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Research Article

DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF FLUNITRAZEPAM IN BULK AND THEIR FORMULATIONS BY UV-VISIBLE SPECTROPHOTOMETRY

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ABSTRACT

UV-Visible spectrophotometric method has been developed and subsequently validated for estimation of Flunitrazepam in tablet dosage form. The proposed method utilized a Shimadzu double beam spectrophotometer using 1.0 cm quartz cells and all determinations were made at a wavelength of 230 nm in a solvent system of methanol: water (1:1). The method was validated for determining linearity, limit of detection and quantitation, accuracy, precision and specificity of this analytical method as per the International Organization for Standardization guidelines (ICH). The drug substance and the drug product were exposed to thermal, photolytic, hydrolytic, and oxidative stress conditions, and the stressed samples were analyzed by the proposed method to demonstrate the specificity of the method. The proposed method was found to be linear in concentration ranges from 3.085-15.380 µg/ml with the linear correlation coefficient of R2=0.999 and the mean recoveries were 98.25% to 99.67%. Although the degradation products of stressed condition had not been identified, the method had been able to detect the changes due to stress condition. The stated method can be used as stability indicating method with high degree of linearity, accuracy and precision for assay of Flunitrazepam in routine pharmaceutical analysis of tablets.

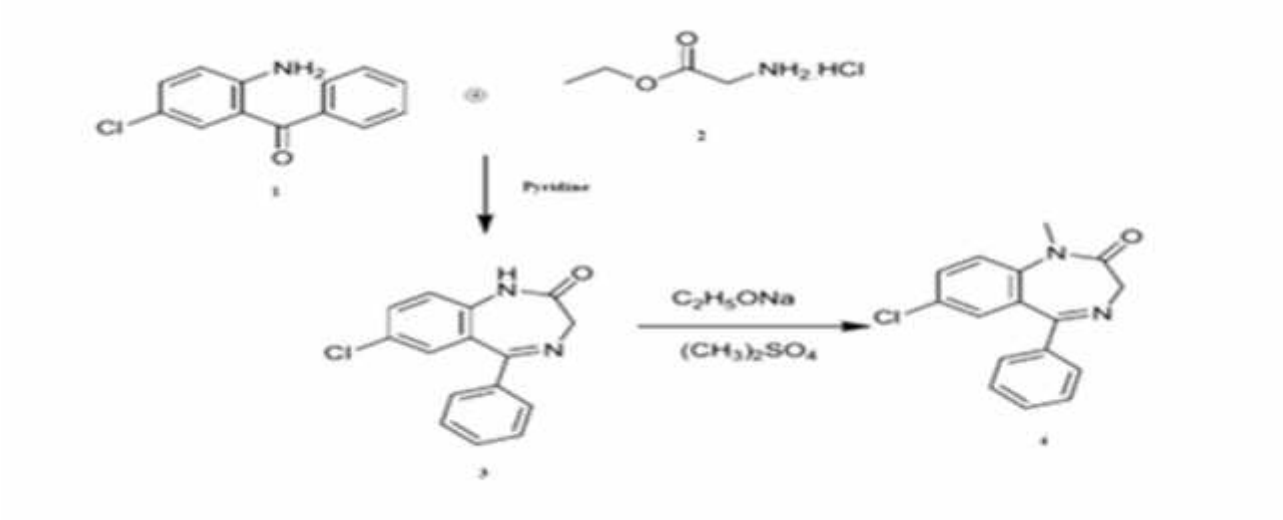
KEY WORDS: Validation; forced degradation, linearity, specificity, UV-Vis spectrophotometer.

