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Development, validation and Comparative statistical Evaluation of HPLC and HPTLC method for determination of Alfuzosin in Human plasma

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ABSTRACT

Rapid, precise , accurate, simple, selective, and sensitive HPLC and HPTLC methods for the determination of Alfuzosin in human plasma have been developed. The method utilizes simple protein precipitation as the sample preparation method in both the techniques. HPLC method was developed using HiQ sil C8 HS column, with mobile phase containing mixture of Acetonitrile: Sodium acetate buffer(0.04M) containing n-hexane sulphonic acid salt (0.005mM) (pH 4.0, adjusted with glacial aceteic acid) (55:45 v/v),at the flow rate of 1ml/min and detection was performed at 244nm. Retention times for Alfuzosin (Alfu) and the internal standard (IS) were 6.7 and 4.32 min, respectively. The calibration curve was linear (r^2>0.99) through the range of 25-45ng/ml. The mean recovery was found to be 96.94% for Alfuzosin. The HPTLC separation was carried out on the Aluminium plates precoated with silica gel 60 F_{254} using Toluene: Methanol: Triethylamine (7:3:0.2%v/v/v)as mobile phase and scanned at 244nm with camag TLC scanner. Quantification was achieved with HPTLC, over the concentration range of 1000 to 1800 ng/band with mean recovery of 97.07% for Alfuzosin. Retention factor (Rf) for Alfuzosin and the internal standard were 0.59 and 0.28 respectively.

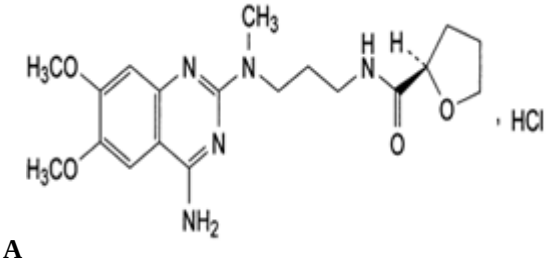
Keywords: HPLC, HPTLC, human plasma, *Alfuzosin*, protein precipitation.

INTRODUCTION

Alfuzosin is (2*RS*)-*N*-[3-[(4-Amino-6,7-dimethoxyquinazolinyl) methylamino] propyl] tetrahydrofuran-2-carboxamide hydrochloride. Alfuzosin is an alpha$_1$-adrenoceptor blocker. It acts preferentially on receptors in the lower urinary tract and is therefore used in benign prostatic hyperplasia to relieve symptoms of urinary obstruction, including acute urinary retention. Alfuzosin is readily absorbed after oral doses and peak plasma concentration is achieved within 0.5 to 3 hours after a dose; bioavailability is about 64%. It is extensively metabolised in the liver, mainly by the cytochrome P450 isoenzyme CYP3A4, to inactive metabolites that are excreted primarily in faeces via the bile. about 11% of a dose is excreted unchanged in the urine[1-2]. Cmax for *Alfuzosin* is 40-45 ng/ml[3].

Several methods are reported to determine the *Alfuzosin* in Pharmaceutical dosage form by using RC-HPLC with ultraviolet detection [4- 7]. Few HPLC and HPTLC methods have been reported for determination of *Alfuzosin* in bulk and pharmaceutical dosage form [8]. No references have been found for determination of *Alfuzosin* in human plasma by using HPLC & HPTLC method.

In the present work, attempt is made to develop a simple, selective, and sensitive HPLC method with UV detection and HPTLC method for determinationof Alfuzosin in human plasma and they are compared . The method utilizes simple, rapid protein precipitation with acetonitrile as the sample preparation technique. The structures for *Alfuzosin* and Internal standards are described in Figure 1[9]The method has been validated as per the Guidelines by *US CDER* [10]. Statistical analysis was carried out for the comaparison of data obtained by 2 techniques using InStat software.



