

Development and Validation of Second-Derivative Spectrophotometry Method for Simultaneous Estimation of Alprazolam and Fluoxetine Hydrochloride in Pure Powder and Tablet Formulation and Its Comparison with HPLC Method

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Abstract

This paper describes validated Second Derivative Spectrophotometry (D2) method for simultaneous estimation of Alprazolam (ALP) and Fluoxetine hydrochloride (FXT) in pure powder and formulation. D2 method, applying the peak zero method, was developed for the determination of ALP and FXT in their combined tablet formulations without prior separation. The solutions of standard and sample were prepared in 0.1M HCl. Quantitative determination of the drugs was performed at 232.14 nm and at 225.25 nm ($N = 4$; $\lambda = 2.8$) for ALP and FXT, respectively. Proposed D2 method was evaluated for the different validation parameters. The specificity test showed that there was no interference from excipients commonly found in the commercial pharmaceutical formulations at analytical wavelength of ALP and FXT. Quantification was achieved over the concentration range 4-14 $\mu\text{g}\cdot\text{mL}^{-1}$ for both drugs with mean recovery of 99.36 ± 0.84 and 99.60 ± 0.93 % for ALP and FXT, respectively. This method is simple, precise, and sensitive and applicable for the simultaneous determination of ALP and FXT in pure powder and formulation. The method was compared to high-performance liquid chromatography (HPLC) method, which was reported for the same drugs. No significant difference was found between the methods for ALP and FXT quantitation.

Keywords:

Alprazolam, Fluoxetine Hydrochloride, Derivative Spectroscopy

1. Introduction

Alprazolam (ALP) is chemically 8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazolo [4,3,- α]-[1,4] benzodiazepine derived from 1,4-benzodiazepines of new generation. It is a benzodiazepine mainly used as anxiolytic in humans, and may be effective in the treatment of depression and panic disorder. It should be borne in mind that neither alprazolam nor any other benzodiazepine is effective when it comes to treating anxiety and strain caused by daily stress. Besides this, ALP is also used to treat panic disturbances with or without agoraphobia [1, 2]. Fluoxetine (FXT) hydrochloride, *N*-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine, is a strong and selective serotonin reuptake inhibitor. It is used for the treatment of unipolar mental depression. FXT is also used in a variety of disorders in addition to depression. Beneficial responses have been reported in obsessive compulsive disorders,

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pain syndromes including diabetic neuropathy and fibrositis, panic disorders and nervous bulimia. The reason behind the combination therapy is based upon the anxiety – depressant continuum [3].

Most of the analytical methods in the literature to determine ALP are aimed at either quantifying ALP in biological fluids [4-9], pharmaceutical formulation [14-16] or to determine its related substances [10-13] and include analysis using high-performance liquid chromatography (LC), LC/tandem mass spectrometry (LC/MS), gas chromatography/MS (GC/MS), Spectrofluorimetry, high-performance thin layer chromatography (HPTLC) and capillary electrochromatography (CEC). Reports are available for determination of FXT using LC, LC/MS, Solid-phase extraction and GC in biological fluids [17-22], and using LC, Capillary electrophoresis, HPTLC, spectrophotometry in pharmaceutical preparations [23-28]. To the best of our knowledge, there is no reported spectrophotometric or pharmacopoeial method for simultaneous determination of ALP and FXT in pharmaceutical formulations, previous to our work. Thus, efforts were made to develop fast, selective and sensitive analytical method for the estimation of ALP and FXT in their combined dosage form using second derivative spectrophotometry (D2) method.

2. Experimental

2.1. Apparatus

Absorbance was measured, and derivative spectra were recorded over the wavelength range 200-400 nm in two matched quartz cells with a 1 cm light path using a double beam Perkin Elmer Lambda 19 (Waltham, Massachusetts, USA) and Chemito 2600 UV-Visible spectrophotometer (Chemito Instruments Ltd., Nasik, India).

2.2. Reagents and Materials

ALP and FXT pure powder were procured as gratis samples from Torrent Pharmaceuticals (Ahmedabad, India) and Sarabhai Pharmaceuticals Ltd (Baroda, India) respectively. Analytical grade hydrochloric acid (HCl) was purchased from E. Merck (Mumbai, India). Membrane filters (nylon 0.45 μm - 47 mm) were purchased from Gelman Laboratory (Mumbai, India).

