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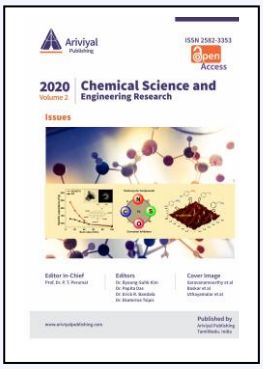

Development and Validation of Three Novel UV Spectrophotometric Methods for Determination of Newly Discovered Combination for the Treatment of Anxiety and Comparison Using Anova

Radharapu Satchitkrishna,* Patel Dhara and Meshram Dhananjay

Department of Pharmaceutical Quality Assurance, Pioneer Pharmacy Degree College, Vadodara, Gujarat, India.

***Corresponding author E-mail address**: patel.dhara.j@gmail.com (R. Satchitkrishna)

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Abstract: Paroxetine HCl and Etizolam is a newly approved combination which is used for treating anxiety. It is necessary to develop a method to estimate both drugs in combined dosage form. For simultaneous estimation of Paroxetine HCl and Etizolam three simple, precise and sensitive UV spectrophotometric methods were developed in the present work. Method 1 first order derivative methods, ZCP selected were 241.50 nm for Etizolam and 256.50 nm for Paroxetine HCl. Method 2 is absorbance correction method, 243 n and 295 nm were used to estimate the both the drugs. Method 3 is dual wavelength method, 230 nm and 250 nm were selected for estimation of Paroxetine HCl and 221.50 nm and 288 nm were selected for estimation of Etizolam. Calibration curve of Paroxetine HCl and Etizolam had shown linearity over the concentration range of 25-150 µg/ml and 1-6 µg/ml, respectively, in all three methods. All the three methods were validated as per ICH guidelines and all the results were found within limits. By using ANOVA three methods were compared and it was found that F_{cal} is less than F_{tab} which indicates that there was no significant difference observed between the assays obtained by all three methods. All the three proposed methods can be used for its intended purpose because they are highly sensitive, accurate and precise.

Keywords: Paroxetine HCl; Etizolam; First order derivative; Absorbance correction; Dual wavelength; Validation.

1. Introduction

Chemically Paroxetine HCl (PAR) is known as (3S, 4R)-3-[(1, 3-benzodioxol-5-yloxymethyl]-4-(4-flurophenyl) piperidine hydrochloride. Pharmacological class of PAR is selective serotonin reuptake inhibitor and it is used as anti-depressant.[1] PAR is prescribed to treat various disorders such as depression, generalized anxiety disorder (GAD), panic disorders and post-traumatic stress disorder (PTSD).[2] Mechanism of action of PAR is that it inhibits the reuptake of serotonin.[3,4] Literature survey shows that less analytical methods were reported for quantification of PAR. Quantification of Paroxetine in biological fluids and finished products is done by using UV spectrometric method,[5] HPLC,[6-10] HPTLC[11] and UPLC.[12] Chemically Etizolam (ETZ) is known as 7-(2-chlorophenyl)-4-ethyl-13-methyl-3-thia-1,8,11,12-tetrazatricyclo [8.3.0.0] trideca-2(6),4,7,10,12-pentaene. It belongs to chemical class of thienotriazolodiazepines. It is used to treat anxiety.[13] Many analytical methods are reported for estimation of ETZ individually or in combination with other drug some of them are HPLC,[14] HPTLC,[15] LC-MS[16,17] and GC-MS.[18,19] However, for simultaneous estimation of PAR and ETZ in combined dosage form no UV spectrometric method is reported until now. So aim of the present work is to develop and validate three novel spectrophotometric methods for simultaneous estimation of PAR and ETZ in combined dosage form.

2. Materials

2.1. Instruments and apparatus

Absorption spectra of both the drugs were recorded by using Shimadzu UV-1800, Japan with Computer software: UV Probe 2.33. Quartz cell of path length 1 cm was used for estimation.