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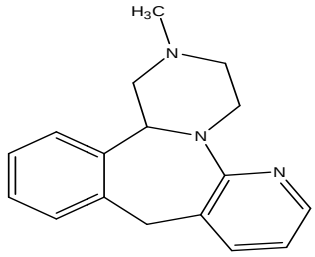

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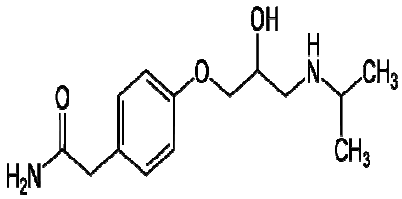

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Fig 1: Structures of *Alfuzosin*(A), Mirtazepine(B), Atenelol (C) respectively

EXPERIMENTAL SECTION

Instruments

For HPLC method, chromatographic separation was performed on a Jasco chromatographic system equipped with a Jasco HPLC pump Model PU2080 plus, Jasco UV-2075 plus detector, Rheodyne injector with 50µl loop volume and Elga water system for HPLC grade water.

For HPTLC method chromatographic separation of drugs was performed on aluminium plates precoated with silica gel 60 F_{254}, purchased from E-Merck, Germany. Samples were applied on the plate as a band with 4 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (10 x 10 cm) at room temperature and a densitometric scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software (Version 1.4.3, Camag). Shimadzu balance(Model AY-120)was used for weighing.

Chemical s and Reagent:

Alfuzosin, Mirtazapine and Atenolol were kindly provided by Cipla Ltd. Mumbai, Matrix Laboratories, Hyderabad and Sun Pharma Baroda, India respectively as gift samples. Toluene, triethylamine, methanol, acetonitrile, glacial acetic acid,(all AR grade), sodium acetate, n-hexane sulphonic acid, were purchased from Sisco Research Laboratories Ltd, Mumbai.

Following chromatographic conditions were selected

A) HPLC Method

Mobile Phase- Acetonitrile: 0.04M Sodium acetate buffer containing n- hexane sulphonic acid salt(0.005mM) (pH 4.0, adjusted with glacial acetic acid) (55:45 v/v).
Flow rate -1.0 mL min^{-1}
Injection volume- 50 µl
Detection wavelength -244 nm
Stationary phase- HiQ sil C8 HS column
Internal Standard- Mirtazepine Hydrochloride

B) For HPTLC Method

Mobile phase -Toluene : methanol : triethylamine (7:3:0.2 v/v/v)
Chamber saturation time-15 min
Detection Wavelength- 244 nm
Internal standard- Atenolol
Stationary phase- Aluminium plates precoated with silica gel 60 F_{254}
Migration distance- 80 mm

Sample preparation

A) HPLC Method:

Working standard solution

10 mg of Alfuzosin was dissolved in 10 mL of methanol to obtain 1000 µg mL^{-1} of standard solution. From this solution 0.01 mL was taken and made the volume to 10 mL so as to obtain the concentrations of 1µg $mL^{-1,}$. From this remove 1, 1.2, 1.4, 1.6, 1.8ml and made the volume to 10 ml so as to obtain the concentrations of 100, 120, 140, 160, 180 ng/ml which were used for preparing the plasma samples. For internal standard, 10 mg of Mirtazepine was dissolved in 10 ml of methanol to obtain 1000 µg/ml. It was diluted appropriately to obtain conc of 1 µg/ml

Sample preparation
Plasma sample was prepared by taking 0.5ml plasma, 0.5ml solution of Alfuzosin (100, 120, 140, 160,180 µg/ml)and 0.5ml of Acetonitrile as precipitating agent were added and, the contents of the tubes were vortexed for 2 min. 0.5 ml stock solution of IS(1 µg/ml) was added, vortexed for 5 min. It was centrifuged for 10 minutes at 2500 rpm. The final plasma solutions were obtained in concentrations of 25, 30, 35, 40, 45 ng/mL. After centrifugation, 50 µl aliquots of each concentration were injected into the HPLC system

B) HPTLC Method:
Working standard solution
10 mg of Alfuzosin was dissolved in 10 mL of methanol to obtain 1000 µg mL^{-1} of standard solution. From this solution 0.01 mL was taken and made the volume to 10 mL so as to obtain the concentrations of 1µg mL^{-1}. from this remove 1,1.2, 1.4, 1.6, 1.8 ml and made the volume to 10 ml so as to obtain the concentrations of 100, 120, 140, 160, 180 ng/ml which were used for preparing the plasma samples. For internal standard, 10 mg of Atenolol was dissolved in 10 ml of methanol to obtain 1000 µg/ml. From this 0.01 ml was diluted to 10 ml with methanol to obtain conc. of 1 µg/ml.