2.3. Preparation of ALP and FXT Mixed Standard Stock Solutions

Stock solution was prepared by weighing ALP (10 mg) and FXT (800 mg). Weighed powder of both drugs were accurately transferred to a same volumetric flask of 100 mL and dissolved in and diluted to the mark with 0.1N HCl to obtain a mixed standard stock solution (solution A) of ALP ($100 \mu\text{g.mL}^{-1}$) and FXT ($8000 \mu\text{g.mL}^{-1}$). Accurately measured solution A was transferred to volumetric flask of 100 mL and diluted to the mark with 0.1N HCl to obtain a mixed standard stock solution (solution B) of ALP ($0.5 \mu\text{g.mL}^{-1}$) and FXT ($40 \mu\text{g.mL}^{-1}$).

2.4. Preparation of Sample solutions

Twenty tablets were weighed and finely powdered. Powder equivalent to 0.25 mg ALP and 20 mg FXT was weighed and transferred in a 25 mL volumetric flask and dissolved in 15 mL of 0.1 mol L^{-1} HCl. The solution was sonicated for 15 min, and the final volume was diluted to the mark with 0.1 mol L^{-1} HCl to obtain solution of ALP ($10 \mu\text{g.mL}^{-1}$) and FXT ($800 \mu\text{g.mL}^{-1}$). This solution was used for estimation of ALP. This solution (0.125 mL) was

further diluted to 25 mL with 0.1 mol L⁻¹ HCl to obtain solution of FXT (10 µg.mL⁻¹). The solutions were filtered through a nylon 0.45 µm membrane filter.

2.5. Selection of wavelength for estimation of ALP and FXT

Standard stock solution of ALP and FXT was diluted appropriately with 0.1 M HCl to obtain solutions containing 4, 6, 8, 10, 12, and 14 µg.mL⁻¹ ALP and FXT respectively. Spectra of these diluted solutions were scanned in the spectrum mode between 200 - 400 nm, with the band width of 2 nm and scan speed of 60 nm/min vs 0.1 mol L⁻¹ HCl as a blank. These zero order spectra of ALP and FXT were treated to obtain corresponding first order and second order derivative spectra with an interpoint distance of 5 nm in the range of 200 – 400 nm.

2.6. Derivative conditions

The second order spectra were scanned by using the spectral mode with the 60 nm.min⁻¹ scan speed. The derivative spectra were recorded by using digital differentiation (convolution method) with 17 to 25 data points with a derivative wavelength difference (Δλ) of 2.8 nm in the range of 200 – 400 nm. No smoothing of the spectra was found to be necessary.

Using memory channels, the second derivative spectra were overlapped. The wavelength 232.14 nm was selected for the quantification of ALP (where the derivative response for FXT was zero). Similar, 225.25 nm was selected for the quantification of FXT (where the derivative response for ALP was zero).

A characteristic wavelength (ZCPs) for ALP and FXT was confirmed by varying the concentration of the one component and while the concentration of the other component was constant, and vice versa.

3. Method of Validation

3.1. Calibration curve (linearity of the method)

Calibration curves were constructed by plotting absorbance vs. concentrations of ALP and FXT, and the regression equations were calculated. The calibration curves were plotted over the six different concentrations in the range 4-14 µg.mL⁻¹ for both drugs. Accurately measured mixed standard solution A of ALP and FXT (0.4, 0.6, 0.8, 1.0, 1.2. and 1.4 mL) were transferred to a series of 10 mL volumetric flasks and diluted to the mark with 0.1 M HCl. Accurately measured mixed standard solution B of ALP and FXT (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mL) were transferred to a series of 10 mL volumetric flasks and diluted to the mark with 0.1 mol L⁻¹ HCl. Absorbance was measured for each solutions at analytical wavelength of ALP and FXT (n = 6).

3.2. Accuracy (% Recovery)

The accuracy of the methods was determined by calculating recoveries of ALP and FXT by the standard addition method. Known amounts of mixed standard solution of ALP and FXT (4.0, 8.0 and 12.0 µg.mL⁻¹) were added to prequantified sample solutions of tablet dosage forms. The amounts of ALP and FXT were estimated by applying values of absorbance to the regression equations of the calibration curve.

