AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Development and Validation of Analytical Methods for Alprazolam and Fluoxetine In Pharmaceutical Dosage Form

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ABSTRACT

Alprazolam (ALP) is benzodiazepine derivative which produce symptomatic relief of anxiety and tension state resulting from a stressful enviourment or emotional factors. The First method was based on simultaneous equation method. In the proposed simultaneous equation method, the signals were measured at 222.0 nm and 227.0 nm corresponding to the absorbance maxima of ALP and FLU in methanol. The method was validated statistically. Recovery study was performed to confirm the accuracy of the method. This method is suitable technique for the reliable analysis of commercial formulation containing combination of ALP and FLU. Simplicity, sensitivity and rapidity of simultaneous equation method render it suitable for routine analysis for ALP and FLU from their combination dosage forms. The Second method was based on absorbance ratio method (Q. analysis). The absorbance was measured at two-wave lengths 222.0 nm (max) of ALP and 227.0 nm, which is an isoabsorptive point, were found adequate for quantification. The method is validated statistically. The recovery more than 99% with low SD suggests the accuracy of the method. In addition, the above-proposed UV spectroscopic methods are simple, easy to apply, low cost, does not use polluting reagents and requires relatively inexpensive instruments.

Keywords - Alprazolam; Fluoxetine; Anxiety, Isoabsorptive

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Received 18 December 2013, Accepted 01 January 2014

Please cite this article in press as: Gorakh J. *et al.*, Development and Validation of Analytical Methods for Alprazolam and Fluoxetine In Pharmaceutical Dosage Form. American Journal of PharmTech Research 2014.

INTRODUCTION

They also are useful in psychoneurotic states characterized by tension, anxiety, apprehension, fatigue, depression symptoms or agitation by gamma amino butyric acid is major inhibitory neurotransmitter in the mammalian CNS. Fluoxetine (FLU) is a antidepressant drug,[3,5,6] Many analytical methods including HPLC[12, 14], GC[15] and Spectrophotometric [13, 16] methods were reported for determination of ALP alone or in combination with other antidepressant drugs[4]. Fluoxetine (FLU) is a antidepressant drug, but this is subtype of selective serotonin reuptake inhibitor (SSRI).Combination therapy of ALP and FLU launched by Cipla, Pharmaceuticals limited, Mumbai for the treatment of antidepressant.

Drug profile of. Alprazolam is an odorless, white crystalline powder with a molecular weight of 308.8. It is soluble in alcohol and chloroform. Practically insoluble in water, but freely soluble in alcohol. Melting Point Reported 228-228.5 °C Observed 228.2 °C, Pka:2.4.

Fluoxetine is a white to off-white crystalline powder that is insoluble in toluene, cyclohexane and hexane. Fluoxetine is very slightly soluble in ethyl acetate, dichloromethane and water and but completely soluble in methanol, ethnol, chloroform and acetone. Melting point Reported:137-140 ^{0}C ,Observed: 138 ^{0}C.

MATERIALS AND METHOD

The Chemicals and reagents used for experimental work are as follows

Alprazolam obtained from M/S KIVI LABS Ltd., Ramangamdi (Baroda), Gujarat. Fluoxetine obtained form M/S KIVI LABS Ltd., Ramangamdi (Baroda), Gujarat. Methanol an R grade obtained from S D Fine Chemicals, Mumbai.Commercially available Pharmaceutical dosage form. Fludep Plus manufactured by Cipla Ltd. Mumbai. Alamflu manufactured by the Sanofi. LTD. Mumbai.

SHIMADZU 1700 UV Double Beam Spectrophotometer with 1cm matched Quartz cell.

Methods:

1. Preparation of Standard solution of Alprazolam and Fluoxetine separately in Methanol.
2. Study of Spectral Characteristics of Alprazolam and Fluoxetine in Methanol. Study of Spectral Characteristics of Alprazolam and Fluoxetine separately in Methanol,the resulting solution had concentrations of 10:20µg/ml.
3. Calibration curve of Alprazolam and Fluoxetine in Methanol by UV Absorption spectroscopy method. Described in Section fig 3 and 4
4. Study of Overlay Spectral characteristics of Alprazolam and Fluoxetine in methanol.

Accurately pipette out 1 ml of stock solution (100µg/ml) separately into 10ml standard flask and volume was made up using Methanol. These solutions were scanned over the entire range from 400 to 210 nm to obtain an overlay spectrum. Fig 5.

5. Preparation and analysis of standard mixture solution of ALP and FLU in methanol by proposed method. Weighed accurately 0.25mg of ALP and 20 mg of FLU transferred in to 50-ml Standard flasks dissolved and made up to the volume in methanol. The solution had a concentration of 1:8mg/ml of ALP and FLU respectively (Solution A).

   Accurately pipette out 5ml of Solution A into 50ml Standard flask and the volume was made up using methanol. These solutions had a concentration of 100:100µg/ ml of ALP and FLU respectively (Solution B).