Sample preparation
Plasma sample was prepared by taking 0.5ml plasma, 0.5ml solution of Alfuzosin (100, 120, 140, 160, 180 µg/ml)and 0.5ml of Acetonitrile as precipitating agent were added and The contents of the tubes were vortexed for 2 min. 0.5 ml stock solution of IS(1 µg/ml) was added, vortexed for 5 min. It was centrifuged for 10 minutes at 2500 rpm. The final plasma solutions were obtained in concentrations of 25, 30, 35, 40, 45 ng/mL. From these solutions 40µl aliquots of each concentration were applied on TLC plate. So as to obtain the final concentrations of 1000, 1200, 1400, 1600, 1800 ng/band.

The calibration curve for alfuzosin was obtained using five calibration standard levels (25, 30, 35, 40, 45 µg/ml) for HPLC and for HPTLC range was 1000, 1200, 1400, 1600, 1800ng/band. Linear regression analysis was done, considering the ratio of the peak area of analyte to internal standard versus concentration applied.

VALIDATION
The method was validated as per *US CDER* guidelines.

Selectivity
Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from different sources spiked at LLOQ (Lower Limit of Quantification).

Calibration/standard curve
Linearity was tested for the range of concentrations 25-45 ng/ml for HPLC and 1000-1800ng/band for HPTLC. Each sample in five replicates was analyzed and peak areas were recorded. The response factor for each concentration was calculated by taking ratio of peak area of *Alfuzosin* and IS. The response factors were then plotted against the corresponding concentrations to obtain the calibration graphs.

Accuracy, precision and lower limit of quantification
The precision of the method were evaluated using the Q.C. samples. Intra-day precision was measured by consecutively analyzing Q.C. samples in one single day. The procedure was repeated for three different days to test the inter-day precision. Accuracy was evaluated using five replicate of LLOQ, LQC, MQC, HQC samples. Accuracy was calculated as percentage accuracy whereas precision was measured in terms of relative standard precision (R.S.D.) of each calculated concentration. The Lower limit of quantification was decided on the basis of lowest concentration on calibration curve.

Recovery
Recovery for *Alfuzosin* was evaluated at three concentration levels corresponding to three Q.C. samples (LQC, MQC and HQC) 30, 35, 40 ng/ml for HPLC and 1200, 1400, 1600ng/band for HPTLC analyzed in triplicate. The %

mean recoveries of Alfuzosin were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at LQC, MQC and HQC levels.

Stability

As per *US CDER* guidelines, stability was checked under different conditions viz.

1. Freeze- thaw stability
2. Short term stability
3. long term stability
4. Stock solution stability
5. Post preparative stability

Freeze-thaw stability of *Alfuzosin* was determined by assaying low and high Q.C. samples that is 30, 40 ng/ml for HPLC and 1200, 1600ng/band for HPTLC in triplicate over three freeze-thaw cycles. First freeze-thaw cycle consisted of 24 hrs freezing at -5^0 C followed by a complete thaw at a room temperature. The next two freeze-thaw cycles were of 12 hrs each frozen state at -5^0 C followed by a complete thaw at a room temperature.