3.3. Method Precision (Repeatability)

The precision of the instruments was checked by repeatedly scanning ($n = 6$) mixed standard solutions of ALP and FXT ($10 \mu\text{g.mL}^{-1}$).

3.4. Intermediate Precision (Reproducibility)

The intermediate precision for the proposed method was determined by estimating mixed standard solution of ALP and FXT for three different concentrations ($4.0, 8.0$ and $12.0 \mu\text{g.mL}^{-1}$) for three times on the same day and on three different days. The results are reported in terms of relative standard deviation (RSD).

3.5. Limit of Detection and Limit of Quantification

The limit of detection (LOD) with signal to noise ratio of 3:1 and the limit of quantification (LOQ) with signal to noise ratio of 10:1 were calculated for both drugs using the following equations as per International Conference on Harmonization (ICH) guidelines [29].

$$\text{LOD} = 3.3 \times \sigma/S$$

$$\text{LOQ} = 10 \times \sigma/S$$

Where σ = the standard deviation of the response and S = the standard deviation of y-intercept of regression line.

3.6. Specificity

The excipients gelatin, hypromellose, hydroxypropyl methylcellulose acetate succinate, sodium lauryl sulfate, sucrose, talc, titanium dioxide, triethyl citrate (Signet Ltd., Mumbai, India) were spiked into a preweighed quantity of drugs to assess the specificity of the methods. The absorbance was measured to determine the quantity of the drugs.

3.7. Robustness

Solutions of both the drugs in 0.1 mol L^{-1} HCl were studied for their stability at ambient temperature for 24 h.

3.8. Analysis of ALP and FXT in Tablet Dosage Forms

Tablets containing ALP (0.25 mg) and FXT (20 mg) of one brand were purchased from local market. The absorbance of sample solutions was measured for quantification at analytical wavelength of ALP and FXT, respectively, by using D2 method as described above. The amounts of ALP and FXT present in sample solution were determined by applying values of absorbance to the regression equations of the calibration curve.

4. Results and Discussion

4.1. D2 Method

The zero order spectra of standard solutions of ALP and FXT were found to be similar in nature and overlapping (Fig.1). It was observed that ALP and FXT contribute significantly at their corresponding λ_{max} values of absorption. Therefore, it was thought that a derivative graphical method could be used to estimate ALP and FXT in the presence of each other.

