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Original Article

DETERMINATION OF ALPRAZOLAM AND FLUOXETINE HCl FROM SPIKED RAT PLASMA USING HPTLC WITH UV DETECTION

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ABSTRACT

Objective: The main aim of this study was to develop a rapid, simple and selective validated high-performance thin-layer chromatographic (HPTLC) method with UV detection for the estimation of alprazolam and fluoxetine HCl from spiked rat plasma.

Methods: In this method, a mixture of acetonitrile and chloroform (2:4 v/v) was employed as the solvent for extraction of alprazolam and fluoxetine HCl from spiked rat plasma samples with good sample recovery. The separation was achieved on a 20 cm x10 cm TLC plate precoated with silica gel 60F254, the 250µm thickness on aluminium sheets employing a mobile phase consisting of ethyl acetate: toluene: methanol: ammonia (4:3:1:0.1v/v/v/v). The densitometric evaluation was performed at 230 nm. The developed method was validated as per the recommendations of USFDA Guidance for Industry: Bioanalytical Method Validation.

Results: The Rf value were observed at 0.48±0.04 and 0.17±0.02 for alprazolam and fluoxetine HCl respectively. The calibration curves were linear in the range of 40-100 ng/µl for both drugs with regression coefficients (r^2) of 0.9910 and 0.9932 for alprazolam and fluoxetine HCl respectively. In the intraday and interday precision study, the % CV was less than 15. Results of recovery studies prove the extraction efficiency of the proposed method. Stability data indicated that both alprazolam and fluoxetine HCl were stable in plasma after three freeze-thaw cycles and upon storage at -20 °C for 1 w.

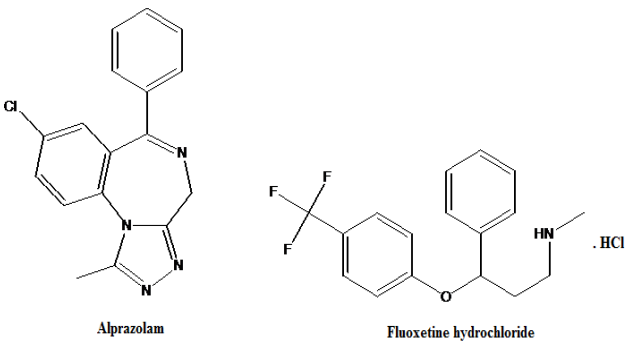
Conclusion: In the proposed method, the rapid, single step extraction of drugs from plasma samples coupled with the simple HPTLC-UV chromatographic conditions makes the method cost effective and suitable for analysis of a large number of binary samples of alprazolam and fluoxetine HCl in plasma.

Keywords: Alprazolam, Fluoxetine HCl, Bioanalytical method, Liquid-liquid extraction, HPTLC, Spiked rat plasma

INTRODUCTION

Alprazolam (fig. 1), chemically known as 8-Chloro-1-methyl-6-phenyl-4*H* [1, 2, 4] triazole [4, 3a] [1, 4]-benzodiazepine, is a synthetic, short-acting anxiolytic of the benzodiazepine class and used to treat anxiety disorders, panic attacks and anxiety caused by depression. It acts by binding to specific sites on the $GABA_A$ receptor, hence possesses anxiolytic, sedative, hypnotic, skeletal muscle relaxant, anticonvulsant and amnestic properties.

Fluoxetine HCl (fig. 1) is (±)-N methyl-3-phenyl-3-[(α, α, α-trifluoro-p tolyl) oxy] propylamine hydrochloride, a selective serotonin reuptake inhibitor (SSRI) antidepressant. It is used to treat major depression (including paediatric depression), obsessive-compulsive disorder (in both adult and paediatric populations), bulimia nervosa (an eating disorder), anorexia nervosa, panic disorder, premenstrual dysphoric disorder (PMDD) and Parkinson's disease [1].



Alprazolam Fluoxetine hydrochloride

Fig. 1: Chemical structures of Alprazolam and Fluoxetine HCl

Combined administration of alprazolam and fluoxetine HCl resulted in an approximate 30% increase in alprazolam plasma concentration relative to plasma concentration following the administration of alprazolam alone. Also psychomotor decrements increased when fluoxetine was administered with alprazolam relative to administration of alprazolam alone.