INTRODUCTION

Method validation may be regarded as one of the most accustomed areas in analytical chemistry. The International Organization for Standardization (ISO) defines validation as the confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled [1]. This enunciation primarily implies that a detailed examination has been carried out and gives evidence that the utility of the analytical method with a high degree of accuracy consequently produces results that are fit for purpose [1]. The use of validated methods is exigent for an analytical laboratory to parade its qualification and proficiency [2]. Stability testing forms a weighty part of the process of the pharmaceutical product development. The motive of stability testing is to provide testimony on how the quality of a drug substance or drug product varies with time under the influence of various environmental factors. Stability testing by forced degradation involves degradation of drug products and drug substances under the influence of

more severe than accelerated conditions such as hydrolysis, temperature, humidity, and light[3,4]. Sense of the stability of molecule helps in selecting proper formulation and package as well as helps in providing proper storage conditions and shelf life, which is fundamental for regulatory documentation [4]. This work deals with development and validation of a new stability indicating spectrophotometric method for the routine appraisement of Flunitrazepam in bulk and tablet dosage form to make the assay test affordable and pecuniary. [6–10] Flunitrazepam ($C_{16}H_{13}ClN_2O$) is a sedative and hypnotic drug of benzodiazepine-2-one group [5-7]. Basically, it is highly lipophilic and rapidly absorbed after oral administration [8]. Reaction between 2-amino -5-chlorobenzophenone and glycine ethyl ester in pyridine leads to 7-chloro-1,3 dihydro-5-phenyl-2H-1, 4-benzodiazepine-2-one, subsequently which is methylated with methyl sulfate in the presence of sodium ethoxide gives Flunitrazepam (Scheme 1) [9]. It is significant in the symptomatic relief of tension and anxiety [6–10].

UV-Vis spectrophotometry is one of the most frequently employed techniques in pharmaceutical analysis which is simple, rapid, specific, precise, accurate and applicable to small quantities of compounds. The main principle of UV-Vis spectrophotometry is the measurement of the amount of ultraviolet or visible radiation absorbed by a substance in solution. Beer-Lambert law is the fundamental law that governs the quantitative spectrophotometric analysis which states that the intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules as it passes through a medium of homogeneous thickness [11].



Scheme 1: Synthesis of Flunitrazepam [9]

1: 2-amino-5-chlorobenzophenone; 2: glycine ethyl ester; 3: 7-chloro-1,3 dihydro-5-phenyl 2H-1, 4-benzodiazepin-2-one; 4: Flunitrazepam

MATERIALS AND METHODS

UV-Vis spectra were recorded on a Specord 250 plus PC double beam spectrophotometer using 1.0 cm quartz cells. All weighing of ingredients were done on electronic balance (A&D Company Ltd, USA). Ultra sonicator (Spinco Biotech Pvt. Ltd, India) was used to aid dissolution. Glassware used in each procedure were bleached entirely with detergent and rinsed thoroughly with double distilled water and dried in hot air oven. Pure drug samples of Flunitrazepam standard were obtained from Emcure Pharmaceuticals Ltd, Pune, India. All other chemicals and reagents used were of analytical grade. All other chemicals were purchased from Merck Fine Chemicals Ltd, Mumbai, India.

Selection and standardization of the solvent: In preliminary trial, five different compositions of solvent were selected e.g. distilled water, methanol, methanol: distilled water (1:1), 0.1 N HCl and phosphate buffer (pH=7.4). Methanol: distilled water (1:1) was selected as the appropriate media for ease of sample preparation, solubility of the drug and cost of the solvent. The wavelength maximum of Flunitrazepam was found to be 230 nm. Stock solution of Flunitrazepam standard was prepared to get an approximate concentration of 5 µg/ml and sonicated for 4 min in bath sonicator.

Linearity: Response function was determined by preparing standard solution at thirteen different concentration levels ranging from 3.085-15.380 µg/ml in UV-Vis spectrophotometric method. The limit of detection (LOD) and limit of quantitation (LOQ) were separately determined based on standard deviation of the Y-intercept and the slope of the calibration curve as per ICH guidelines [12]. LOD=3.3 (standard deviation of Y-intercept/slope of the curve) and LOQ=10 (standard deviation of Y-intercept/slope of the curve).

Accuracy: Accuracy of the method was determined by performing the recovery experiment. This experiment was performed at five levels equivalent of 80% to 120% of nominal concentration. Three replicate samples of each concentration level were prepared and the percentage recovery at each level (n=15) were determined.

