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DEVELOPMENT OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND FLUOXETINE HYDROCHLORIDE IN PHARMACEUTICALTABLET DOSAGE FORM

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ABSTRACT

A new, simple, accurate and precise reversed phase high performance chromatographic method with UV detection was developed for the simultaneous quantitative estimation of Alprazolam (APZ) and Fluoxetine hydrochloride (FLX) in combined tablet dosage form. The method was employed on reversed phase liquid chromatographic analysis using C18 column (4.6 × 250 mm, 5 µm). The Eluent was monitored by absorbance at wavelength 225 nm using a mixture of Acetonitrile and phosphate buffer (50:50 v/v) at pH 4.5 with a flow rate of 1.0 mL min^{-1}. Calibration curves were found to be linear in the concentration range of 0.5-1.50 µg mL^{-1} and 40-160 µg mL^{-1} for APZ and FLX respectively. The proposed method was validated by testing its linearity, recovery, repeatability and it was successfully employed for the rapid and specific simultaneous quantitative estimation of APZ and FLX in combined tablet dosage form.

KEYWORDS: Alprazolam, Fluoxetine Hydrochloride, RP-HPLC, Method development, Validation.

INTRODUCTION

Alprazolam (Fig.1) is 8-Chloro-1-methyl-6-phenyl-4H-1,2,4-triazolo(4,3-a)(1,4)benzodiazepine. Medicinally it is a short-acting drug in the benzodiazepine class used to treat anxiety disorders and as an adjunctive treatment for depression. It is also used as a medication to aid the beginning of treatment with SSRI type drugs, since these can cause anxiety in the initial stages of use.[1] Benzodiazepines produce a variety of effects by modulating the $GABA_A$ subtype of the GABA receptor, the most prolific inhibitory receptor within the brain.[2]

Fluoxetine hydrochloride (Fig.2) is N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy]-propan-1-amine hydrochloride. Pharmaceutically it is antidepressant agent belonging to the selective serotonin reuptake inhibitors (SSRIs), is used to treat depression, bulimia nervosa, premenstrual dysphoric disorder, panic disorder and post-traumatic stress. According to the amines hypothesis, a functional decrease in the activity of amines, such as serotonin and nor epinephrine, would result in depression; a functional increase of the activity of these amines would result in mood elevation.[3]

Literature survey reveals the availability of HPLC and spectroscopic methods for the individual analysis of alprazolam and fluoxetine. However, no single method is reported for their simultaneous estimation in combined dosage form. Present work emphasizes simple, reproducible, precise and RP-HPLC quantitative method for simultaneous estimation of alprazolam and fluoxetine in combination, using UV-spectroscopy and HPLC techniques.

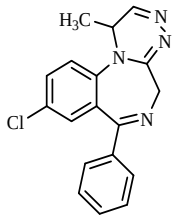

Fig 1: Alprazolam

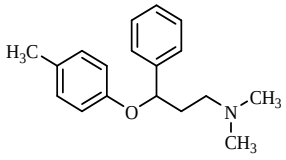

Fig 2: Fluoxetine Hcl

MATERIALS AND METHODS

1. Chemicals and reagents

Pharmaceutical grade of FLX and APZ were kindly supplied by Sun Pharmaceutical Industries, Silvasa, Gujarat, India and Unichem labrotraries, Baddi, Himachal Pradesh, India respectively. HPLC grade water, methanol and acetonitrile were purchased from Merck Chemicals, Mumbai. Analytical grade of hydrochloric acid, *o* -phosphoric acid and potassium dihydrogen phosphate were used in present research work. Commercially available Fluwel (Bestochem Formulation, India) tablet claimed to contain 0.25 mg

alprazolam and 20 mg fluoxetine were procured from local market.

2. Chromatographic system and conditions

Chromatographic work was carried on Shimadzu HPLC system (Kyoto, Japan) having inbuilt degasser unit (DGU 14A), mixer unit (FCV 10AL) attached to the solvent delivery unit with low-pressure gradient pump (LC-10 AT), Rheodyne injector port (2E, 7725i, 20 µl loop), column oven (CTO 10AS) and UV/VIS detector (SPD 10 AVp). The mobile phase contains acetonitrile and potassium dihydrogen phosphate buffer adjusted to pH 4.5 with *o* -phosphoric acid in the ratio 50:50 (v/v) at the flow rate of 1.0 ml/min. The mobile phase was filtered through 0.2 µm membrane filter. Detection was monitored at 225 nm at ambient temperature.