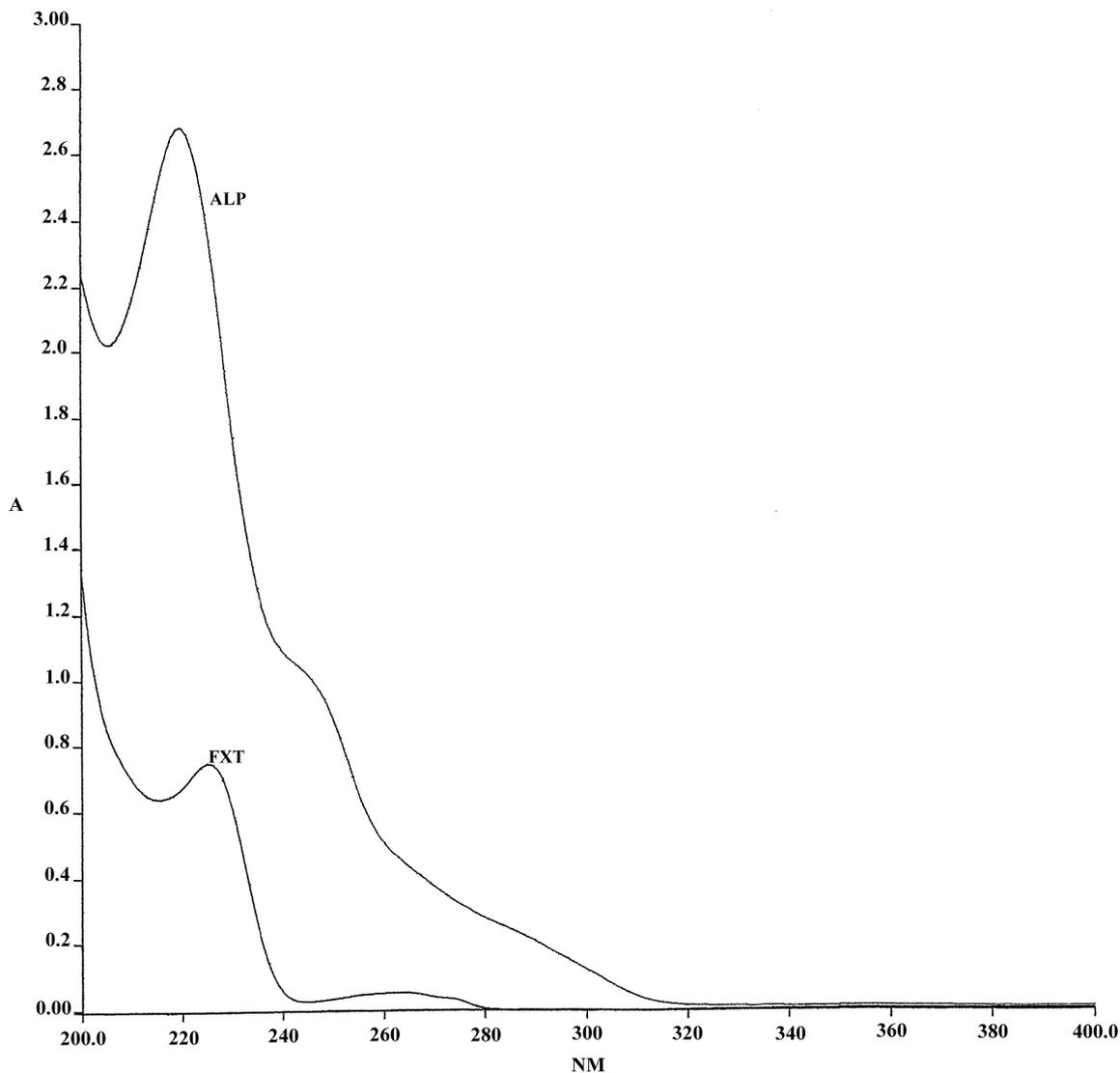


Fig.1. Overlain zero order spectrums of ALP and FXT in 0.1 mol L⁻¹ HCl.

The derivative spectra of different orders were obtained from the zero order spectra using digital differentiation. The principle advantages of derivative spectroscopy are the improvement of resolution of overlapping absorption bands and the accuracy and precision compared to UV absorption methods; therefore, derivative spectroscopy has been used in quantitative analysis when the analyte to be determined present in admixture with other components [30, 31]. Fig. 2 shows that the Second derivative could be used for determination of ALP and FXT. When the second derivative used the spectra present well defined bands for determination of the analytes, and the sensitivities are greater. Thus second derivative was selected and the other derivatives were discarded because they showed insufficient resolution and do not present analytical advantages.

The type of solvent, degree of deviation, range of wavelength, and N value were chosen in order to optimize the conditions. The solvent selected was 0.1 mol L⁻¹ HCl because it allowed sufficient spectral resolution to be obtained for the application of the peak zero method. The derivative wavelength difference $\Delta(\lambda)$ depends on the measuring wavelength range and N values. Generally, the noise decreases by increasing $\Delta\lambda$. The optimal wavelength range should be chosen because broad peaks become sharper, the ratio of signal-to-noise

(S/N) increases, and the sensitivity of the method increases by the degree of low pass filtering or smoothing. Therefore, a series of N value (N = 1 – 9) was tested in the second order UV spectrum of ALP and FXT in 0.1 mol L⁻¹ HCl. Optimum results were obtained in the measuring wavelength range 200 – 300 nm, N = 4 (λ = 2.8) for D2 method. A value of 4 was selected because it presents maximum sensitivity without affecting the S/N.