Precision: The precision of the instrument, relative standard deviation (RSD) was checked by repeated scanning of samples (n=6) for Flunitrazepam standard without changing the parameter of the proposed spectrophotometric method. Intra-assay and inter-assay precision (RSD) data were obtained by repeatedly analyzing, in one laboratory on one day and several days within a week, aliquots of a homogeneous sample, respectively.

Assay: The assay of four marketed brands (F1, F2, F3 and F4) was carried out with this proposed method at 230 nm.

Specificity: Specificity detects quantitatively the analyte in presence of component that may be expected to be present in the sample matrix. Commonly used excipients, such as purified talc, magnesium stearate, Povidone K-30 and lactose were mixed at appropriate amount and dissolved in the solvent system in amount equivalent to the weight of excipient present in portion of sample preparation as per the assay method. At first different excipient concentration level (80-120% of nominal concentration present in assay preparation) were spiked with nominal concentration of drug substance followed by nominal concentration of excipient at different concentration level of drug substance (80-120% of nominal concentration present in assay preparation) and then absorbance was measured and calculation done to determine quantity of drugs.

The responses of the standard Flunitrazepam, marketed product and excipient of stressed condition were also compared with the response of the same solution of unstressed condition to establish the stability indicating nature of the developed UV-Vis method as part of the forced degradation studies.

Forced degradation study: Here, the forced degradation of each drug substance and the drug product was carried out under neutral/acid/base hydrolytic, oxidative, photolytic and thermolytic stress conditions [13]. Only thermal degradation of drug substance and drug product was carried out in solid state. Solutions were prepared by dissolving drug substance or drug product in distilled water, aqueous HCl/NaOH/H2O2 solution, or solvent to obtain a concentration of 50 µg/ml and later diluted with the solvent of methanol: distilled water (1:1) to achieve an approximate concentration of 5 µg/ml. Finally, a spectral scan ranges from 200-400 nm in UV-Vis spectrophotometer was performed to take absorbance at different days for observing degradation. Neutral hydrolysis of drug substance and drug product in solution state was conducted with distilled water at room temperature and 80° temperature for 10 d. Acid hydrolysis of drug substance and drug product in solution state was

directed with 0.1 N HCl at room temperature and 60° temperature for 8 d. Base hydrolysis of drug substance and drug product in solution state was guided by 0.1 N NaOH solution at room temperature and 60° temperature for 3 d. For oxidative stress, sample solutions of drug substance and drug product in 3% H2O2 were kept at room temperature and 60° temperature for 8 d. Drug substance and drug product observed for photolytic stress were bestowed at light and dark for 15 d. For thermal stress, solid samples of drug substance and drug product were entrusted in a controlled-temperature oven at room temperature and 60° temperature for 15 d. A preparation of placebo was applied to all stressed condition.

RESULTS AND DISCUSSION

The described method has been validated for response function, accuracy, repeatability and intermediate precision. The nominal concentration of standard solutions was approximately 5 µg/ml. The results of UV analysis have been shown in Tables 1-4. The proposed method was found to be linear with a linear correlation coefficient of 0.999 and the linear regression equation y=0.125x+0.006 (Table 1 and fig. 1). The minimum concentration levels at which Flunitrazepam can be reliably detected (LOD) and quantified (LOQ) were found to be 0.155 µg/ml and 0.380 µg/ml, respectively (Table 1).