3. Standard stock solutions

Accurately weighed amount of APZ (100 mg) and FLX (100 mg) were transferred to 100 ml of volumetric flasks separately and diluted up to the mark with 0.1 M methanolic hydrochloric acid.

An aliquot portion of the standard stock solution of APZ and FLX were further diluted with mobile phase to get the series of concentration of 0.5-1.5 µg/ml for APZ and 40-160 µg/ml for FLX. 20 µl injections were made for each concentration and chromatograph under the optimized conditions described above. The peak area was plotted against the corresponding concentrations to obtain the calibration graphs.

4. Sample preparation

Twenty tablets contents were accurately weighed, their mean weight was determined and they were mixed and finely powdered. A portion equivalent to about one capsule was accurately weighed three different laboratory mixtures of APZ and FLX were prepared by appropriately weighing the quantities of drug samples so as to get the concentration of 80 µg/ml of FLX and 1µg/ml of APZ. The sample solution was then filtered using 0.45µ filter (Millipore, Milford, MA). A 20 µl volume of sample solution was injected into HPLC. The peak areas for the drugs were measured at 225 nm and amounts of APZ and FLX were determined using the related linear regression equations.

5. Method validation

The developed method was validated according to the ICH guidelines.[4] The system suitability was evaluated by six replicate analyses of APZ and FLX mixture at a concentration of 1:80 µg/ml. The acceptance criteria were a R.S.D. of peak areas and retention times less than 2%, Theoretical plate numbers (N) at least 2500 for each peak and tailing factors (T) less than 1%.

5.1 Linearity

Calibration curves were found to be linear in the concentration range of 0.5-1.50 µg mL^{-1} and 40-160 µg mL^{-1} for APZ and FLX respectively. The peak areas versus concentrations of drugs were plotted and a linear least-square regression analysis was conducted to determine the slope, intercept and coefficient of determination (R^2) to demonstrate the linearity of the method.

5.2 Accuracy and Precision

To study the reliability and suitability of the developed method, recovery experiments were carried out at three levels 80, 100 and 120%. Known concentrations of commercial capsules were spiked with known amounts of standard laboratory sample of APZ and FLX. At each level six determinations were performed and the results obtained were compared with expected results. Recovery for pharmaceutical formulations should be within the range 100±5%. The percent R.S.D. of individual measurements was also determined. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentrations of APZ and FLX were analyzed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision). Every sample was injected in triplicate. The repeatability of sample application and measurement of peak area for active compounds were expressed in terms of percent RSD.

5.3 LOD and LOQ

All chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional excipients peaks. Marketed formulations were analyzed to determine the specificity of the optimized method in the presence of common capsule excipients. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from the signal-to-noise ratio. LOD and LOQ were calculated using 3.3 σ/*s* and 10 σ/*s* formulae, respectively, where, σ is the standard deviation of the peak areas and *s* is the slope of the corresponding calibration curve.

5.4 Robustness

To evaluate robustness of HPLC method a few parameters were deliberately varied. The parameters included variation of flow rate, percentage of buffer in the mobile phase and pH of mobile phase.

RESULTS AND DISCUSSION

During the optimization of HPLC method, two columns (HI-Q Sil C18 5µm; 250 mm × 4.6 mm and Inertsil ODS C18 5µm; 150×4.6 mm), two organic solvents (acetonitrile and methanol), two buffers (acetate and phosphate) at two different pH values (3 and 4.5) were tested. Initially, acetonitrile (100%), Acetonitrile: Water (70:30 v/v), Acetonitrile: Buffer (70:30v/v) (pH = 3), Acetonitrile: Buffer (70:30v/v) (pH = 4.5), In order to decrease the analysis time, column length was reduced from 250 to 150 mm. The mobile phase conditions were optimized so the peak from the first-eluting compound did not interfere with those from the solvent, excipients. Other criteria, *viz.* time required for analysis, appropriate

k range (1 < k < 10) for eluted peaks, assay sensitivity, solvent noise were also considered. Finally a mobile phase consisting of a mixture of acetonitrile: phosphate buffer pH 4.5 adjusted with *o*-phosphoric acid in ratio 50:50 (v/v), was selected as mobile phase to achieve maximum separation and sensitivity. Flow rates between 0.8 to 1.4 ml/min were studied. A flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed phase C18 column, the retention times for APZ and FLX were observed to be 4.04, 5.19 mins respectively. Total time of analysis was less than 10 mins. The chromatogram at 225 nm showed a complete resolution of all peaks [Fig.1].