Short term stability was determined by analysing of two Q.C.(LQC & HQC) samples stored for 4 hrs at room temperature and long term stability involved storage of two (LQC & HQC) Q.C samples for 14 days at 4^0 C. For stock solution stability, the stock solutions of the drug and IS were stored for period of 5 days in refrigerator at 4^0 C and then for 6 hrs at room temperature. Post preparative stability, where stability of the spiked samples for MQC of *Alfuzosin* and IS were determined after the storage for 5 hrs at room temperature. All these Q.C. samples were then evaluated in triplicate and the results were compared with the freshly prepared samples of same concentrations.

Statistical Analysis:

Unpaired t-test has been applied for the comparison of data obtained by two techniques HPLC and HPTLC. Unpaired test was done using InStat software.

RESULT AND DISCUSSION

Chromatographic characteristics

A) HPLC Method:

A mobile phase consisted of Acetonitrile: 0.04M Sodium acetate buffer (PH-4,55:45v/v) offered a good separation at flow rate 1ml/min and at run time of 6.7min for alfu and 4.32min for IS as shown in Fig 2,3,4 respectively.

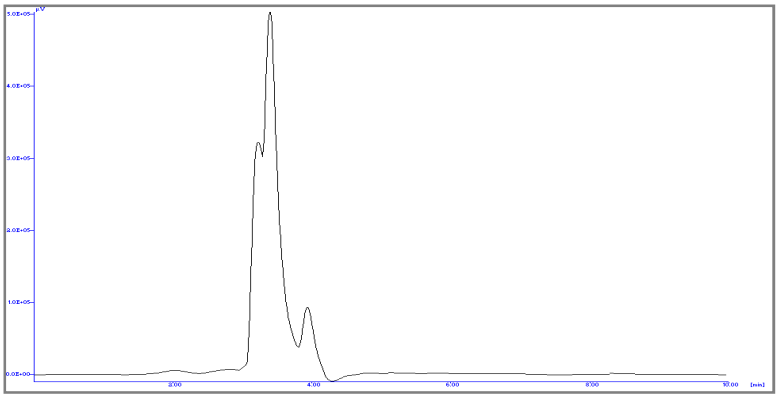

Fig 2: Typical chromatogram of blank human plasma

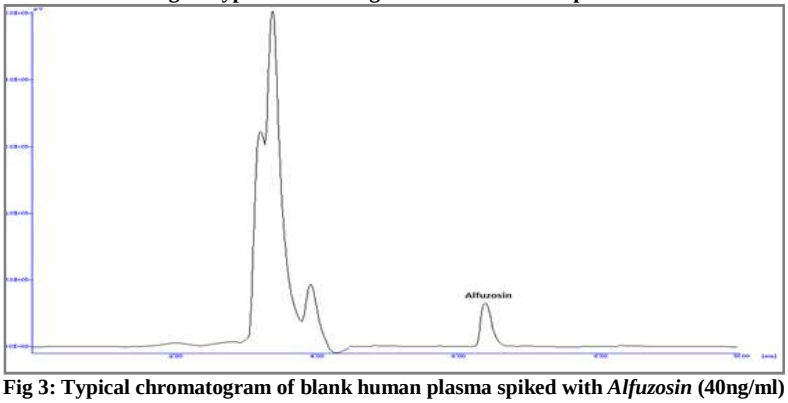

Fig 3: Typical chromatogram of blank human plasma spiked with *Alfuzosin* (40ng/ml)

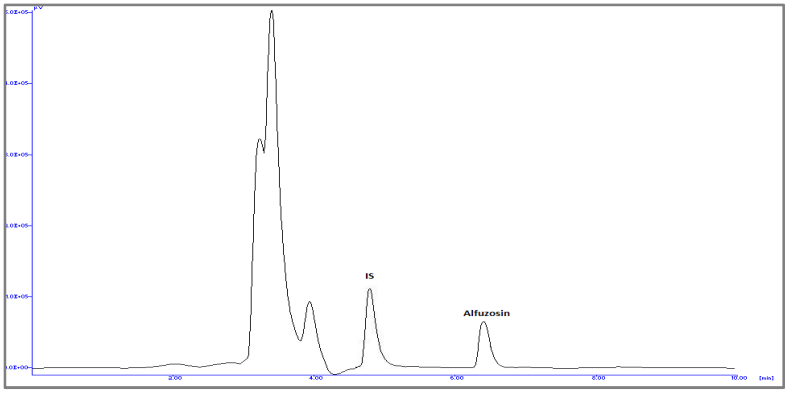

Fig 4: Typical chromatogram of blank human plasma spiked with *Alfuzosin* (40ng/ml) and IS (1µg/ml)