   Accurately pipette out 0.5 ml,1ml,2ml of solution B into three 10 ml standard flask and the volume was made up using methanol and the resulting solution had concentrations of 2.5:5,5:10,10:20µg/ml. The absorbance of each solution was measured at 222.0 nm and 227.0 nm of ALP and FLU respectively and the concentration of each component is calculated as per simultaneous equation method .The results are tabulated in Table: 1

6. Simultaneous Estimation of Alprazolam and Fluoxetine in Dosage forms. Twenty tablets containing each of 0.25mg ALP and 20 mg of FLU were accurately weighed and finely powdered in a glass mortar. A weight equivalent to 10mg of ALP and 5 mg of Flu was accurately weighed and transferred to a 10 ml standard flask. 4 ml of methanol was added and swirled gently for a period of 10 min. The clear supernatant solution was then transferred to 10ml standard flask through Whatmann No 1 filter paper. The residue was further extracted twice with 2 ml each of methanol and passed through the same filter paper and the volume was finally made up to 10ml with methanol. The resulting solution had a concentration of 1 mg and 0.5mg/ml (solution A)

   Accurately pipette out 1 ml of the above solution and transferred to a 10 ml standard flask and made up to volume with methanol. The final solution had a concentration of 100 µg/ ml and 50µg/ ml of ALP and FLU respectively (solution B).

   Accurately pipette out 1 ml of solution B into a 10 ml Standard flask and volume was made up using methanol to obtain 10: 5 µg/ ml of ALP and FLU respectively. The absorbance of this solution was measured at 222.0 nm and 227.0 nm. The concentration of each drug is calculated using simultaneous equation method. The results of analysis are tabulated in Table: 2.

VALIDATION OF THE METHOD.

Linearity and Range:

Calibration curves were prepared for both the drugs at 222.0 nm and 227.0 nm separately and the entire calibration data at the selected analytical wavelengths were summarized in the Table: 3. The Data reveals that Beer's law is obeyed in the concentration range of 1 to 10 µg/ml at 222.0 nm and 2 to 18µg/ml 227.0 nm.

Repeatability / Precision:

The experiment preparation of calibration curve was repeated five times in a day for intra day and five different days for inter day precision. The average % RSD of intra day and inter day measurement was found to be 1.03% and 1.07562% respectively at 222.0 nm for ALP and 227.0 nm for FLU the % RSD of intra day and inter day measurement was found to be 1.522 and 1.1893% respectively Table: 4 and Table: 5.

Limit of Detection and Limit of Quantification (LOD and LOQ):

It is the smallest quantity of the analyte that is significantly different from the blank. Validating an analytical method, it is only necessary to establish the detection limit and the limit of quantification if samples with low concentration near these limits to be assayed. The values of LOD and LOQ for both the drugs at selected wavelength are represented in Table: 6.

Recovery Studies:

Accuracy of the proposed method was determined by performing recovery studies. A fixed amount of each drug from the dosage forms was taken and pure standard drug at three different concentrations within Beer's range was added and the total concentration was found by the proposed method. The determination with each concentration was repeated three times and average percent recovery of the added standard was calculated. The results are tabulated in Table: 7.

Method 2: Absorbance ratio method

1. Preparation of standard of solution of ALP and FLU in methanol.
2. Study of spectral characteristics of ALP and FLU in methanol.
3. Calibration curve ALP and of FLU in methanol by UV absorption method mentioned in Fig no 6 and 7.
4. Study of overlay spectral characteristics of ALP and FLU in methanol. Accurately pipette 1ml each of solution B. Separately and transferred in to 10 ml standard flask and volume were made up using methanol. These solutions were scanned over the entire range from 210-300nm to obtain overlay spectrum Fig 8. From the overlay spectra two wave lengths

are selected one at 223.7 nm which is an iso absorptive point because at this point both drugs shows same absorbance and other at 222.0nm corresponding to the maximum absorbance (λ max) of ALP .

5. Calibration curve of ALP and FLU at 223.7 -isoabsorptive point. Accurately pipette 0.5 – 4ml of solution B separately in to each eight 10ml standard flasks and volume were made up using methanol. The absorbance of each solution was measured at 223.7 nm for ALP and FLU with methanol as blank. The data and absorptive for both drugs at isoabsorptive point is presented in Table: 8 and calibration graphs were shown in Fig 9 and 10.
6. Preparation and analysis of standard mixture solution of ALP and FLU in methanol. Weighed accurately 0.25mg of ALP and 20 mg of FLU transferred in to 50-ml Standard flask dissolved and made up to the volume in methanol. The solution had a concentration of 1:8mg/ml of ALP and FLU respectively (Solution A).

   Accurately pipette out 5ml of Solution A into 50ml Standard flask and the volume was made up using methanol. This solution had a concentration of 100:100µg/ ml of ALP and FLU respectively (Solution B).