2.2. Reagents and materials

Paroxetine gift sample was provided by Zydus Cadila Healthcare pvt ltd, Dhabhasa, Vadodara, Gujarat. Etizolam gift sample was provided by Centaur Pharmaceuticals, Vakola, Mumbai, Maharashtra. Other reagents and glassware's were provided by Pioneer Pharmacy Degree College, Vadodara, Gujarat.

2.3. Selection of common solvent

Solubility of both the drugs was performed by using various solvents such as distilled water, methanol, ethanol and acetonitrile. It was found that both the drugs were soluble in methanol so it was selected as solvent.

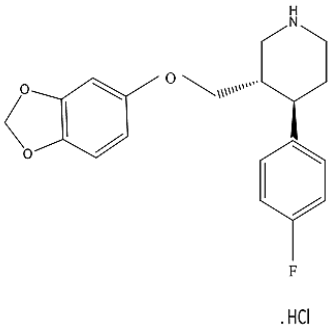

Fig. 1. Chemical structure of PAR

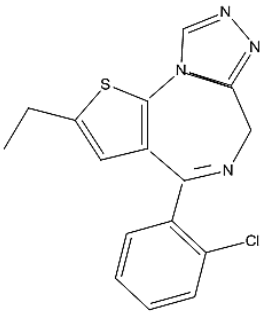

Fig. 2. Chemical structure of ETZ

2.4. Preparation of standard stock solution of PAR and ETZ

100 mg of PAR and ETZ were weighed accurately and transferred to separate 100 ml volumetric flask and diluted by filling the methanol upto mark. Concentration of the obtained primary stock solution was 1000 µg/ml. From the above solutions secondary stock solution of PAR and ETZ were obtained. Concentration of these solutions was 250 µg/ml and 100 µg/ml respectively. From the secondary stock solution various working solutions were prepared.

2.5. Selection of λ_{max} of PAR and ETZ

Working solutions of ETZ and PAR was scanned by using UV between the ranges of 200-400 nm. λ_{max} of PAR and ETZ was found to be 295 nm and 243 nm. Three methods were developed for simultaneous estimation of PAR and ETZ in combined dosage form. Three methods were[1] First order derivative[2] Absorbance correction method and[3] Dual wavelength method.

3. Methodology

3.1. Method 1: First order derivative spectrophotometric method

For first order derivative method, working solution of both the drugs was prepared. For PAR working solutions from 25-150 µg/ml was prepared by transferring the mentioned amount (1, 2, 3, 4, 5, 6 ml) of secondary stock solution to 10 ml volumetric flask and the dilution of this solution was done by filling the methanol up to the mark. In the same way ETZ working solutions from 1-6 µg/ml was prepared by transferring the mentioned amount (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 ml) of secondary stock solution to 10 ml volumetric flask and the dilution of this solution was done by filling the methanol up to the mark. Then zero order spectra of the above solutions were recorded. Then these spectra were derivatized to first order spectra. From overlain first order spectra of ETZ (3 µg/ml) and PAR (75 µg/ml) ZCP points of PAR and ETZ were obtained. Wavelength selected as the ZCP for ETZ was 241.50 nm where PAR gives the substantial absorbance while for PAR 256.50 nm wavelengths were selected as its ZCP where ETZ was giving substantial absorbance. From the derivatized spectra of mixtures estimation of PAR was done at 241.50 nm (ZCP of ETZ) and estimation of ETZ was done on 256.50 nm (ZCP of PAR). Then the calibration curve of both the drugs was obtained by plotting the graph between concentration Vs absorbance and the concentration of both the drugs was estimated.

3.2. Method 2: Absorbance correction method

In these method two wavelengths for one drug was selected from which one wavelength was λ_{max} of one drug at which the other drug will also give some substantial absorbance (λ_1). On other wavelength first drug will give no absorbance and another drug will give substantial absorbance (λ_2). Hence, this method is modification of simultaneous equation method. In this method, it was observed that PAR was giving substantial absorbance at 295 nm (λ_{max} of PAR) while ETZ was practically nil. Therefore, estimation of PAR can be done at 295 nm without interference of ETZ. At 243 nm ETZ, PAR and formulation was giving substantial absorbance. For estimation of ETZ absorbance of PAR was subtracted from formulation absorbance so that the absorbance of ETZ was obtained. Obtained absorbance of ETZ is known as corrected absorbance of ETZ. The concentration of ETZ was calculated from calibration curve at 243 nm by using