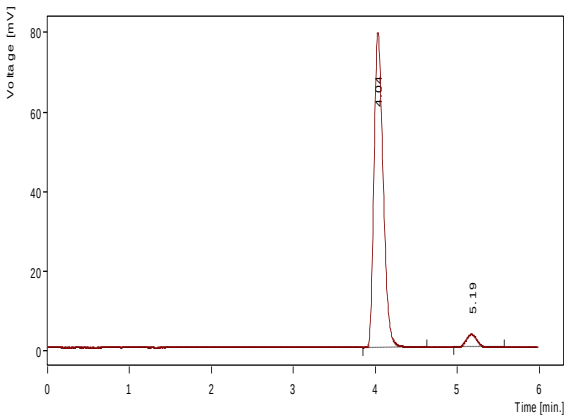

Fig.1: Chromatogram of APZ and FLX

Validity of the analytical procedure as well as the resolution between different peaks was insured by the system suitability test. All critical parameters tested met the acceptance criteria on all days. As shown in the chromatogram, all two analytes were eluted by forming symmetrical single peaks well separated from the solvent front [Fig.1].

Linearity was obtained for all the drugs. The correlation coefficients (r^2) were found to be greater than 0.999 (n=6) in all instances. The results of calibration studies are summarized in [Table 1]. The proposed method afforded high recoveries for APZ and FLX tablets. Results obtained from recovery studies presented in [Table 2]. Precision of the analytical method was found to be reliable based on % RSD (< 2%) corresponding to the peak areas and retention times. As can be seen in [Table 3], the % RSD values were less than 2, for intra-day and inter-day precision. Hence, the method was found to be precise for all the two drugs.

In all deliberately varied conditions, the SD of retention times of APZ and FLX were found to be well within the acceptable limit. The tailing factor for all peaks was found to be < 1.5 [Table 4]. The validated method was used in the analysis of marketed conventional tablet Fluwel and ALBIZ-FX with a label claim: 20 mg FLX and 0.25 mg APZ per tablet. Representative chromatogram is shown in [Fig. 3]. The results for the drugs assay show a good agreement with the label claims [Table 5].

The developed HPLC method is simple, specific, accurate and precise for the simultaneous determination of APZ and FLX from tablet. The developed method provides good resolution between APZ and FLX. It was successfully validated in terms of system suitability, linearity, range, precision, accuracy, specificity, LOD, LOQ and robustness in accordance with ICH guidelines. Thus, the described method is suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs either as such or in combination.

Simultaneous Estimation of Alprazolam and Fluoxetine in Their Combined Dosage Form by

V. HPLC METHOD

Assay procedure[4] for Fluoxetine and Alprazolam has been described in USP and was carried out using:

Fluoxetine HCl

Mobile phase - Triethylamine buffer: Tetrahyrofuran: Methanol (6:3:1 v /v)
Detector - UV-Visible
Column - C18

Alprazolam

Mobile phase-Acetonitrile: Chloroform: Butyl Alcohol: water: Glacial acetic acid (25:25:35:15: 0.5 v/v)
Detector - UV-Visible
Column - C18

Using C18 column different mobile phase were tried and these results are shown in Table 6.

Standard solution of Fluoxetine and Alprazolam

2.5 mg of Alprazolam and Fluoxetine HCl equivalent to 200 mg of Fluoxetine was dissolved in 25 mL of solvent and the final concentration of was made to 1: 80 (APZ:FLX). The λmax was determined on Shimadzu UV-Visible spectrophotometer (model UV-1601) in the range 200-400 nm. The detection wavelength was selected at 225.0 nm

SELECTION OF MOBILE PHASE

Procedure

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. The standard solution containing mixture of FLX and APZ was run with different individual solvents and their combinations until a good separation and stable peak was obtained. Each mobile phase was filtered through 0.22 micron membrane filter. From various mobile phases tried, mobile phase containing Acetonitrile: Phosphate Buffer (50: 50 v/v) (pH- 4.5) was selected, since it give sharp, well resolved peaks with symmetry within limits and significant reproducible retention time for FLX and APZ. The chromatograms are shown in Figure 1.