B) HPTLC Method:

A mobile phase consisted of Toluene: methanol: triethylamine (7:3:0.2 v/v/v) offered a good separation. Retention factor (Rf) *for* Alfu and the internal standard were 0.27±0.02 and 0.59±0.03 respectively as shown in Figure 5 and 6 respectively.

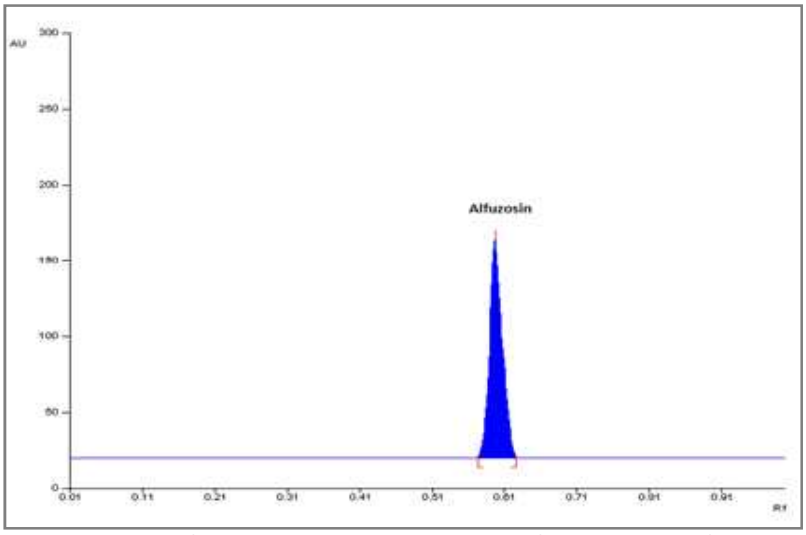

Fig.5: Densitogram of blank human plasma spiked with Alfu1000 ng/band (Rf-0.59±0.03)

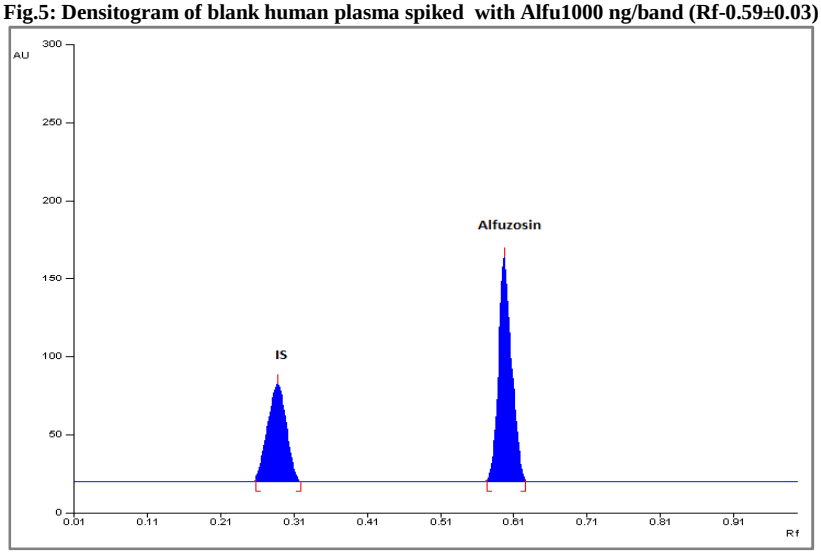

Fig.6: Densitogram of blank human plasma spiked with Alfu 1000 ng/band (Rf- 0.59) and IS, 1000 ng/band (Rf-0.28)

Selectivity

The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from different sources spiked at LLOQ of 25ng/ml for HPLC method and 1000ng/band for HPTLC method in which no interference by

endogenous components was noted. % RSD (Relative standard deviation) for 6 replicates spiked at LLOQ was found to be 1.37% for HPLC method and 1.47% for HPTLC method.