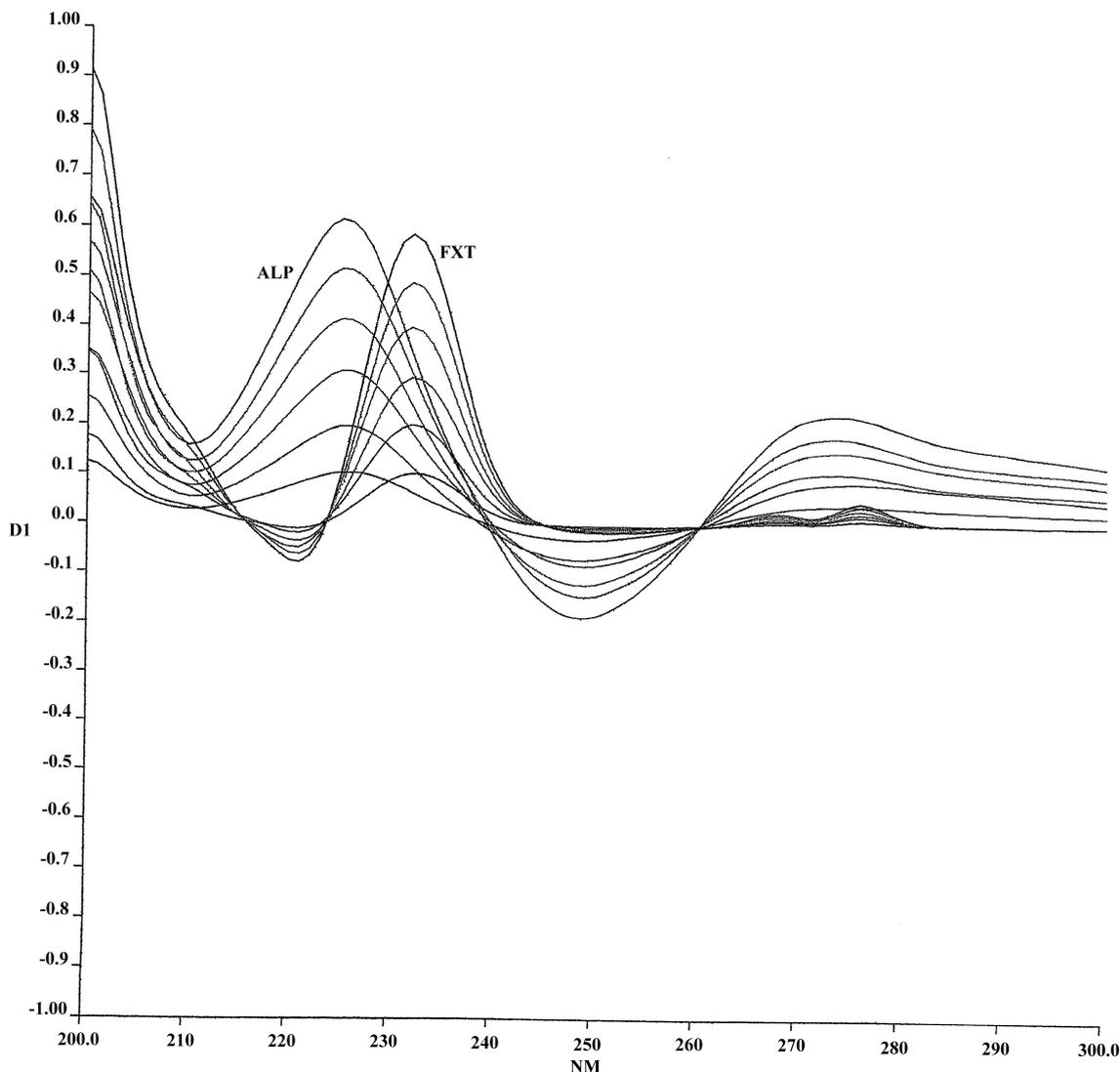


Fig.2. Overlain first derivative spectrums of ALP and FXT in 0.1 mol L⁻¹ HCl.