TABLE 1: CALIBRATION CURVE OF FLX AND APZ

Sr. No.	Concentration (µg/mL) 1:80		Peak Area (Mean % SD) n=3	
	APZ	FLX	APZ	FLX
1	0.50	40	15.36 ± 0.2	317.85 ± 4.58
2	0.75	60	22.00 ± 0.15	456.29 ± 6.15
3	1.00	80	30.64± 0.33	623.30 ±1.73
4	1.25	120	44.05 ± 0.60	879.68 ± 0.72
5	1.50	160	60.29 ± 2.02	1235.6± 40.60

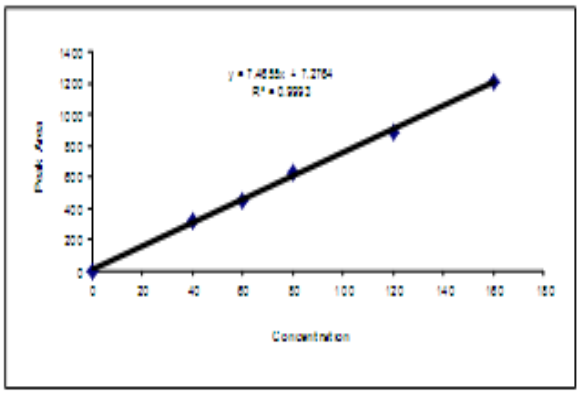

Fig. No. 2: Standard calibration curve for FLX

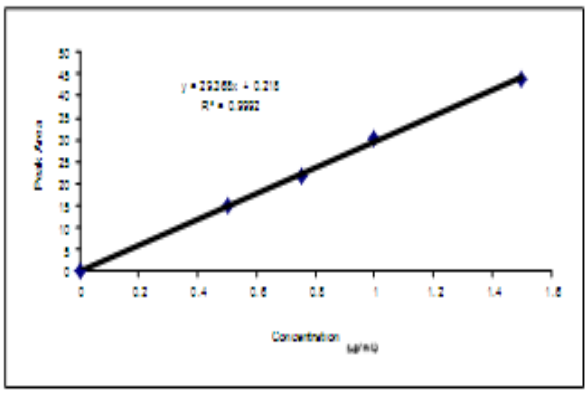

Fig. No. 3: Standard calibration curve for APZ

IV. STUDY OF SYSTEM SUITABILITY PARAMETERS

The system suitability is pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard solutions.

PREPARATION OF STANDARD STOCK SOLUTIONS

Mixed standard solution

Aliquot portions of standard stock solutions of fluoxetine and alprazolam were mixed and diluted appropriately to get final concentration of 1: 80 mg/ mL.

Procedure

The previously filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20 µl standard drug solution was injected separately and system suitability parameters were recorded as shown in Table 2.

TABLE 2: STATISTICAL DATA FOR SYSTEM SUITABILITY PARAMETERS OF FLUOXETINE AND ALPRAZOLAM

Sr.No.	RT (min)		Assymetry		No. of theoretical plates per meter		Capacity factor		Peak Area		Resolution
	APZ	FLX	APZ	FLX	APZ	FLX	APZ	FLX	APZ	FLX	
1	5.18	4.03	1.1	1.42	63283	56594	4.18	3.05	30.39	621.46	4.76
2	5.18	4.05	1.1	1.42	63283	56638	4.18	3.03	30.6	623	4.93
3	5.17	4.03	1.11	1.4	63120	56222	4.17	3.03	30.6	626.7	4.79
4	5.18	4.04	1.1	1.42	63283	56594	4.18	3.05	29.95	607.63	4.76
5	5.18	4.04	1.1	1.4	63446	56408	4.19	3.04	30.02	631.8	4.82
Mean	5.178	4.038	1.102	1.412	63283	56491.2	4.18	3.04	30.31	622.11	4.81
SD	0.004	0.008	0.004	0.010	115.25	174.69	0.007	0.01	0.31	9.025	0.070
CV	0.08	0.20	0.40	0.77	0.18	0.30	0.16	0.32	1.02	1.45	1.46

