plasma spiked with 25.0 ng/ml alfuzosin and with internal standard. The analytes were well separated from co-extracted material under the present chromatographic conditions at retention time of ~1.5 and ~2.3 min for atenolol and alfuzosin respectively. The total run time was ~4 min. The peaks were of good shape and, completely resolved one from another. The

chromatogram of extracted plasma samples did not show any co-eluting interference peak with the analyte or IS. There were no interfering peaks present in six different randomly selected samples of drug free human plasma used for analysis at the retention time of either analyte or IS. Several compounds (salicylic acid, paracetamol, caffeine, mefenamic acid, ibuprofen and nicotinamide) did not produce any interference with the drug or the internal standard during analysis.

#### Sensitivity

The limit of detection and quantification were 0.025 and 0.1 ng/ml respectively. The precision and relative errors for LLOQ (n=15) were 3.5% and 6.5%,

respectively.

#### Recovery and linearity

Recovery from plasma was calculated by comparing the peak areas of pure standards prepared in methanol, and injected directly into the analytical column with those of precipitated plasma samples containing the same amount of the test compound (n = 6 each). Mean recoveries of alfuzosin were ranged from 96.7-100.1 % with coefficients of variation 1.34-2.74 % of at three different concentrations ranging for alfuzosin (0.3, 12.5 and 25 ng/ml) (Table 1). The mean recovery of IS was 42.8%. These results indicate that the method was reliable and reproducible within the acceptance ranges.

**Table 1: Representative calibration curve for HPLC assay of alfuzosin in plasma\***

Calibration curve	Slope	y intercept	r <sup>2</sup>
Day 1 (n=5)	0.054526±0.001214	0.006987±0.00511	0.99987
Day 2 (n=5)	0.055539±0.001347	0.007383±0.00537	0.99981
Day 3 (n=5)	0.061535±0.001559	0.01435±0.002034	0.99943

\*Eight non-zero calibration standards were included in each calibration curve.

Calibration curves were linear over the concentrations range from 0.1 to 25 ng/ml for alfuzosin (r = 0.999 or better) with coefficients of variation less than 5%. The best-fit calibration curve were achieved with linear equation  $y=mx+c$  with 'x' weighting factor. The mean linear equation of calibration curve (n=15) for the analyte was  $y= 0.057544 (\pm 0.0032)x + 0.009594 (\pm 0.005304)$ , where y was the peak area ratio of the analyte to the I.S. and the x was the concentration of the analyte. Precision and relative error of back calculated concentrations of standard solutions for alfuzosin are mentioned in Table-2.

#### Accuracy and precision

Within and between-day precision and accuracy were evaluated with different concentration of alfuzosin. Within and between-day precision (% CV) were less than 3.3 and 2.9 respectively. Within and between day relative error (bias, %) were -7.1 and 2.46 %, respectively (Table

3). Accuracy was expressed as percent error (relative error)  $[(\text{measured concentration} - \text{spiked concentration}) / \text{spiked concentration}] \times 100$  (%). Calculating within- and between-day % CV values quantitated precision.

#### Dilution integrity

The dilution integrity was also conducted to assess whether upper concentration limit (25 ng/mL) can be extended or not. Quality control sample in (six replicate) at concentration of 40 ng/mL were diluted by two times with blank plasma and the assay, precision and accuracy were determined as described in text earlier. For Alfuzosin, the concentration found was  $40.52 \pm 0.32$  ng/ml and bias was 1.3%. The result indicated that samples whose concentrations were greater than upper limit of the standard curve could be re-analysed by appropriate dilution.