The second derivative spectra (D2) of ALP and FXT were found to be appropriate for the determination of ALP and FXT by having separated ZCPs in 0.1 mol L⁻¹ HCl. The D2 spectrum of ALP has zero absorption at 225.25 nm, where FXT gives significant derivative response, while the D2 spectrum of FXT has zero absorption at 232.14 nm, where ALP gives the significant derivative response. Therefore, 232.14 nm was selected for estimation of ALP and 225.25 nm was selected for the estimation of FXT. (Fig. 3)

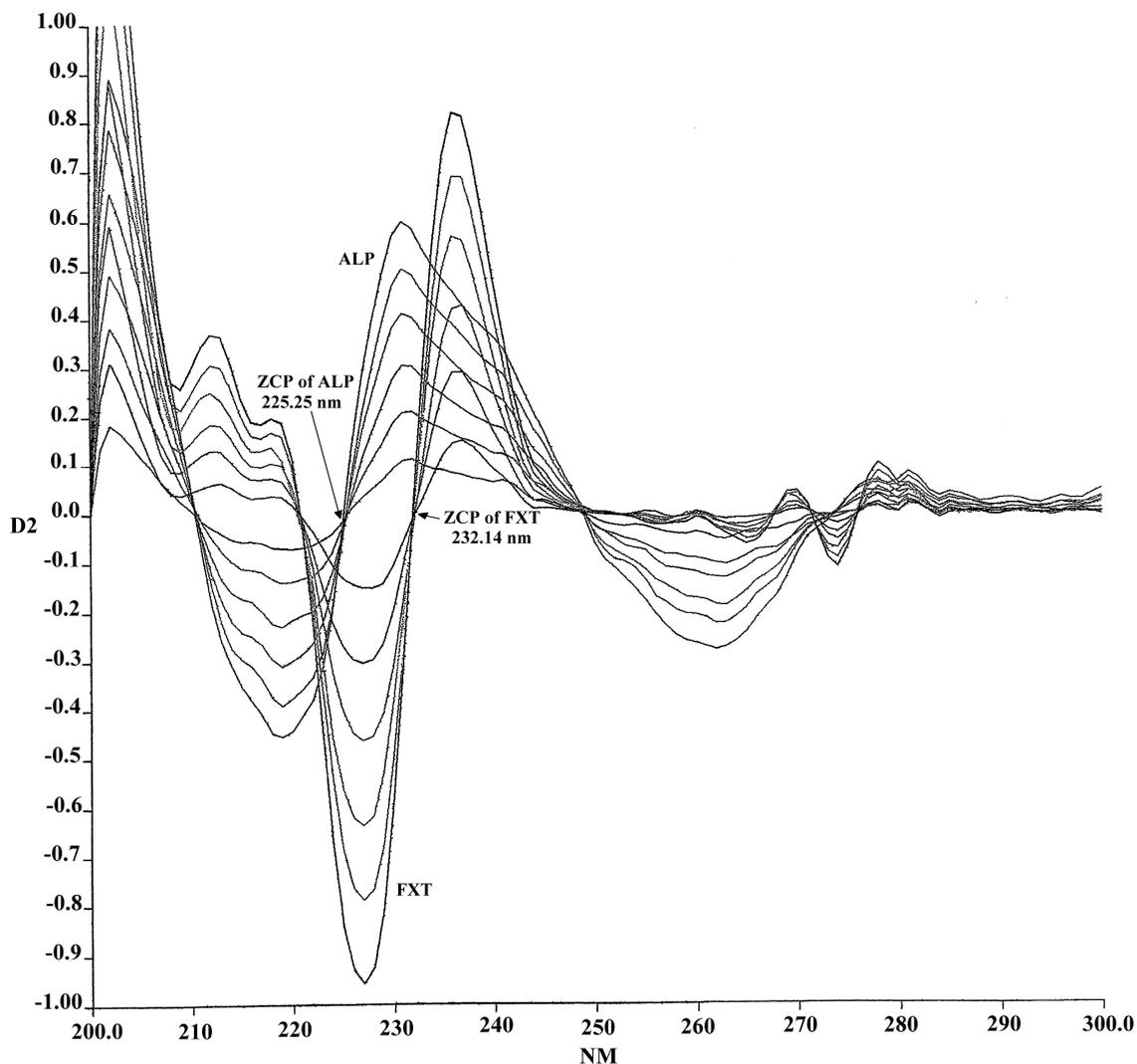


Fig. 3. Overlaid second derivative spectrums of ALP and FXT in 0.1 mol L⁻¹ HCl..

4.2. Validation of Proposed Method

4.2.1. Linearity

Linear correlation was obtained between absorbance and concentration of ALP and FXT in the range of 4-14 $\mu\text{g}\cdot\text{mL}^{-1}$ for both the drugs, respectively. Data of regression analysis are summarized in Table 1.