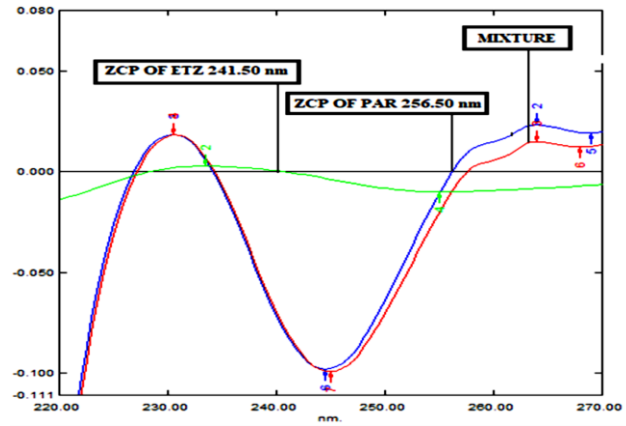

Fig. 3. Overlain first order derivative spectra of ETZ (3 µg/ml) and PAR for (75 µg/ml).

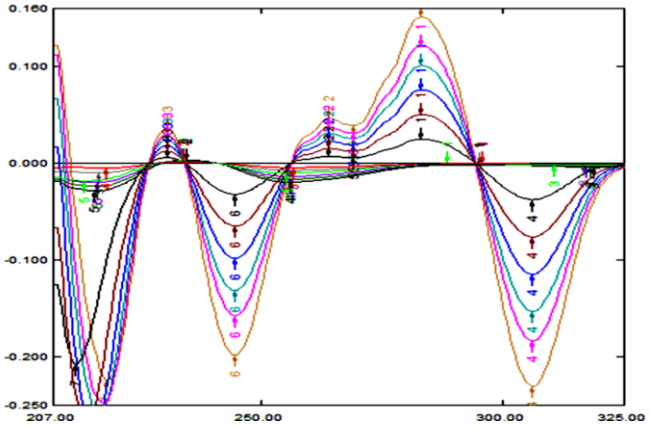

Fig. 4. Overlain first order derivative spectra of standard ETZ (1-6 µg/ml) and PAR (25-150 µg/ml).

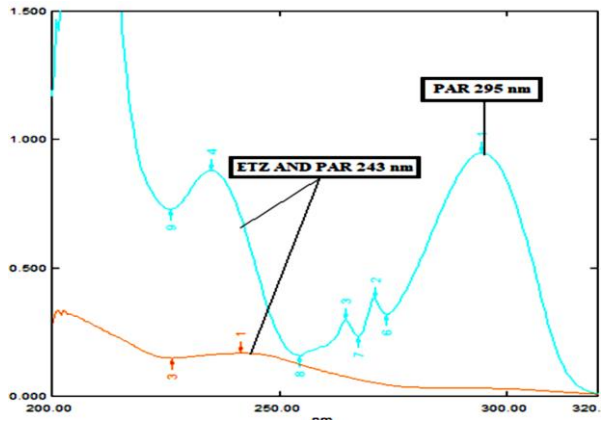

Fig. 5. Overlain spectra of ETZ (3 µg/ml) and PAR (75 µg/ml).

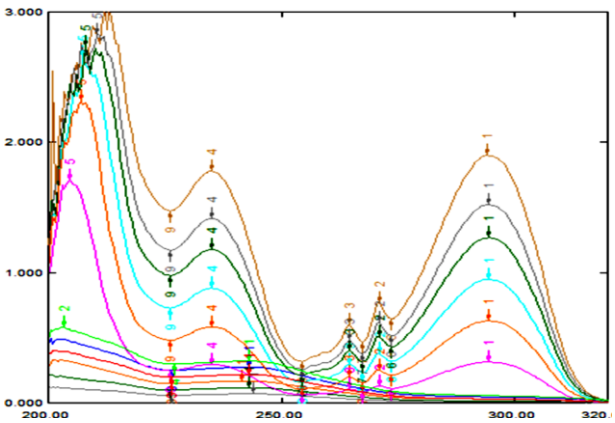

Fig. 6. Overlain spectra of ETZ (1-6 µg/ml) and PAR (25-150 µg/ml).