TABLE 1: LINEARITY, LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTATION (LOQ)

λmax (nm)	Regression equation (y=mx+c)	Linearity range (µg/ml)	Slope	SD	Correlation coefficient	LOD (µg/ml)	LOQ (µg/ml)
230	0.130x+0.009	3.08 to 15.3	0.126	0.005	0.999	0.155	0.380

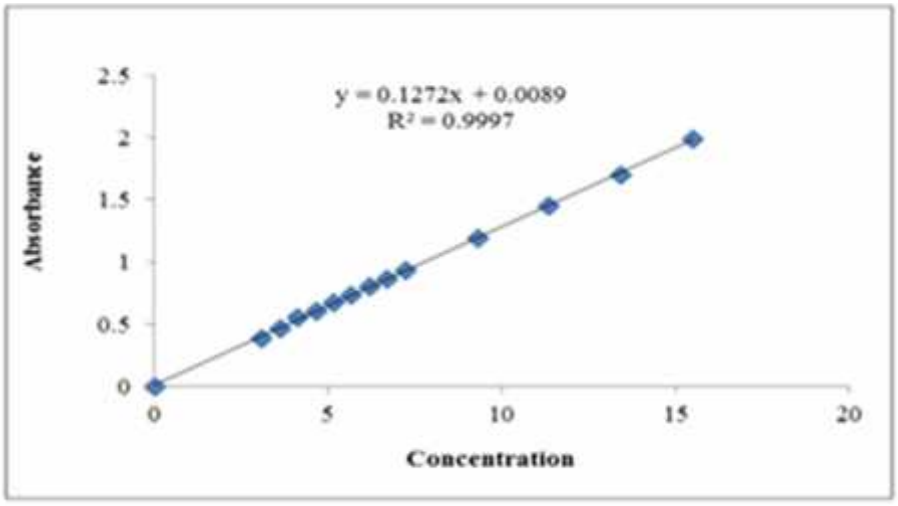


Fig. 1: Calibration curve of linearity Study

The mean recoveries were 98.25-99.67% substantiated the method as accurate (Table 2).

TABLE 2: ACCURACY STUDY

S. no.	% Recovery level	% Recovery mean±SD
1	80%	98.25
2	90%	99.38
3	100%	99.65
4	110%	99.12
5	120%	99.67

The repeatability and intraday assay precision (RSD) was <1% and the interday assay precision was <2% revealed the proposed method as precise (Table 3).

TABLE 3: PRECISION STUDY

Sample No.	% Assay (repeatability)	Intra-day assay			Inter-day assay			
		1st h	3rd h	8th h	1st day	3rd day	5th day	7th Day
1	101.82	99.17	100.66	99.92	100.50	101.87	99.34	101.59
2	101.88	99.33	100.52	99.90	97.00	100.96	100.71	101.02
3	101.84	99.42	100.39	100.21	96.88	97.10	99.96	100.68
4	101.71	99.55	100.21	99.58	98.94	101.15	101.09	99.02
5	101.85	99.55	100.55	99.60	100.29	100.24	99.20	102.16
6	101.33	99.51	100.55	99.70	100.22	100.50	100.24	99.67
Mean±SD	101.74±0.21	99.42±0.15	100.48±0.16	99.82±0.24	98.97±1.68	100.30±1.66	100.09±0.74	100.69±1.17
%RSD	0.21							

Potency assay tests of four different brands of Flunitrazepam were performed by the proposed method. According to USP 29, Flunitrazepam tablets must contain 95-105% of the labeled amount of drug. All the brand products met the standard criteria with the new analytical method (Table 4).

TABLE 4: DRUG CONTENT

Sample No.	mg/tablet	Percentage	Sample No	mg/tablet	Percentage
F1	4.91	98.0%	F3	5.03	99.8%
F2	4.98	99.4%	F4	4.85	100.2%

Specificity is the ability of the method to measure the analytic response accurately in the presence of all potential components. Specificity of the proposed method is also confirmed by the stress study of the sample. The study was performed to validate stability indicating capability of the developed method and to identify the key factors which will impact the stability of the drug product. The

specificity was determined according to ICH Guidelines by subjecting a sample solution and solid to accelerated degradation by acidic/alkaline/neutral hydrolytic, oxidative, photolytic, and thermal stress conditions to evaluate the interference of degradation products in the quantitation of Flunitrazepam. The reported method did not provide data on specificity for their estimation in the presence of formulation excipients, degrades or impurities. The absorbance obtained with the mixture of the excipients showed no interference with the absorbance of standard (Table 5 and fig. 2.).