   Accurately pipette 0.5 ml, 1ml, and 2ml of solution B into three 10 ml standard flasks and volume were made up using methanol. The absorbance of each solution was measured at 222.0 nm and 223.7 nm and concentration of each drug was calculated by proposed method. The result of analysis is tabulated in Table: 9

   Simultaneous estimation of ALP and FLU in dosage forms. Twenty tablets containing each of 0.25 mg ALP and 20 mg of FLU were accurately weighed and finely powdered in a glass mortar. A weight equivalent to 5mg of ALP and 10 mg of FLU was accurately weighed and transferred to a 10 ml standard flask. 4 ml of methanol was added and swirled gently for a period of 10 min. The clear supernatant solution was then transferred to 10ml standard flask through a what Mann no 1 filter paper. The residue was further extracted twice with 2 ml each of methanol and passed through the same filter paper and the volume was finally made up to 10ml with methanol. The resulting solution had a concentration of 1 mg and 8mg/ml (solution A)

   Accurately pipette out 1ml of the above solution and transferred to a 10 ml standard flask and made up to volume with methanol. The final solution had a concentration of 100 µg/ ml and 100µg/ ml of ALP and FLU respectively (Solution-B).

   Accurately pipette out 1ml of solution B into a 10 ml Standard flask and volume was made up using methanol to obtain 10: 5 µg/ ml of ALP and FLU respectively. The

absorbance of this solution was measured at 222.0 nm and 227.0 nm. The concentration of each drug is calculated using simultaneous equation method. The results of analysis are tabulated in Table: 10.

VALIDATION OF THE METHOD.

Linearity and range:

The absorbance ratio method showed good linearity for ALP in the range of 2 to 10 µg/ml with correlation co-efficient, intercept and slope 0.9985, 0.0782, 0.0835 respectively and for FLU the linearity range was also found in the range of 4 to 24 with correlation co-efficient, intercept and slope. 0.9996, 0.0076, 0.0379 Table: 11.

Repeatability / Precision:

The preparation of calibration graph of ALP and FLU at 222.0 nm was repeated 5 times in a day for intraday precision and on five different days for interday precision. The average % RSD tabulated in Table: 12

Limits of Detection and Quantification:

Calibration curve was prepared for 5 times at 222.0 nm and SD of the intercept was calculated for both the drugs. The LOD and LOQ are calculated. The values are given in Table: 13.

Recovery Studies:

Accuracy of the proposed method was determined by performing recovery studies. A fixed amount of each drug from dosage forms were taken and pure standard drug at three different concentrations within Beer's range was added the total concentration was found by the proposed method. The determination with each concentration was repeated three times and average percent recovery of the added standard was calculated. The results are tabulated in Table: 14

RESULTS AND DISCUSSION

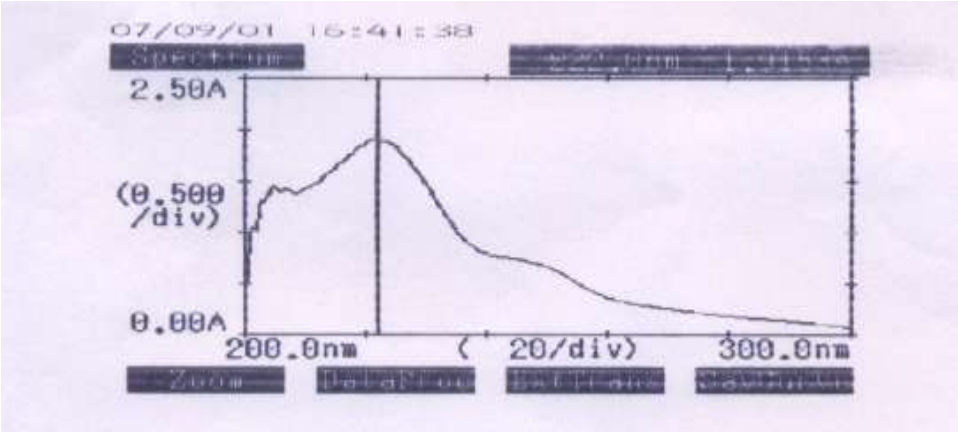

Figure. 1 Absorption spectra of ALP at 222.0 nm.

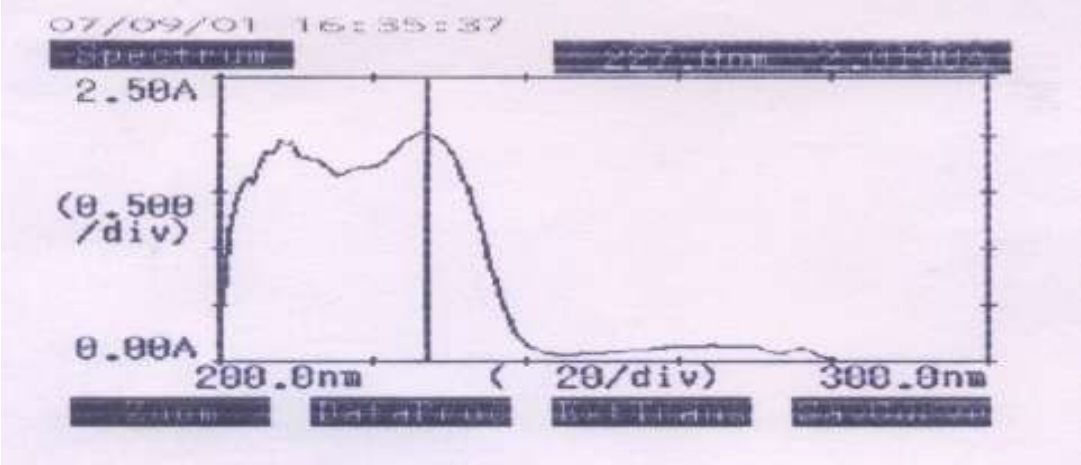

Figure. 2 Absorption spectra of FLU at 227.0 nm.