V. APPLICATION OF PROPOSED METHOD FOR ESTIMATION OF FLUOXTEINE AND ALPRAZOLAM IN LABORATORY MIXTURE

Preparation of standard drug solution

Preparation of Laboratory mixture (standard)

Accurately weighed quantity of APZ 2.5 mg and FLX HCl equivalent to FLX 20.0 mg were separatly transferred to 25 ml volumetric flask, shaken vigorously for five minutes and volume was made up to mark. The standard stock solution of APZ and FLX were mixed and diluted properly to obtain a Laboratory mixture containing a concentration ratio of 1: 80.

Preparation of Laboratory mixture (sample)

Three different laboratory mixtures of APZ and FLX were prepared by appropriately weighing the quantities of drug samples so as to get the concentration of 80 µg/ml of FLX and 1µg/ml.of APZ.

The amount of Fluoxetine and Alprazolam in laboratory mixture was calculated using following formula:

$$\% \text{ Estimation} = \frac{At}{As} \times \frac{Ds}{Dt} \times \frac{Ws}{Wt} \times 100$$

Where,

At = Area count for sample solution
As = Area count for standard solution
Ds = Dilution factor for sample
Dt = Dilution factor for standard
Ws = Weight of standard (mg)
Wt =Weight of sample (mg)

APPLICATION OF PROPOSED METHOD FOR ESTIMATION OF FLUOXETINE AND ALPRAZOLAM IN TABLET FORMULATION

PREPARATION OF STANDARD SOLUTION

Same procedure as laboratory mixture was used.

PREPARATION OF SAMPLE SOLUTION

20 tablets of fluwel were weighed and average weight was determined. They were finely powdered and mixed thoroughly and same procedure as laboratory mixture was used.

Amount of drug in tablet was calculated using following formula-

$$\%\text{Label claim} = \frac{At}{As} \times \frac{Ds}{Dt} \times \frac{Ws}{Wt} \times \frac{A}{LC} \times 100$$

Where,

At = Area count for sample solution
As = Area count for standard solution
Ds = Dilution factor for standard
Dt = Dilution factor for sample
Ws = Weight of standard (mg)
Wt = Weight of sample (mg)
LC = Label claim
A = Average weight

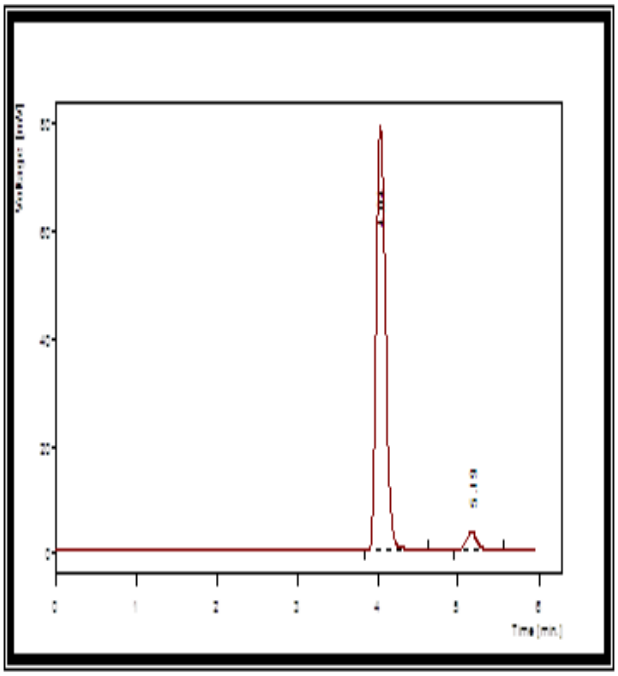

Fig. 4: Chromatogram of Fluoxetine and Alprazolam In Tablet Dosage Form

Recover studies

Preparation of sample solution

An accurately weighed quantity of preanalysed tablet powder equivalent to 20 mg of FLX was taken in 25 ml volumetric flask; to it standard solution of FLX and APZ was added in different proportions[5] Then volume was adjusted up to the mark with the mobile phase. Solution was filtered through whatman filter No. 41. The aliquot portion of the filtrate was further diluted to get final concentration (1:80). The amount of drug contributed by tablet powder was deduced from the total amount of respective drugs estimated and the resultant quantities were assumed to be recovered from the added pure drugs. The content of drug was calculated using same formula as in marketed formulation.