Several methods are reported for the estimation of alprazolam employing HPLC-UV, LC-MS/MS, and GC-MS individually and in combination with other drugs [2-4]. Literature survey also reveals different methods like LC-MS/MS, RP-HPLC with fluorescence detection and pre-column derivitisation for UV-sensitivity enhancement for estimation of fluoxetine HCl individually and in combination with other drugs [5-8]. Few HPTLC methods are also reported for estimation of alprazolam and fluoxetine HCl individually and in combination with other drugs [9-12]. Also, a single HPLC and HPTLC method is reported for the simultaneous estimation of alprazolam and fluoxetine HCl in tablet formulation [13]. However, no HPTLC method has been reported so far for the simultaneous estimation of alprazolam and fluoxetine HCl in plasma.

Therefore, it was thought desirable to develop a simple, accurate, cheap and fast procedure that could be applied for the simultaneous determination of alprazolam and fluoxetine in plasma with protein precipitation and liquid-liquid extraction as the only sample preparation step involved prior to HPTLC analysis.

MATERIALS AND METHODS

Chemicals and reagents

Active pharmaceutical ingredient (API), working standards of alprazolam and fluoxetine HCl, were obtained from Aarti Drugs Pvt Ltd., India. Ethyl acetate, toluene, methanol, ammonia AR grade ammonia grade were obtained from Fisher Scientific, India and

Himedia Lab Pvt. Ltd, India. TLC plates precoated with silica gel $60F_{254}$ with 250µm thickness on aluminium sheets were purchased from Merck India Pvt. Ltd., India. Rat plasma used for the research work was obtained from albino wistar rats purchased from National Toxicology Centre, Pune, India.

Instrument

Camag TLC plates with precoated silica gel plate $60F_{254}$ (20 x 10 cm), 250 µm thickness (E. Merck, Darmstadt, Germany) was used as stationary phase. The sample application was done by Camag 100 µl syringe using Camag Linomat 5 applicator. The sample was sprayed in the form of narrow bands of 6 mm length at a constant rate 0.2µl/s. Linear ascending development was carried out in 20 x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The densitometric scanning was performed using Camag TLC scanner 3 supported by win CATS software (Version 1.4.3.6336 Camag). Evaluation of chromatograms was done by using peak areas.

Methods

Preparation of Stock solution, Calibration standards (CS) and quality control (QC) samples

A stock solution of concentration 1000 µg/ml each of alprazolam and fluoxetine HCl were prepared in methanol. In 10 ml centrifuge tubes, 1.0 ml of stock solution of alprazolam and fluoxetine HCl (1000ug/ml each) were added with 1.0 ml drug-free plasma. The samples were incubated at RT (28 °C) for 2 h. Protein precipitation and extraction was carried out using acetonitrile (2 ml) and chloroform (4 ml) with vigorous vortex shaking for 2 min and centrifuged at 5000 rpm for 10 min. The organic phase was recovered and evaporated to dryness at RT. The residual mass was reconstituted with 10 ml methanol. Calibration standards (40-100 ng/µl each) were prepared. Lastly, the quality control samples at three different concentration levels; Lower Limit of Quantitation LLOQ: 40 ng/µl; Low QC sample: 50 ng/µl; Middle QC sample: 70 ng/µl; High QC sample: 100 ng/µl of both alprazolam and fluoxetine HCl respectively were prepared from the reconstituted solutions. A fixed volume (1µl) of the prepared standards were applied on TLC plates and developed under the optimized HPTLC conditions.

Plasma sample preparation

Fresh blood samples were withdrawn through the retro-orbital route of rats and collected in Eppendorf tubes previously rinsed with sodium citrate solution as an anti-coagulant. The blood samples were centrifuged at 5000 rpm for 15 min for separation of plasma. The separated plasma was employed for the study. Different solvents were tried for the extraction of alprazolam and fluoxetine HCl from plasma. Initially n-hexane, n-butanol were tried, but the sample recovery was very low. When chloroform (4 ml) was used, recovery was significantly improved. Acetonitrile was also added to increase the precipitation of plasma proteins. Finally, a mixture of acetonitrile and chloroform (2:4 v/v) was employed as the final solvent for extraction of alprazolam and fluoxetine HCl with good sample recovery.