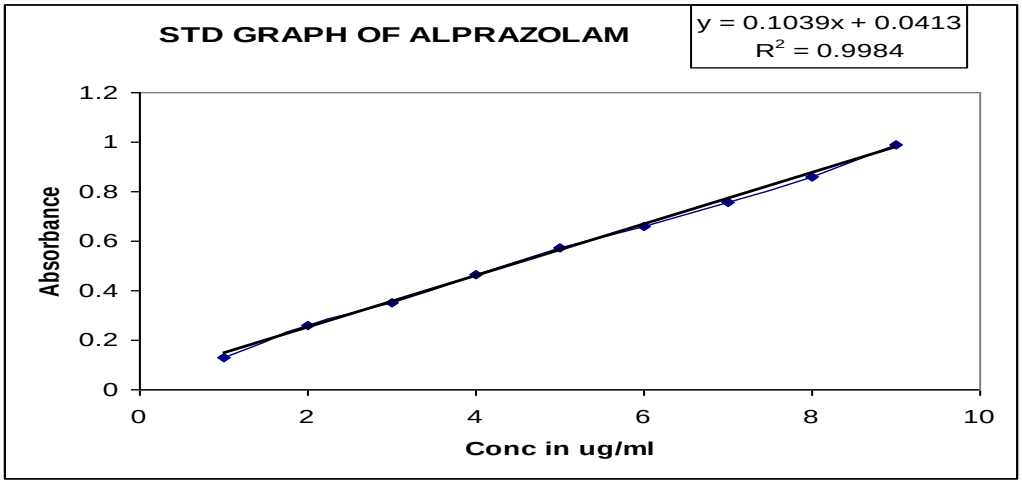

Figure. 3 Calibration curve for ALP at 222.0 nm.

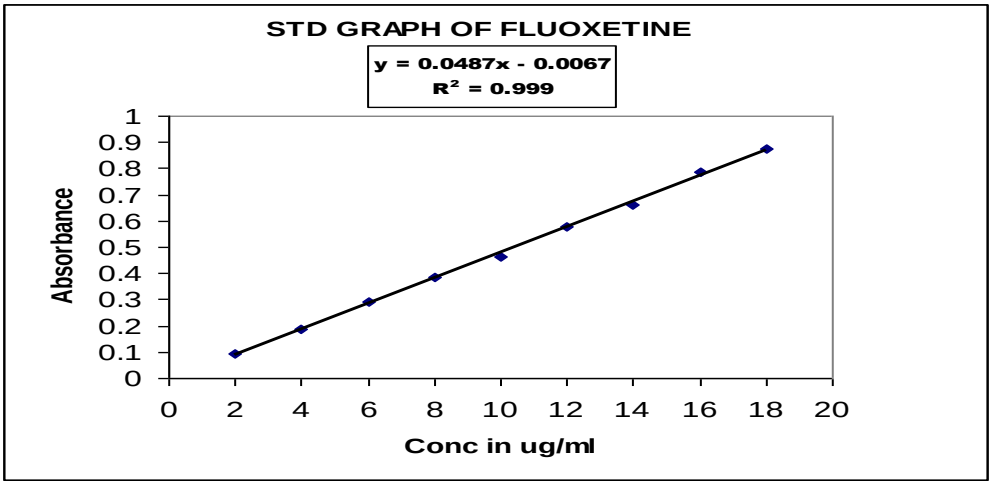

Figure. 4 Calibration curve for FLU at 227.0 nm.

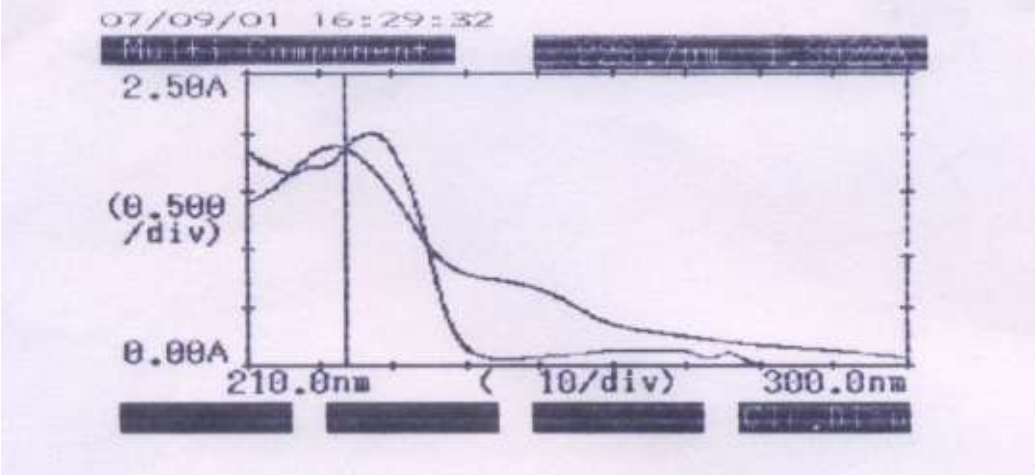

Figure. 5 over lay spectra of the both the drugs at selected wavelength.