Table 1. Assay results for capsule using the proposed methods

Formulation	Proposed Methods	Label claim (mg/cap)		Amount found (mg/cap)		% Label claim Assay ± SD	
		PAR	ETZ	PAR	ETZ	PAR	ETZ
Capsule	METHOD 1	12.5	0.5	12.50	0.50	100.13 ± 0.0005	100.55 ± 0.0005
	METHOD 2	12.5	0.5	12.50	0.52	100 ± 0.0015	100 ± 0.0005
	METHOD 3	12.5	0.5	12.50	0.52	100.77 ± 0.0011	100.38 ± 0.0069

corrected absorbance.

Corrected absorbance =Total absorbance – interfering absorbance.

The concentration of two drugs(X and Y) in the mixture can be calculated using following equations:

$$Cy = A_2 / ay_2 \quad (1)$$

$$Cx = A_1\text{-}ay_1 * Cy/ax1 \quad (2)$$

Where, A_1 and A_2 are the absorbance of mixture at λ_1 and λ_2 respectively, ay_1 and ay_2 are absorptivity of y at λ_1 and λ_2 respectively, ax1 is absorptivity of X at λ_2, CX is concentration of X, CY is concentration of Y.

3.3. Method C: Dual wavelength method

The utility of dual wavelength data processing program is to calculate the unknown concentration of a component of interest present in a mixture containing both the components of interest and an unwanted interfering component by the mechanism of the absorbance difference between two points on the mixture spectra. This is directly proportional to the concentration of the component of interest, independent of the interfering components. The pre-requisite for dual wavelength method is the selection of two such wavelengths where the interfering component shows same absorbance whereas the component of interest shows significant difference in absorbance with concentration. Working solution of PAR and ETZ was scanned in UV between 200-400 nm. Overlain spectra of both the drugs were obtained from which two wavelengths for PAR and ETZ were selected. For ETZ two wavelengths that are 230 nm and 250 nm was selected. The absorbance difference of ETZ was zero but PAR and mixture has shown some significant absorbance difference at 230 nm and 250 nm. However, the difference obtained from absorbance of PAR and mixture at 230 nm and 250 nm was same. For PAR two wavelengths that are 221.50 nm and 288 nm was selected. The absorbance difference of PAR was zero but ETZ and mixture had shown some significant difference at 221.50 nm and 288 nm. However, the difference obtained from absorbance of ETZ and mixture at 221.50 nm and 288 nm was same. Hence, the estimation of ETZ was done by calculating the absorbance difference at 221.50 nm and 288 nm while estimation of PAR was done by calculating the absorbance difference at 230 nm and 250 nm.

3.4. Analysis of Par and Etz in Capsule

For estimation of both the drugs in the commercial formulations, twenty capsules were weighed and average weight was calculated. The powder equivalent to 12.5 mg Paroxetine HCl and 0.5 mg of Etizolam were transferred to 100 ml volumetric flask consisting of 30 ml methanol. Then methanol was filled up to the mark of volumetric flask. Concentrations obtained are 5 µg/ml (ETZ) and 125 µg/ml (PAR). These solutions were scanned according to the wavelength selected in different methods. In method 1 PAR solution is scanned at 241.50 nm and ETZ solution is scanned at 256.50 nm. In method 2 PAR solutions is scanned at 295 nm (λ max of PAR) and ETZ solution is scanned at 243 nm (λ_{max} of ETZ). In method 3 absorbance difference of PAR is calculated at 230 nm and 250 nm while ETZ is calculated at 221.50 nm and 288 nm. Absorbance obtained from three methods was put into their respective calibration curve equations and concentration is obtained. From this obtained concentration %label claim was found.