HPTLC chromatographic conditions

Merck TLC plates coated with silica gel 60 F_{254} (0.25 mm thickness) on aluminium sheets were used as stationary phase. The mobile phase consisting of a mixture of ethyl acetate: toluene: methanol: ammonia (4:3:1:0.1, v/v/v/v) was used throughout the analysis. Plates were pre-washed using methanol as solvent and activated at 120 °C for 30 min in hot air oven. Chamber saturation time was optimized at 20 min and plate development time was fixed at 10 min with migration distance of 8 cm. Slit dimension was constant at 5.0 x 0.45 mm with a scan speed of 20 mm/s. From the overlain spectra of alprazolam and fluoxetine HCl, 230 nm was selected as the scanning wavelength for densitometric evaluation [14].

Method validation

The developed method was validated as per the recommendations of USFDA Guidance for Industry: Bioanalytical Method Validation [15-16]. Selectivity was studied at the Lower Limit of Quantification (LLOQ) of 40ng/µl by comparing the blank responses of plasma from six different sources with the peak areas afforded by the LLOQ samples.

Accuracy and precision were studied by analyzing five replicates of the Lower Quality Control (LQC), Middle-Quality Control (MQC) and Higher Quality Control (HQC) samples. The concentration of alprazolam and fluoxetine HCl in the QC samples was determined by referring to the area ratio of the drug to standard drug dilution, obtained from the QC samples, the calibration equation generated on the same day. The accuracy was estimated as the mean % BIAS while the precision was measured in terms of % CV. The recovery of the extraction procedure was calculated by comparing the peak areas of the processed QC samples to those of corresponding standard dilutions.

Stability was evaluated under various conditions viz. three freeze-thaw cycles, stability at room temperature for 6 h and long-term stability at -20 °C for one week. For the determination of freeze-thaw stability, five replicates of LQC and HQC samples were frozen at -20 °C for a minimum of 24 h and then allowed to thaw unassisted at room temperature. For short-term room temperature stability, five replicates of LQC and HQC samples were kept at room temperature for 6 h. Long term stability at -20 °C for one week was determined by using five replicates of LQC and HQC. The precision and accuracy of stability samples was found, and the % CV and % BIAS were calculated [16-17].

RESULTS AND DISCUSSION

Different mobile phase systems like ethyl acetate: toluene: methanol, ethyl acetate: toluene: methanol: glacial acetic acid, toluene: methanol: ammonia were tried in order to determine the best conditions for the effective separation of alprazolam and fluoxetine HCl. The mobile phase consisting of ethyl acetate: toluene: methanol: ammonia (4:3:1:0.1 v/v/v/v) was selected as it gave high resolution with compact spots at Rf value 0.48±0.04 and 0.17±0.02 for alprazolam and fluoxetine HCl respectively. Also from the overlain spectra, it was observed that both alprazolam and fluoxetine HCl exhibited fairly good absorbance at about 230 nm which was selected as the analytical wavelength for densitometric analysis.

Method validation

Linearity

Linearity was established by analysing seven concentrations of alprazolam and fluoxetine HCl, both in the concentration range of 40-100 ng/µl respectively, and then plotting the peak areas against the corresponding concentrations (table 1).

Table 1: Assay parameters and regression characteristic of the binary mixture determined by the proposed method

Parameters	Alprazolam	Fluoxetine HCl
Wavelength (nm)	230	230
Rf	0.48±0.04	0.17±0.02
Concentration range	40-100 ng/µl	40-100 ng/µl
Intercept(a)	215.757	31.242
S_a[a]	1.169	0.785
Slope(b)	10.916	5.329
S_b[b]	1.110	0.888
RSD% of slope	10.172	16.673
r^2	0.9910	0.9932

Regression equation: A= a+bc, where A is the absorbance, a is the intercept, b is the slope and c is the concentration.

Linearity of the calibration curves were validated by the high value of the regression coefficients (r>0.99).