**Table 3: Extraction recovery of alfuzosin and atenolol from plasma**

Analyte	Concentration (ng/mL)	Concentration found (mean ± SD) ng/mL	% recovery (mean ± SD)	Mean recovery
Alfuzosin (n=5)	0.3	0.292±0.004	97.2±1.3	98.0±0.9
	12.5	12.083±0.331	96.7±2.7	
	20.0	20.027±0.205	100.1±1.0	
Atenolol (n=5)	750.0	320.75±8.78	-	42.8±1.2

**Stability**

The stability tests of the Qc samples were designed to cover anticipated conditions that clinical samples may experience. Stability data were summarized in Table-5. Briefly, three freeze-thaw cycles and ambient temperature storage of the freeze quality control samples up to 12 h prior to sample preparation appeared to have no effect on the quantitation of analyte. Twelve hour room temperature

storage and freeze-thaw cycles for low, mid and high quality controls samples indicated that alfuzosin was stable in human plasma under experimental condition. QC samples were stable for at least 30 days, which are frozen at approximately  $-70 \pm 5$  °C. Results from auto injector stability indicate that the alfuzosin is stable when kept in the auto sampler for up to 24 h at  $5 \pm 1$  °C.

**Table 4: Accuracy and precision of the HPLC method for determining alfuzosin concentrations in plasma samples**

Within-batch precision (n = 10)			
Concentration added (ng/mL)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Bias (%)
0.3 (Low)	0.299±0.008	2.74	-0.3
12.5 (Med)	11.838±0.389	3.28	-5.3
20.0 (High)	18.574±0.593	3.19	-7.1
Between-batch precision (n = 30)			
Concentration added (ng/mL)	Concentration found (mean ± S.D.)(ng/mL)	Precision (%)	Bias (%)
0.3 (Low)	0.305±0.008	2.77	1.52
12.5 (Med)	12.808±0.337	2.65	2.46
20.0 (High)	19.679±0.560	2.85	-1.60

**Table 5: Stability of the samples**

Sample concentration (ng/mL)	Concentration found (ng/mL)	Precision (%)	Bias (%)
Short term stability for 12 h (n=3) in plasma			
0.3 (Low)	0.281±0.021	7.55	-6.2
12.5 (Med)	12.860±0.418	3.25	2.9
20.0 (High)	20.207±1.219	6.03	1.1
Three freeze and thaw cycles (n=3)			
0.3 (Low)	0.309±0.009	2.98	-1.7

Sample concentration (ng/mL)	Concentration found (ng/mL)	Precision (%)	Bias (%)
12.5 (Med)	12.293±0.916	7.45	1.36
20.0 (High)	19.431±1.422	7.32	-2.8
<b>Auto-sampler stability for 24 h (n=3), at 5 ± 1 °C</b>			
0.3 (Low)	0.292±0.010	3.36	-2.6
12.5 (Med)	12.750±0.178	1.39	2.0
20.0 (High)	19.684±0.521	2.64	-1.6
<b>30-days stability at -70 ± 5 °C (n=3)</b>			
0.3 (Low)	0.285±0.019	6.67	-5.1
12.5 (Med)	12.844±0.249	1.93	2.8
20.0 (High)	19.862±0.504	2.53	-0.6

### CONCLUSION

A simple, highly sensitive, fast, accurate and economical HPLC method for the quantitation of alfuzosin in human plasma has been developed and validated as per guide line [13]. The previously reported methods required large volume of sample<sup>(3)</sup> and large volume of organic mobile phase. The present liquid extraction based isocratic HPLC method with its LLOQ (0.1ng/ml) value makes it more sensitive and precise than the other previously reported methods<sup>(3,6,8,9,10)</sup>. With the

use of atenolol as internal standard and BDS Hypersil C-18 column, the total chromatographic run time was reduced to 4 min., which enables the analysis of more than 350 samples in 24 hr. Good validation data were achieved for the assay precision, linearity, accuracy and recovery. The validated HPLC method described for the estimation of alfuzosin can be applied to bioequivalence studies. In conclusion, this paper describes a very simple, rapid and sensitive HPLC method for the quantitation of alfuzosin in plasma with dilution integrity.

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%4.8 2.74 - 3.28%

%1.6 -1.60 - 2.46%

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