Calibration/standard curve

A) Calibration curve (linearity) of the HPLC method

Calibration curve was constructed by plotting response factor Vs concentration of *Alfuzosin* solutions, and the regression equation was calculated. The calibration curve was plotted over the concentration range of 25-45ng/ml. Correlation coefficient obtained was 0.993, a mean slope of 0.062, mean y-intercept of 0.261 was obtained.

B) Calibration curve (linearity) of the HPTLC method

Calibration curve was plotted over a concentration range of 1000 to 1800 ng/band for *Alfuzosin*. A correlation coefficient of 0.999, a mean slope of 0.001 and mean y-intercept of 0.285 was obtained.

Accuracy, precision and lower limit of quantification

A) HPLC Method: The method showed good accuracy and precision in plasma samples for LLQC,LQC, MQC and HQC Table 1 shows the results for intra- and inter-day precision and Table 2 shows the results for accuracy of *Alfuzosin* in plasma samples. %CV for intraday precision 3.11 ± 0.55 and for inter-day precision 2.49 ± 0.75 respectively. The % mean accuracy of for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels were in the range of 97.57 to 99.9, LLOQ was found to be 25ng/ml

Table 1: Intra-day, inter-day precision of in human plasma QC samples

Theoretical Conc. (ng/ml)	Observed Conc.(mean ng/ml ± SD)	Precision (%CV)
Intra-day		
25	23.26 ± 0.91	3.93
30	27.78 ±0.81	2.94
35	33.41± 0.96	2.87
40	38.28 ± 1.03	2.71
Average		**3.11 ± 0.55**
Inter-day		
25	23.22 ± 1.09	2.85
30	28.46 ±0.95	3.33
35	33.66 ± 0.54	1.60
40	38.34 ± 0.83	2.17
Average		2.49 ± 0.75

Table 2: Accuracy of in human plasma QC samples

Theoretical Conc. (ng/ml)	Observed Conc.(mean ng/ml ± SD)	Accuracy (%)
25	24.94 ± 0.13	99.8
30	29.95±0.72	99.83
35	34.15 ±0.65	97.57
40	39.96 ± 0.27	99.9
		99.27 ± 1.13

Table 3: Intra-day, inter-day precision of in human plasma QC samples

Theoretical (ng/band)	Observed (mean ng/band± SD)	Precision (%CV)
inter-day		
1000	987.02 ± 6.19	0.62
1200	1187.68 ± 8.94	0.75
1400	1387 ± 5.31	0.38
1600	1587 ± 5.85	0.36
Average		0.52 ± 0.18
Intra-day		
1000	988.06 ±4.81	0.61
1200	1187.8 ± 6.8	0.57
1400	1385±4.52	0.32
1600	1590± 3.08	0.19
Average		0.42 ± 0.20

HPTLC Method:

The method showed good accuracy and precision in plasma samples for LLOQ, LQC, MQC and HQC Table 3 shows the results for intra- and inter-day precision and Table 4 shows the results for accuracy for *Alfuzosin* in plasma samples. %CV for intraday precision 0.42 ± 0.20 and for inter-day precision 0.52 ± 0.18 respectively. The %

mean accuracy of for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels were in the range 98.32 to 99.1, LLOQ was found to be 1000ng/band

Table 4: Accuracy of in human plasma QC samples

Theoretical Conc. (ng/ml)	Observed Conc.(mean ng/ml ± SD)	Accuracy (%)
1000	991.31 ± 13.90	99.1
1200	1179.84 ± 20.10	98.32
1400	1375.91± 18.32	98.21
1600	1592.2 ± 7.03	99.5
	Average	98.78± 0.62

Recovery

A) HPLC Method:

Table 5 shows the results of the recovery tests for LQC, MQC and HQC levels. The extraction recovery in plasma samples ranged from 96.50 to 97.66 % for *Alfuzosin* at three concentration levels. The mean recovery for *Alfuzosin* was found to be 96.94 %.