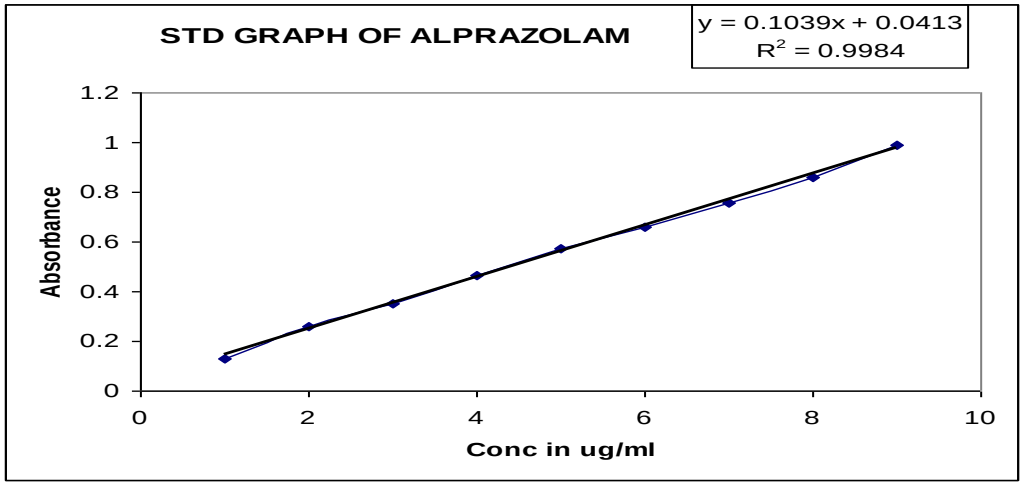

Figure. 6 Calibration curve for ALP at 222.0.

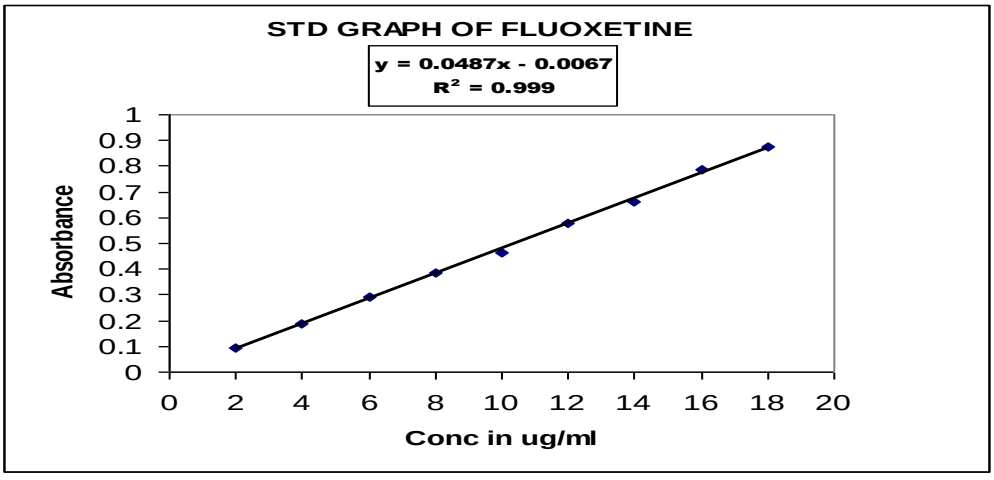

Figure. 7 Calibration curve for FLU at 227.0 nm.

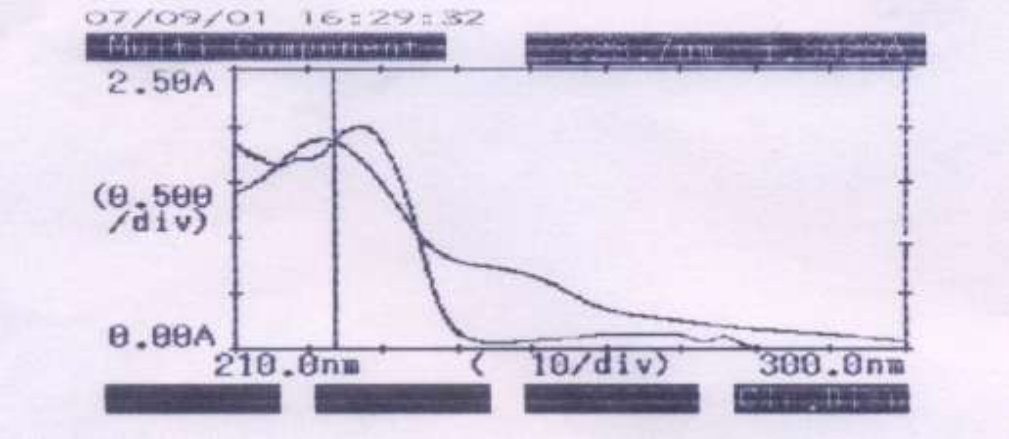

Figure. 8 Overlay spectra of ALP and FLU in method of showing Iso absorptive point.