3.5. Validation Parameters

According to ICH guideline (Q2 R1), the above mentioned three methods (*Method 1: First order derivative spectrophotometric method, Method 2: Absorbance correction method and Method C: Dual wavelength method*) were validated

Table 2. Application of the standard addition technique to the analysis of PAR and ETZ in capsule by the proposed methods

Method	Drugs	Level	Conc. present (µg/ml)	Spiked Conc. (µg/ml)	Total Conc. taken (µg/ml)	Mean of total Conc. found (µg/ml)	Amt recovered (µg/ml)	%Recovery ± SD	%RSD
Method 1	PAR	50%	50	25	75	74.94	24.85	99.41 ± 0.3958	0.39
		100%		50	100	99.74	49.65	99.30 ± 0.3428	0.34
		150%		75	125	124.36	74.27	99.03 ± 0.1319	0.13
	ETZ	50%	2	1	3	2.98	0.99	99.33 ± 0.4792	0.48
		100%		2	4	3.99	1.98	99.47 ± 0.2396	0.24
		150%		3	5	4.98	2.99	99.70 ± 0.2767	0.27
Method 2	PAR	50%	50	25	75	74.98	24.98	99.94 ± 0.1855	0.18
		100%		50	100	99.98	49.86	99.73 ± 0.1606	0.16
		150%		75	125	124.96	74.37	99.16 ± 0.6537	0.10
	ETZ	50%	2	1	3	2.99	0.99	99.36 ± 1.1322	1.13
		100%		2	4	3.98	1.98	99.01 ± 0.5661	0.56
		150%		3	5	4.98	2.97	99.14 ± 0.6537	0.65
Method 3	PAR	50%	50	25	75	74.78	24.66	98.65 ± 0.3780	0.38
		100%		50	100	99.98	49.98	99.93 ± 0.3273	0.32
		150%		75	125	124.60	74.48	99.30 ± 0.2182	0.21
	ETZ	50%	2	1	3	2.97	0.98	99.44 ± 0.4036	0.40
		100%		2	4	3.98	1.98	99.44 ± 0.6728	0.67
		150%		3	5	4.97	2.98	99.44 ± 0.7769	0.78

Table 3. Summary of validation parameters by developed methods

Parameters	First order derivative method		Absorbance correction method		Dual wavelength method	
	PAR	ETZ	PAR	ETZ	PAR	ETZ
Working wavelength (nm)	241.50	256.50	295	243	Abs diff at 230 & 250.	Abs diff at 221.50 & 288.
Concentration range (µg/ml)	25-150	1-6	25-150	1-6	25-150	1-6
Regression équation	Y = 0.005x – 0.005	Y = 0.120x + 0.054	Y = 0.012x + 0.007	Y = 0.051x + 0.016	Y = 0.006x – 0.007	Y = 0.042 + 0.010
Slope	0.005	0.120	0.0120	0.0509	0.006	0.042
Intercept	0.0058	0.0016	0.049	0.0017	0.0010	0.0009
Correlation coefficient (r^2)	0.999	0.998	0.998	0.999	0.999	0.998
LOD (µg/ml)	1.00	0.07	1.30	0.11	1.00	0.07
LOQ (µg/ml)	3.03	0.22	3.95	0.33	3.03	0.22
			Precision (% RSD)			
Repeatability (n=6)	0.17	0.13	0.24	1.13	0.31	0.78
Intraday (n=3)	0.13-0.26	0.10-0.28	0.12-0.24	0.34-1.30	0.09-0.22	0.54-0.75
Interday (n=3)	0.09-0.20	0.10-0.19	0.10-0.33	0.46-0.92	0.09-0.19	0.31-0.62
% Label claim Assay ± SD (n=6)	100.13 ± 0.0005	100.55 ± 0.0005	100 ± 0.0005	100 ± 0.0015	100.77 ± 0.0011	100.38 ± 0.0069

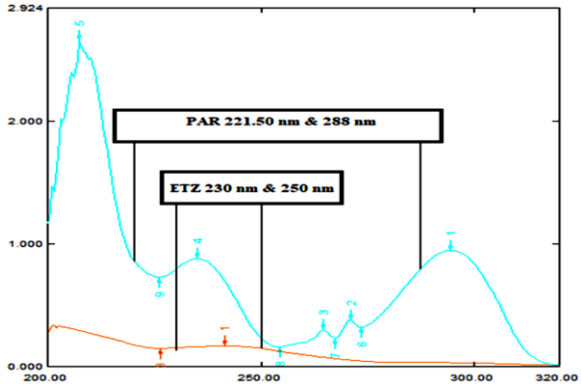

Fig. 7. Overlain spectra of ETZ (3 µg/ml) and PAR (75 µg/ml).