4.2.2. Accuracy

The recovery experiments were carried out by the standard addition method. The recoveries obtained were 99.36 ± 0.84 and 99.60 ± 0.93 % for ALP and FXT, respectively. The high values indicate that both methods are accurate.

4.2.3. Method Precision

The RSD values for ALP and FXT were found to be 0.228 and 0.296 %, respectively. The RSD values were found to be below 1% which indicate that the proposed methods are repeatable.

Table 1: Regression analysis of calibration curves for ALP and FXT for the proposed Derivative Spectrophotometric (D2) method.

Parameter	D2 method	
	ALP	FXT
Concentration range	4-14 $\mu\text{g/mL}$	4-14 $\mu\text{g/mL}$
Slope	0.04676	-0.07260
Standard deviation of the slope	0.00013	0.00018
Intercept	-0.07407	0.16102
Standard deviation of the intercept	230.65	507.56
Correlation coefficient	0.9998	0.9999

4.2.4. Intermediate Precision

The RSD values were found to be below 2% which indicate that the proposed methods are reproducible (Table 2).

Table 2: Summary of validation parameters for the proposed D2 method.

Parameter	D2 method	
	ALP	FXT
LOD ^a	0.045 $\mu\text{g/mL}$	0.033 $\mu\text{g/mL}$
LOQ ^b	0.151 $\mu\text{g/mL}$	0.108 $\mu\text{g/mL}$
Accuracy, %	99.36-100.29	99.60-100.53
Repeatability (RSD ^c , %, n ^d = 6)	0.228	0.296
Precision (RSD, %)		
Interday (n=3)	0.02 - 0.22	0.03 - 0.83
Intraday (n=3)	0.15 - 0.57	0.297-0.526

^a LOD = Limit of detection.

^b LOQ = Limit of quantification.

^c RSD = Relative standard deviation.

^d n = Number of determination

4.2.5. LOD and LOQ

LOD for ALP and FXT were found to be 0.045 and 0.033 $\mu\text{g.mL}^{-1}$ respectively. LOQ for ALP and FXT was found to be 0.151 and 0.108 $\mu\text{g.mL}^{-1}$ respectively. These data show that microgram quantity of both drugs can be accurately determined.

4.2.6. Specificity

Excipients used in the specificity studies did not interfere with the estimation of either of drugs by the proposed methods. Hence, method was found to be specific for estimation of ALP and FXT.

4.2.7. Robustness

Absorbance variation was found to be less than 1%. Also, no significant change in absorbance was observed during 24 h. No decomposition was observed after 24h. Hence, methods were found to be robust for estimation of ALP and FXT.

4.3. Assay of the tablet dosage form

The proposed validated method was successfully applied to determine ALP and FXT in their tablet dosage forms. The results obtained for ALP and FXT were comparable with the corresponding labeled amounts (Table 3).

Table 3: Assay results for the combined dosage form using the proposed D2 method.

Parameter	ALP \pm SD ^a (n ^b = 5), %	FLU \pm SD ^a (n ^b = 5), %
A	99.89 \pm 0.40	100.29 \pm 0.39

^a SD = Standard deviation.

^b n = Number of determination.

4.4. Comparison of the Proposed Method

The proposed D2 method was compared with HPLC method [32] for determination of ALP and FXT in tablet dosage forms. The assay results obtained by these methods were used for statistical comparison to evaluate the validity of developed D2 method. For ALP the calculated *F* value was found to be 1.89 which is less than the tabulated *F* value (5.05) at 95% (*P* = 0.05) confidence interval. For FXT the calculated *F* value were found to be 1.66 which is less than the tabulated *F* value (5.05) at 95% (*P* = 0.05) confidence interval. Therefore, there was no significant difference among the methods.

5. Conclusion

Proposed method was found to be precise and accurate. The methods can be used for the routine simultaneous analysis of ALP and FXT in pharmaceutical preparations. In spite of the low concentration of ALP, method was successfully used to estimate the amount of ALP and FXT present in the tablet without the need for addition of internal standard or prior separation. Moreover, the proposed method has the advantages of simplicity, convenience and quantification of ALP and FXT in combination and can be used for the assay of their dosage form.

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