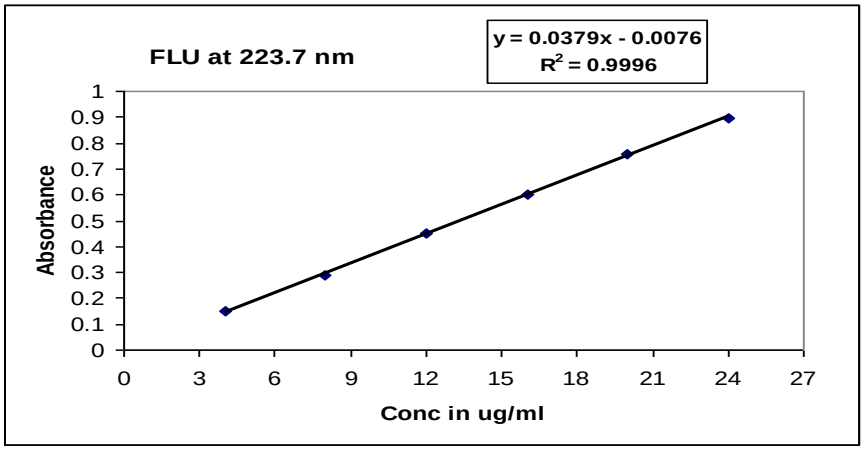

Figure. 9 Calibration curve for ALP at 223.7 nm.

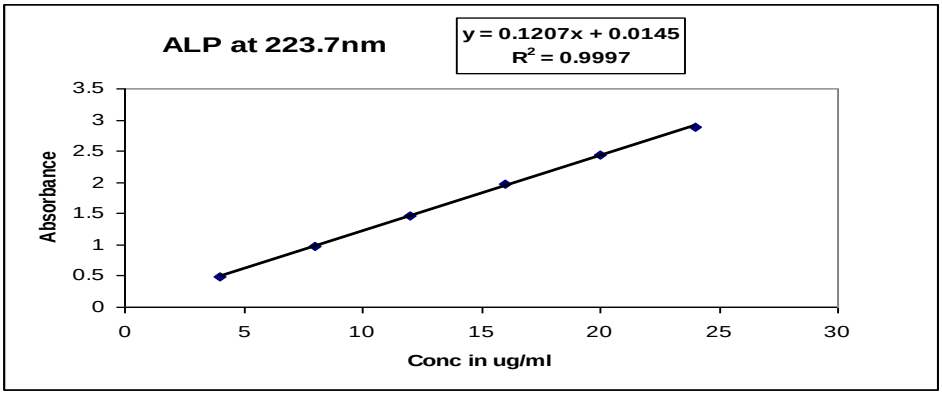

Figure. 10 Calibration curve for FLU at 223.7 nm.

Table 1 Result of analysis of standard mixture by simultaneous equation method

Mixed Std Cons µg/ml	*Abs at 222.0nm	*Abs at 227.0nm	Conc. found µg/ml		Average recovery %	
			ALP	FLU	ALP	FLU
2.5: 5	0.245	0.218	2.49	5.002	99.2	100.2
5:10	0.498	0.567	4.980	10.058	99.6	100.05
10:20	1.027	1.148	9.981	19.96	99.8	99.8

* Average of five Experiments.

Table 2: Results of analysis of Pharmaceutical Dosage forms by simultaneous Equation method.

Formulation	Label Claim Conc. In µg/ ml		*Absorbance at 222.0 nm	*Absorbance at 227.0 nm	Amt of Drug found in Conc. In µg/ ml		% Label Claim	
	ALP	FLU	A_1	A_2	ALP	FLU	ALP	FLU
Fludep Plus	0.25	20	0.0871	0.1147	449.62	500.08	99.24	100.08
Alamflu	0.25	20	0.7955	0.1095	450.02	599.96	100.04	99.96

* Average of Three experiments

Table 3: Calibration data Absorptivity values for ALP and FLU at 222.0 nm and 227.0 nm.