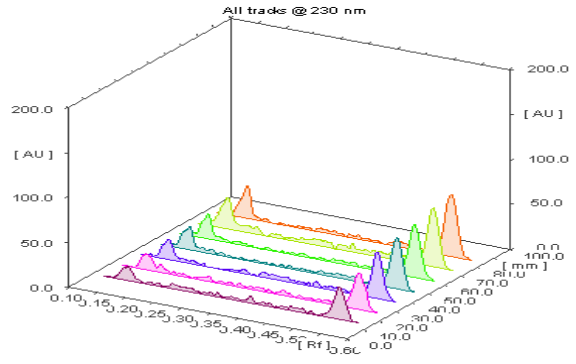


Fig. 2: 3D chromatogram of calibration standards (40-100 ng/µl) for each drug detected at 230 nm

The calibration range for the proposed method was established based on the reported pharmacokinetic data available in literature and the concentrations present in pharmaceutical products. Fig. 2 represents the 3D chromatogram for each concentration detected at 230 nm.

Selectivity

The selectivity of the method was investigated by analyzing blank rat plasma. The blank plasma sample was checked for its interference using the proposed liquid-liquid extraction procedure under the optimized HPTLC conditions and compared with a spiked sample concentration of alprazolam and fluoxetine HCl at LLOQ (40ng/µl) in rat plasma. There was no interference of the components of the biological matrix (plasma) in quantitation of both the drugs.

Sensitivity

During the validation studies, it was found that the peak areas for the LLOQ samples were more than five times the blank responses obtained using six different plasma sources, as shown in table 2.

Table 2: Sensitivity data for ALP and FLU at LLOQ

S. No.	Nominal conc. (ng/µl)	
	Alprazolam	Fluoxetine HCl
1	34.8	36.8
2	41.2	36.3
3	36.5	32.8
4	37.0	39.0
5	35.7	41.6
Avg.	36.92	37.30
SD	2.5645	3.2741
% CV	6.94	8.77
% Bias	6.57	6.45

This proves that the method is selective at the LLOQ of 40ng/µl. The representative densitogram of blank plasma shows no interference at the Rf values of both drugs (fig. 3). The representative densitogram of alprazolam and fluoxetine HCl extracted from plasma at LLOQ is shown in fig. 4.

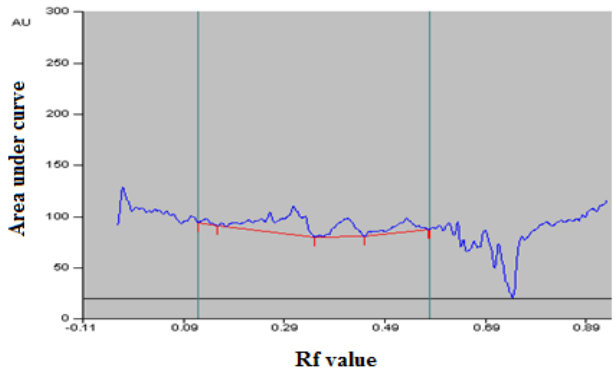


Fig. 3: Densitogram of blank rat plasma

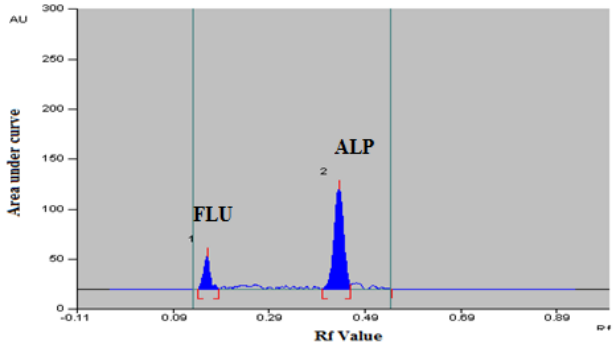


Fig. 4: Typical densitogram of QC sample at LLOQ (40ng/µl)

The lower limit of quantitation was found to be 36ng/and 37ng/µl for alprazolam and fluoxetine HCl respectively. The %CV was found to be 6.94% for alprazolam and 8.77% for fluoxetine HCl, % bias was found to be 6.57% and 6.45% for alprazolam and fluoxetine HCl respectively, and is within the acceptable limits.