TABLE 5: SPECIFICITY STUDY

Fixed drug substance spiked with different concentration of excipient			**Fixed excipient spiked with different concentration of drug substance**		
Nominal drug substance concentration (µg/ml)	**Excipient concentration (µg/ml)**	**Absorbance**	**Nominal excipient concentration (µg/ml)**	**Drug substance concentration (µg/ml)**	**Absorbance**
5.160	101.41 113.10 125.8 140.48 151.16	0.6711 0.6798 0.6780 0.6772 0.6863	126.8	4.125 4.643 5.159 5.675 6.190	0.5419 0.6095 0.676 0.7405 0.8009
Regression equation, y=0.0002x+0.6515 RSD=0.804%			Regression equation, y=0.1254x+0.0268 R^2=0.9995		

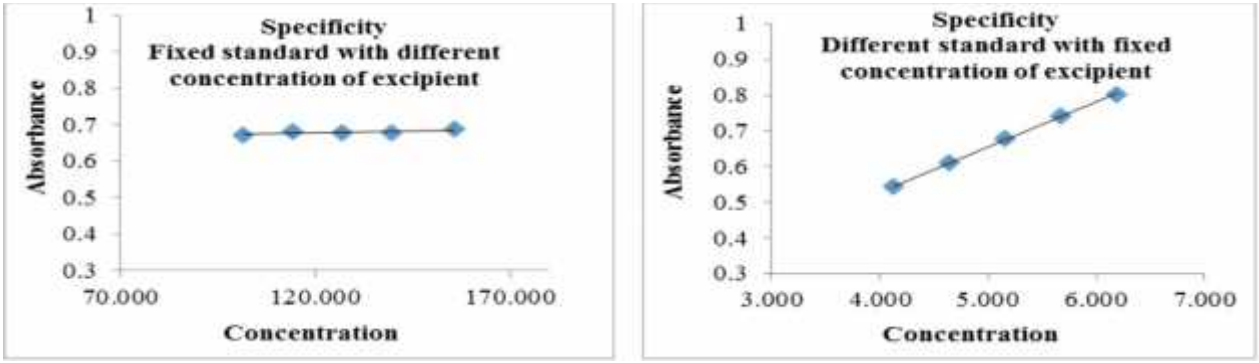


Fig. 2: Specificity study

The percentage assays of Flunitrazepam standard and marketed product under stressed condition at different observation days are listed at Table 6.

TABLE 6: FORCED DEGRADATION STUDY

Degradation type	Experimental conditions	Maximum degradation (d)	% Assay of standard	% Assay of Product
Hydrolysis	Neutral hydrolysis at RT*	$_{10}$th	90.85	103.47
	Neutral hydrolysis at 80° C	$_{10}$th	129.89	100.88
	Acid hydrolysis at RT*	$_{10}$th	90.57	93.25
	Acid hydrolysis at 60° C	$_{5}$th	92.65	82.28
		$_{8}$th	110.89	141.20
	Base hydrolysis at RT*	$_{3}$rd	60.65	67.92
	Base hydrolysis at 60° C	$_{1}$st	35.78	71.65
Oxidation	3% H2O2 at RT*	$_{8}$th	79.47	99.69
	3% H2O2 at 60° C	$_{8}$th	105.25	107.37
Photolytic (Solution state)	Dark	15th	107.55	105.78
	Light	15th	101.46	141.35
Thermal (Solid state)	RT*	15th	102.35	99.28
	60° C	15th	110.65	103.43