The % recovery was then calculated by using formula

$$\% \text{ Recovery} = \frac{A}{B + C} \times 100$$

Where,

A = Total amount of drug estimated
B = Amount of drug found on preanalyzed basis
C = Amount of pure drug added

The results of recovery studies and statistical data are recorded in Table 3

TABLE 3: STATISTICAL DATA FOR RECOVERY STUDY

Sample	Statistical data	% Label claim					
		APZ			FLX		
Standard laboratory mixture	Mean	98.00			99.18		
	SD	0.80			0.47		
	% RSD	0.81			0.48		
Marketed formulation sample	Mean	96.70			99.46		
	SD	1.93			0.12		
	% RSD	1.97			0.12		
Recovery studies	Level	80 %	100 %	120 %	80 %	100 %	120 %
	Mean	99.60	96.40	98.00	98.33	99.83	98.66
	SD	0.61	0.88	0.39	0.88	0.76	0.38
	% RSD	0.65	0.95	0.40	0.95	0.82	0.41

III. VALIDATION PARAMETERS

i. Accuracy

It was ascertained on the basis of recovery studies performed by standard addition method. The results of recovery studies and statistical data are recorded in table 3.

ii. Precision

Precision of an analytical method is the degree of agreement among individual results when the method is applied repeatedly to multiple readings of homogeneous sample. It is expressed as S.D. or R.S.D. of series of measurements. It was ascertained by replicate estimation of drugs by proposed method.

iii. Ruggedness

The ruggedness studies were carried out under two different conditions:

- Intra and Inter days
- Analyst

Days: a] Intraday studies

It was performed by using same procedure as under marketed formulation analysis and the results were recorded at 3 hrs. interval within a day. The % label claim was calculated using same formula as for marketed formulation analysis. Results and statistical data are shown in table 4.

TABLE 4: STATISTICAL DATA OF INTRADAY STUDY

Sr. No.	Wt. of tablet powder (g)	Peak area of Std.		Peak area of sample		% Label claim	
		APZ	FLX	APZ	FLX	APZ	FLX
1.	0.115	29.00	660.00	27.85	619.58	95.20	93.06
2.	0.114			27.36	613.37	94.34	92.93
3.	0.113			28.28	618.44	94.00	94.53
					Mean	94.51	93.50
					S.D.	0.61	0.88
					C.V.	0.65	0.95

b] Interday studies

Same procedure was performed as under marketed formulation analysis and results of same sample were recorded on different days. The label claim was calculated using same formula as for marketed formulation analysis. Results and statistical data are shown in table 5.

TABLE 5: STATISTICAL DATA OF INTERDAY STUDY

Sr.No.	Wt. of tablet powder (g)	Peak area of Std.		Peak area of sample		% Label claim	
		APZ	FLX	APZ	FLX	APZ	FLX
1.	0.115	29.00	660.00	28.90	624.95	98.79	93.87
2.	0.114			27.52	614.87	98.00	92.35
3.	0.113			28.78	619.86	98.38	93.10
					Mean	98.38	93.11
					S.D.	0.39	0.76
					C.V.	0.40	0.82

c] Different analyst

The sample solutions were prepared by three different analysts and same procedure was followed as described earlier.[6] The % label Claim was calculated as done in marketed formulation estimation. Results and statistical data are shown in Table 6.

TABLE 6: STATISTICAL DATA OF DIFFERENT ANALYST

Sr.No.	Wt. of tablet powder (g)	Peak area of Std.		Peak area of sample		% Label claim	
		APZ	FLX	APZ	FLX	APZ	FLX
1.	0.120	29.00	660.00	27.62	625.57	94.41	93.96
2.	0.117			27.52	620.5	95.07	93.20
3.	0.115			28.1	622.8	96.05	93.54
					Mean	95.17	93.57
					S.D.	0.82	0.38
					C.V.	0.86	0.41

IV. LINEARITY AND RANGE

According to USP tablet powder equivalent to 80, 90, 100, 110 and 120% of label claim was taken and dissolved in mobile phase, diluted appropriately to obtain the concentration in the range of 80-120% of the test concentration. The chromatograms of the resulting solutions were recorded.[7]

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