Precision and accuracy

The evaluation of accuracy and precision showed that the % CV and % bias was less than ±15 as required by the USFDA Guidance guidelines. Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day)studies and accuracy for set of quality control (QC) samples at 40, 50, 70, 100 ng/µl (LLOQ, LQC, MQC, HQC) in replicate (n = 5) (table 3). The coefficient of variance (%CV) and percent bias (%BIAS) were within the acceptable limits. The % BIAS was calculated using the formula:-

$$\% \text{ BIAS} = \frac{\text{True conc.} - \text{Observed conc. (for both drugs)}}{\text{True conc. (for both drugs)}} \times 100$$

Recovery

The recovery data was determined in triplicate at two concentrations (LQC and HQC) as recommended by FDA guidelines. The recovery at these two concentrations, 50 and 100 ng/µl was found to be 87.10%, 94.42% for alprazolam and 87.42%, 93.29% for fluoxetine HCl (table 3). The % recovery was calculated using following formula:-

$$\% \text{ Recovery} = \frac{\text{Area of fresh extracted sample (both drugs)}}{\text{Area of fresh unextracted sample (both drugs)}} \times 100$$

Stability study

From the stability studies, it was observed that the % CV and % RSD was less than 15, as shown in table 4, which falls within the

acceptable limits. This indicates that the deviation of the results from nominal concentration may be attributed to the variability of the assay and implies that the drugs are stable after the stability cycles.

Table 3: Assessment of the accuracy, precision and % recovery of Alprazolam and Fluoxetine HCl

Level		Alprazolam				Fluoxetine HCl			
		LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC
Conc.		40	50	70	100	40	50	70	100
Intra	Mean conc.* (ng/band)	35.66	44.76	65.56	95.36	37.30	44.56	62.7	94.65
day	% CV	2.16	2.03	2.67	1.99	8.77	3.99	3.32	3.40
	% Bias	11.00	10.48	6.42	4.40	6.45	11.2	10.4	5.35
Inter	Mean conc.* (ng/band)	37.08	45.9	66.14	95.66	36.92	45.64	65.26	96.28
day	% CV	3.35	4.38	1.95	1.62	6.94	4.48	3.35	3.64
	% Bias	7.42	8.20	5.51	4.34	6.57	8.72	6.77	3.72
% Recovery*		-	87.10	-	94.42	-	87.42	-	93.29

*n = 5

Table 4: Results of stability studies for alprazolam and fluoxetine HCl

Stability study		Alprazolam		Fluoxetine HCl	
		*LQC	*HQC	*LQC	*HQC
Short term stability	% CV	1.48	3.97	2.76	2.01
	% Bias	8.86	3.18	10.28	2.82
Freeze thaw stability	% CV	1.27	1.12	2.37	1.65
	% Bias	12.9	2.24	9.48	4.16
Long term stability	% CV	4.30	1.76	1.45	1.01
	% Bias	9.84	1.24	9.20	2.82

*n = 5

The results of the study indicated that the proposed HPTLC-UV method has a high degree of sensitivity, selectivity with an acceptable degree of accuracy and precision. As there are no reported methods for the simultaneous estimation of these two drugs in biological fluids, the developed method can prove to be suitable for such applications.

CONCLUSION

The present work presents a simple, rapid, sensitive and economical method for determination of alprazolam and fluoxetine HCl in rat plasma. A good separation of the analytes in this combination was achieved by using the mobile phase containing ethyl acetate: toluene: methanol: ammonia (4:3:1:0.1 v/v/v/v). The method utilizes protein precipitation with liquid-liquid extraction using a mixture of acetonitrile and chloroform as the only sample preparation step prior to HPTLC analysis. The rapid, single step, sample preparation coupled with the simple HPTLC-UV chromatographic conditions makes the method cost effective and suitable for analysis of a large number of plasma samples containing the drugs. The method was fully validated to meet the requirements for linearity, sensitivity, accuracy and precision as per US FDA guidelines. The method can be suitably employed for the bioavailability and bioequivalence study of alprazolam and fluoxetine HCl.

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CONFLICT OF INTERESTS

Declare none

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