*RT: Room Temperature

To develop the stability indicating method, the UV spectrums of Flunitrazepam under several stressed condition were studied on UV-Vis spectrophotometer (fig. 3 and 4). When Flunitrazepam standard was subjected to neutral hydrolysis at room temperature, some minor degradants was generated (fig. 3A). On the other hand, neutral hydrolysis at 60° gave a result of highly increased value with 135.93% in the assay (fig. 3B). There was no notable degradation for neutral hydrolysis of marketed product (fig. 3A and 3B). Again, a minor degradation was remarked for acid hydrolysis of both Flunitrazepam standard and marketed product when reacted with 0.1 N HCl at room temperature (fig. 3C). However, after 5 days of observation, approximately 16% of Flunitrazepam standard and 17% of marketed product were found to be degraded due to acid hydrolysis at elevated temperature of 60°. Subsequently, after 7 d of observation the amount of Flunitrazepam and marketed product were highly increased with 112.93% and 142.35%, respectively in the assay (fig. 3D). A major degradation was observed for base hydrolysis. Here, after 3rd day more than 20% of Flunitrazepam was degraded when reacted with 0.1 N NaOH at room temperature (fig. 3E). Nevertheless, after 1st day of observation more than 20% of standard Flunitrazepam and marketed products were degraded because of base hydrolysis at elevated temperature of 60 ° (fig. 3F). Only 20% of Flunitrazepam standard was degraded due to oxidation at room temperature (fig. 3G). During photolytic stress in light and dark, at 15th day the amount of marketed product kept in light was increased by 142.28% (fig. 4J). After 15th day of observation the % assay of Flunitrazepam standard under thermal stressed condition at room temperature (fig. 4K) and 60° temperature (fig. 4L) were 102.2% and 111.72%, respectively. Hence, a brief thermal stress did not generate any degradants for marketed product (fig. 4K and 4L). Flunitrazepam has shown a broad UV-Vis absorption profile at 230 nm (fig. 3). Some data showed a high increased value of drug product and substance. Such as, neutral hydrolysis on Flunitrazepam standard at 60°, acid hydrolysis at 60°, photolytic stress on marketed product at light and thermal stress on Flunitrazepam standard at 60°. These may occur due to formation of other side/degraded products which may have similar absorption profile as Flunitrazepam. Acid hydrolysis of Flunitrazepam gives rise 2,4-dichloro-10-methylacridin-9-(10H)-one and 2,4-dichloroacridin-9-(10-H)-one[14]. Again, photolysis of Flunitrazepam gives rise nor Flunitrazepam, FNZ 3, FNZ 4, FNZ 6, FNZ 7 etc. [15].

The absorbance pattern of Flunitrazepam was also changed with time and temperature. These all factors resulted in an overall effect on the assay of Flunitrazepam content in standard as well as marketed product. Although the degradation products had not been identified, the method had been able to detect the

changes due to stress condition. Absorbance of excipient increased at approximately 230 nm to 270 nm during basic hydrolysis which increases at 60° (fig. 3F). The study also demonstrated that the excipient has no major effect in other stressed conditions on Flunitrazepam standard and marketed product (fig. 3 and 4).