Table 5: Recovery of in human plasma Q.C. samples

QC Levels(ng/band)	% R.S.D.	Recovery (%)
30	1.164	96.67
35	1.952	97.66
40	2.183	96.50
Average	**1.766**	**96.94%**

B) HPTLC Method:

Table 6 shows the results of the recovery tests for LQC, MQC and HQC levels. The extraction recovery in plasma samples ranged from 95.43 to 97.89 % for *Alfuzosin* at three concentration levels. The mean recovery for *Alfuzosin* was found to be 97.07 %.

Table 6: Recovery of in human plasma Q.C. samples

QC Levels(ng/bamd)	% R.S.D.	Recovery (%)
1200	0.96	95.43
1400	1.21	97.89
1600	1.32	97.95
Average	**1.16**	**97.07**

Stability

It was performed to evaluate the influence of storage conditions from the sample collection to analysis. Table 7 and 8 represents the results of stability studies. Results indicated that *Alfuzosin* is stable in human plasma for the given stability conditions.

A) HPLC Method

Table 7: Stability of in human plasma Q.C. samples

Stability	Conc. (ng/ml)	Mean Stability (%)	% CV
Freeze thaw stability	30	100.29	3.96
(three cycles)	40	98.72	1.99
Short term stability	30	100.93	1.92
(for 4h at RT)	40	100.9	2.06
Long term stability	30	102.46	1.70
(for 14 days at 4^0 C)	40	101.83	1.75
Stock solution stability (for 5 days at 4^0 C, 6hrs at RT)	30	100.32	1.17
	35	102.11	1.78
	40	99.9	0.92
Post preparative stability (for 5hrs RT)	35	103.54	1.71
	1(IS)	104.87	2.13

B) HPTLC Method:

Table 8: Stability of in human plasma Q.C. samples

Stability	Conc. (ng/band)	Mean Stability (%)	% CV
Freeze thaw stability (three cycles)	1200	100.14	0.40
	1600	100.07	0.13
Short term stability (for 4h at RT)	1200	99.84	0.53
	1600	99.79	0.46
Long term stability (for 14 days at 4^0 C)	1200	99.66	0.53
	1600	100.02	0.58
Stock solution stability (for 5 days at 4^0 C, 6hrs at RT)	1200	97.71	1.59
	1400	99.69	0.34
	1600	98.86	1.10
Post preparative stability (for 5hrs RT)	1400	99.76	0.28
	1000(IS)	100.85	1.21

Statistical Analysis:
Unpaired t – test applied for comparision of recovery data obtained by HPLC and HPTLC methods.

The two-tailed P value is 0.8687 at 95% confidence level, which is large than 0.05, thus, there is no significant difference in the recovery data obtained by two techniques.

DISCUSSION

To the best of our knowledge, no HPLC & HPTLC method has been reported for determination of Alfuzosin in human plasma. In this study, rapid and sensitive and economic HPLC and HPTLC methods have been developed for the determination of Alfuzosin in human plasma by simple protein precipitation technique and they are compared. Validation results proved that the developed methods perform well with selectivity, precision, accuracy, stability and linearity for the concentration range expected to be found in human plasma. The validated method covers the wide range of linearity over 25-45 ng/ml for HPLC and 1000- 1800ng/band for HPTLC method and is therefore suitable for the determination of in human plasma at different therapeutic dose levels. The mean recovery was found to be 96.94%% and 97.07% for HPLC and HPTLC methods respectively. Unpaired t-test has been applied for the comparison of recovery data of *Alfuzosin* standard and spiked plasma obtained by two techniques and there is no significant difference in data obtained by two techniques. The proposeds methods can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis. The developed methods are able to measure concentration in plasma, for dose regulation and bioavailability studies.

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