3.6. Accuracy.

By using standard addition method interference of the excipients was checked by calculating the %recovery of drug. In this method standard solution of PAR and ETZ were added to sample solution and the standard drug recovered was calculated in terms of mean recovery with upper and lower limits with its %RSD.

3.7. Precision/Repeatability.

By keeping the parameter of proposed methods constant solutions of PAR and ETZ was scanned (n=6) and absorbance were recorded.

3.8. Intermediate precision.

In this intraday and interday precision is measured. Three concentrations of PAR and ETZ was scanned on the thrice a day for intraday and for interday same concentrations was scanned on three different days. The results of intraday and interday precision were calculated in terms of %RSD.

3.9. Limit of detection (LOD) and Limit of Quantification (LOQ).

By using 3 s/m and 10 s/m LOD and LOQ was calculated respectively where, S is the standard deviation of intercept (n=6) of the sample and m is the slope of the corresponding calibration curve.

Table 4. One way ANOVA for PAR

Source of variation	Sum of Squares	Degree of freedom	Mean of Square	F_{cal}	P-Value	F_{tab}
Between Groups	0.709433	2	0.354717	0.819558	0.459417	3.68232
Within Groups	6.492217	15	0.432814	-	-	-
Total	7.20165	17	-	-	-	-

Table 5. One way ANOVA for ETZ

Source of variation	Sum of Squares	Degree of freedom	Mean of Square	F_{cal}	P-Value	F_{tab}
Between Groups	1.362144	2	0.681072	2.506256	0.115059	3.68232
Within Groups	4.076233	15	0.271749	-	-	-
Total	5.438378	17	-	-	-	-

3.10. Anova

This statistical tool is used to check the variation between the three developed methods used for the simultaneous estimation of PAR and ETZ in combined dosage form.

4. Results and Discussions

Method A: First order derivative spectrophotometric method

First order spectra show more resolution than zero order spectra in terms of zero crossing points. Fig. 3 and 4 shows the overlain first order spectra of PAR and ETZ respectively. At 241.50 nm ETZ has zero crossing point and PAR was estimated. At 256.50 nm PAR has zero crossing point and ETZ was estimated.

Method B: Absorbance correction method

Fig. 5. and 6 shows the overlain spectra of PAR and ETZ respectively. At 243 nm ETZ and PAR gives absorbance while at 295 nm ETZ becomes zero. Hence, these two wavelengths were selected. By using equation 1 and 2 estimation of PAR and ETZ in sample solution was done.

Method C: Dual wavelength method

Fig. 7. shows the four selected wavelengths two for each drug where the drugs showed zero absorbance difference and their overlain spectra. Hence these wavelengths were used each other drugs by preparing calibration curves of absorbance difference for each drug. Results of assay, accuracy and summary of validation parameters of various methods was shown in table 1, table 2 and table 3 respectively.

Statistical Comparison of the Results of the Developed Three Methods

By using one way ANOVA variation between the three developed methods were checked and no significant variation was observed because F_{cal} is less than F_{tab}. Results of one way ANOVA are shown in table 4 and 5.

5. Conclusions

Three UV methods (First order derivative, Absorbance correction and Dual wavelength) were developed for the simultaneous estimation of PAR and ETZ in Capsule without prior separation. During estimation of both the drugs from the formulation another excipients present in the formulation had not shown any interference. Developed methods were also successfully applied to formulation. Result of all the validation parameters were found within limits. Comparison of three methods was done by using ANOVA and there is no significance difference found between these methods. These methods are simple, accurate, precise and cost effective so it can be used for routine analysis of PAR and ETZ in combined dosage form.

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Conflicts of Interest

The authors declare no conflict of interest.

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