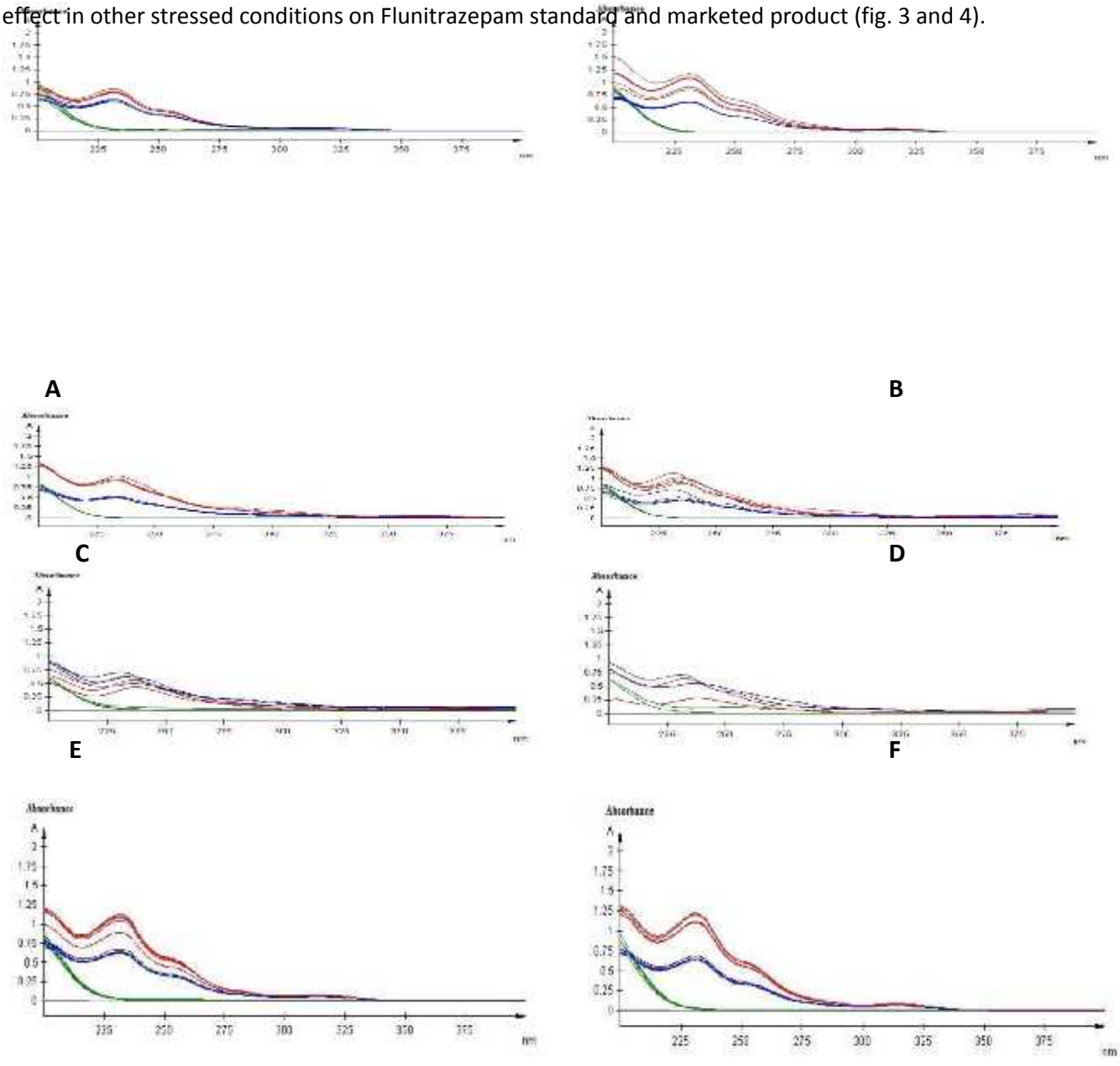
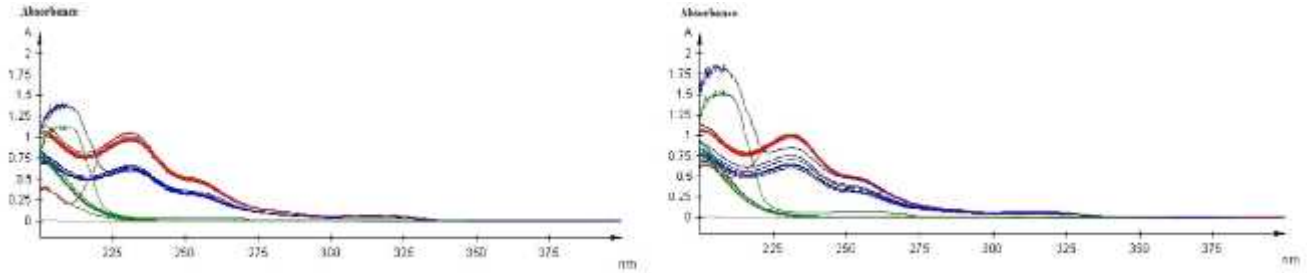
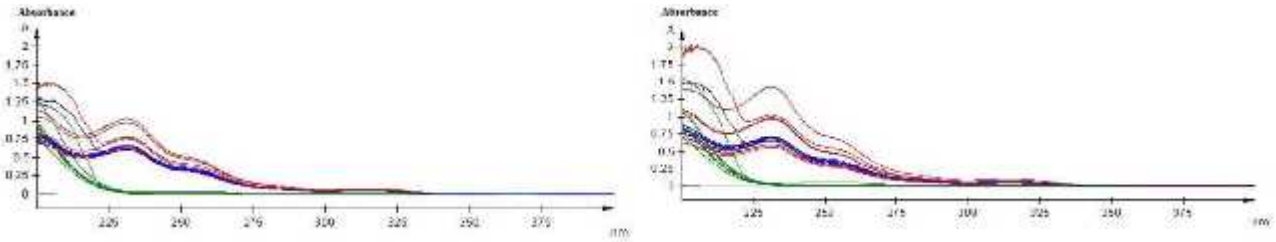