Parameters	At 222.0 nm		At 227.0 nm	
	ALP	FLU	ALP	FLU
Beer's law limit (µg/ ml)	1 to 10	2 to 18	1 to 10	2 to 18
Absorptivity	122.38	113.41	36.48	42.51
Molar Absorptivity	2.1236Lmol^{-1}cm^{-1}	2.7044 Lmol^{-1}cm^{-1}	2.0298 Lmol^{-1}cm^{-1}2.	1.2594 Lmol^{-1}cm^{-1}
Regression Equation	Y= 0.1039X+0.0413	Y = 0.0371X-0.0072	Y = 0.0834X + 0.0711	Y = 0.0487X - 0.0067
Slope	0.1039	0.0371	0.0834	0.0487
Intercept	0.0413	0.0072	0.0711	0.0067
Correlation Co efficient (R^2)	0.998	0.9999	0.9978	0.999
Beer's law limit (µg/ ml)	1 to 10	2 to 18	1 to 10	2 to 18

Table. 4: Precision Data of ALP at 222.0 nm.

Conc. of ALP in µg/ ml	* Absorbance at 222.0 nm	% RSD_1	% RSD_2
1	0.132	2.485	3.446
2	0.259	0.646	1.315
3	0.351	1.661	0.483
4	0.465	1.287	1.645
5	0.575	1.339	0.090
6	0.661	2.518	1.671
7	0.756	0.309	0.249
8	0.859	0.698	0.457
9	0.989	0.867	0.481
Avg % RSD	____	1.522	1.1893

*Average of five experiments. % RSD1 – Intra day precision, % RSD2 – Inter day precision.

Table. 5: Precision Data of FLU at 227.0 nm.

Conc. of FLU in µg/ ml	* Absorbance at 227.0 nm	% RSD_1	% RSD_2
2	0.093	1.754	2.434
4	0.189.	1.174	1.881
6	0.291	1.791	2.227

8	0.387	0.181	2.686
10	0.466	0.794	1.756
12	0.576	2.824	0.764
14	0.664	1.514	1.262
16	0.785	1.786	0.346
18	0.874	1.667	1.128
Avg % RSD	____	1.503	1.7562

*Average of five experiments. % RSD_1 – Intra day precision, % RSD_2 – Inter day precision

Table 6: LOD and LOQ values for ALP and FLU at 222.0 nm and 227.0 nm

	ALP	FLU
SD	0.0043	0.0032
LOD µg/ ml	0.5089	0.283
LOQ µg/ ml	1.864	0.784

SD - Standard Deviation of Intercept of five experiments.

Table 7: Recovery studies of ALP and FLU by simultaneous equation Method.

Formulation	Label Claim in mg		Conc. of pure drug added in mg		* Amount of drug found in mg		% Recovery ± SD		Avg. % ± SD	
	ALP	FLU	ALP	FLU	ALP	FLU	ALP	FLU	ALP	FLU
Fludep Plus	0.25	20	_	_	0.21	18	95.65 0.1132	95.63 0.1431		
	_	_	5	5	0.23	19	99.64 0.1928	99.82 0.2307		
	_	_	10	10	0.25	16	100.24 0.2145	96.72 0.1835		
	_	_	15	15	0.26	21	102.46 0.2353	99.21 0.2176	99.62 0.1296	99.72 0.2278
Alamflu	0.25	20	_	_	0.24	20	100.48 0.2637	101.23 0.1848		
	_	_	5	5	0.22	19	98.32 0.3721	102.41 0.2551		
	_	_	10	10	0.23	20	99.92 0.0982	100.01 0.2186		
	_	_	15	15	0.27	22	100.46 0.2551	100.01 0.6721	99.99 0.3027	99.99 0.2279

*Average of five determinations

Table 8: Calibration data and Absorptivity of ALP and FLU at Iso absorptive point 223.7 nm.

ALP Conc. in µg/ ml	*Abs 223.7nm	Absorptivity	FLU Conc. In µg /ml	*Abs 223.7 nm	Absorptivity
1	0.132	176.0	2	0.090	180
2	0.259	174.0	4	0.176	176
3	0.351	170.66	6	0.258	172
4	0.465	174.0	8	0.355	177.5
5	0.575	175.0	10	0.428	171.2

6	0.661	180.66	12	0.542	180.33
7	0.756	177.42	14	0.633	180.85
8	0.859	173.57	16	0.711	179.81
9	0.989	175.52	18	0.859	177.77
Mean		175.40	-	-	175.80
SD		0.4188	-	-	0.4805
CV		0.12165	-	-	0.1547
SE		0.1230	-	-	0.1545

*Avg of five experiments.

Table 9: Result of analysis of standard mixture by absorbance ratio method.

Mixed std. Conc. µg/ml	* Abs at 222.0 nm	* Abs at 223.7 nm	* Conc. found µg/ml		Avg. recovery %	
			ALP	FLU	ALP	FLU
2.5:5	0.0764	0.112	2.411	5.02	96.4	100.02
5:10	0.145	0.304	5.06	9.99	100.06	99.9
10:20	0.317	0.461	10.03	20.08	100.03	100.8

*Average of five experiments

Table: 10 Results of Analysis of Pharmaceutical Dosage form by absorbance ratio method.