Fig. 3: Overlay UV spectrum at different pH and oxidative stress effect on Flunitrazepam.





K **L**

Fig. 4: Overlay UV spectrum

The proposed UV-Vis spectrophotometric method was validated successfully in accordance to ICH Q2B guideline. Based on the UV spectral results attained from the analysis of forced degraded samples using the proposed method, it can be concluded that the method is specific for determination of Flunitrazepam in presence of degrades. The method has linear response in stated range with a lower value of LOD and LOQ and is found to be accurate and precise. The stated method can be used as stability indicating method for assay of Flunitrazepam in tablet dosage form.

REFERENCES

1. Pedro A. Key aspects of analytical method validation and linearity evaluation. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(23):2224-4.
2. Isabel T, Marc DL, Erik VB. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends Anal Chem 2004; 23:535-52.
3. Raghu N, Udhav NK, Murlidhar SS. Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product. J Pharma Biomed Anal 2005; 39:147-55.
4. Blessy M, Ruchi DP, Prajesh NP. Development of forced degradation and stability indicating studies of drugs-A review. J Pharma Anal 2014; 4:159-65.
5. US Pharmacopoeia 29-NF24. Vol. 30(1); 2006. p. 673.
6. Laurence LB, John SL, Keith LP. Goodman & Gilman's: The Pharmacological Basis of Therapeutics. 11th ed. New York: Pergamon Press; 2006. p. 402-5.
7. Bertram GK, Susan BM, Anthony JT. Basic & Clinical Pharmacology. 12th ed. New York: Lange Medical Publication; 2012. p. 373-386.
8. John MBJ, John HB. Wilson and Gisvold's Text book of Organic Medicinal and Pharmaceutical Chemistry. 12th ed. Lippincott Williams & Wilkins Publication; 2011. p. 443-7.
9. Andrejus K. Essentials of Medicinal Chemistry. 2nd ed. A Wiley-Interscience Publication; 1998. p. 288.
10. Roland B, Erwin S. Benzodiazepines affect channel opening of GABAA receptors induced by either agonist binding site. Mol Pharma 2005; 67:1005-8.
11. Siladitya B, Subhajit G, Fahad A, Saayak S and Sritoma B. UV-Visible spectrophotometric method development and validation of assay of paracetamol tablet formulation. Anal Bioanal Techniques 2012; 3:1-6.
12. ICH Q2B Validation of Analytical Procedures-Methodology. Consensus Guidelines, ICH Harmonized Tripartite Guidelines 1996.
13. ICH Q1A (R2) Guideline: Stability Testing of New Drug Substances and Products, Comments for its Application, 2003.
14. Gallardo Cabrera C, Goldberg de Waisbaum R, Sbarbati Nudelman N. Kinetic and mechanistic studies on the hydrolysis and photodegradation of Flunitrazepam and alprazolam. J Phys Org Chem 2005; 18:156-61.
15. Jakimska A, Śliwka Kaszyńska M, Nagórski P, Kot Wasik A, Namieśnik J. Environmental fate of two psychiatric drugs, Flunitrazepam and sertraline: phototransformation and investigation of their photoproducts in natural waters. J Chromatogr Sep Tech 2014; 5:253.