Formulation	Label Claim Conc. in mg		*Abs at 222.0nm	*Abs at 227.0 nm	Amt of Drug found Conc. in mg		% Label Claim	
	ALP	FLU	A_1	A_2	ALP	FLU	ALP	FLU
Fludep Plus	0.25	20	0.078	0.182	4.821	9.967	96.40	99.6
Alamflu	0.25	20	0.094	0.197	4.902	10.002	98.04	100.2

*Average of five experiments.

Table 11: Calibration data for ALP and FLU at 223.7 nm.

Parameters	ALP at 223.7nm	FLU at223.7nm
Beer's law limit in µg/ml	2 to 10	4 to 24
Molar Absorptivity	2.7042 L mol^{-1} cm^{-1}	2.0298 L mol^{-1} cm^{-1}
Regression equation	Y=0.0835X+0.0782	Y=0.0379X-0.0076
Slope	0.0835	0.0379
Intercept	0.0782	0.0076
Co-relation co-efficient	0.9985	0.9996

Table 12: Precision data for ALP and FLU by absorbance ratio method.

Mixed std conc. µg/ml	* Amount found conc. in µg/ml		Avg. recovery %		% RSD_1		% RDS_2	
	ALP	FLU	ALP	FLU	ALP	FLU	ALP	FLU
2.5:5	2.397	5.032	99.24	100.02	1.374	0.287	0.741	0.883
5:10	4.881	10.185	99.8	101.1	0.564	0.329	1.280	0.503
10:20	8.981	2.057	99.3	100.02	1.182	1.066	1.264	0.1641
Avg. % RSD					1.029	0.5667	1.0403	0.4902

*Avg. of 3 determination RSD_1 = intra day precision RSD2 = inter day precision.

Table 13:LOD and LOQ values for ALP and FLU at 223.7 nm.

	ALP	FLU
* SD	0.00174	0.00113
LOD (µg/ml)	0.384	0.229
LOQ (µg/ml)	1.341	0.785

* SD = Standard deviation of intercept of five experiment.

Table 14: Recovery studies of ALP and FLU by absorbance ratio method.

Formulation	Label Claim in mg		Conc. of pure drug added in mg		* Amount of drug found in mg		% Recovery ± SD	
	ALP	FLU	ALP	FLU	ALP	FLU	ALP	FLU
Fludep Plus	0.25	20	_	_	0.22	19	96.42 0.3162	99.6 0.5771
	_	_	5	5	0.23	18	99.75 0.2621	99.72 0.8216
	_	_	10	10	0.25	20	100.24 0.6821	100.81 0.5211
	_	_	15	15	0.24	21	99.8 0.2710	100.2 0.0846
Alamflu	0.25	20	_	_	0.23	18	98.04 0.3227	100.2 0.6347
	_	_	5	5	0.22	19	100.12 0.5410	99.96 0.7851
	_	_	10	10	0.26	20	100.24 0.2314	99.81 0.1264
	_	_	15	15	0.24	19	99.34 0.3579	100.21 0.2164

* Avg of three experiment.

Specific accurate precise and simple UV spectroscopic methods were developed for simultaneous determination of ALP and FLU from their dosage forms. This method was suitable technique for the reliable analysis of commercial formulation containing combination of ALP and FLU. Simplicity, sensitivity and rapidity of simultaneous equation method render it suitable for routine analysis ALP and FLU from their combination dosage form.

The First method was based on simultaneous equation method, the signals were measured at 222.0 nm and 227.0 nm corresponding to the absorbance maxima of ALP and FLU in methanol. Concentration of each drug was obtained by using the simultaneous equation. The method was validated statistically. This method is suitable technique for the reliable analysis of commercial formulation containing combination of ALP and FLU.

The Second method was based on absorbance ratio method (Q. analysis). The absorbance was measured at two-wave lengths 222.0 nm (λ_{max}) of ALP and 227.0 nm, which is an isoabsorptive point, were found adequate for quantification. The proposed method is a modification of

simultaneous equation method. The method is validated statistically. Recovery study was also performed to confirm the accuracy of the method. The recovery more than 99% with low SD suggests the accuracy of the method. Low values of % RSD for intraday and interday suggest the method is precise enough for routine QC analysis of both the drugs.In addition, the above-proposed UV spectroscopic methods are simple, easy to apply, low cost, does not use polluting reagents and requires relatively inexpensive instruments.

CONCLUSION

This method was suitable technique for the reliable analysis of commercial formulation containing combination of ALP and FLU. Simplicity, sensitivity and rapidity of simultaneous equation method render it suitable for routine analysis ALP and FLU from their combination dosage form. The marketed products Fludep plus and Alamflu were analyzed to developed new methods for simultaneous estimation of ALP and FLU in combined dosage form. The results obtained by the proposed method were found to be satisfactory. The UV spectroscopic methods demonstrated herein, are applicable to the estimation of ALP and FLU in pure as well as in existing dosage forms. In order to ensure that the data generated each of the above methods are both accurate and precise. The experiments have been performed on calibrated equipments using suitable reference standards. To prove and documents the reliability of the methods, validation as per ICH guide lines have been carried out to a possible extent.

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