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Validation of HPLC Method Used For the Estimation of Degradation Products of Oxazepam

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Abstract

A HPLC method was validated which shows good separation for Oxazepam, its impurities and degradation products The drug substance, its impurities and degradation products were found well separated with gradient conditions having run time of 60 mins by using Zorbax Extended C-18 column from Agilent (250 x 4.6 mm, 5µ). The flow rate was kept 1.0 mL.min^{-1}. The gradient mobile phase consisted of A= 0.02M di-potassium hydrogen phosphate pH 10.5 and B= Acetonitrile (100%). Detection was performed at 235 nm using PDA detector. The method was validated for Specificity, LOD, LOQ, Linearity, Precision and Accuracy as per ICH guidelines [1, 2]. The stability indicating capability of the method was established by performing forced degradation study. The method was found to be reliable for its intended purpose.

Keywords:

Oxazepam, HPLC, Validation, degradation products

1. Introduction

Oxazepam is the active metabolite from the wide range of 1,4-benzodiazepines and it is used particularly for the treatment of anxiety, insomnia, agitation and tension[3]. The chemical structure of Oxazepam is shown below in Fig 1. It is almost white crystalline powder which is practically insoluble in water and slightly soluble in ethanol (96 percent).

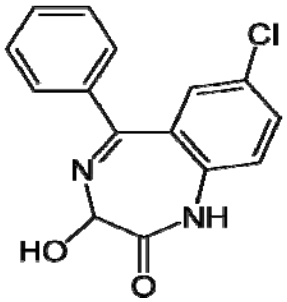

Fig 1. Chemical structure of Oxazepam

The quality of this type of compound is controlled by various tests given in official compendia such as European pharmacopoeia [4] and British Pharmacopoeia [5]. The Related substance method by HPLC in British Pharmacopoeia [5] was found to be suitable for separating all the impurities and degradation products formed during the stability testing,

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hence the same was used in the analysis of Oxazepam and validated it for specificity, Linearity, LOD, LOQ, Accuracy, Precision and Robustness [1,6]. The stability testing provides the evidence of the effect of the environmental condition such as acidic, basic, oxidation, heat and light on the quality of the drug substance and drug product. Nowadays the various drug authorities in the pharmaceuticals are requesting for the determination of the degradation products which are more difficult task. A validation of the method as per ICH guidelines [1,2] is demanded if slight changes are made in the process parameter to achieve the yield or even if the make of the reagent is changed from the original.

2. Experimental

2.1. Instrumentation

Agilent 1100 series HPLC system (USA) consisted of quaternary pump, an automatic injector, a PDA detector and a column oven. The system control, data collection and processing was done by Agilent ChemStation^{TM} software. The weighing was done on Mettler Toledo AB204-S balance (Switzerland) and pH was adjusted using pH meter from Thermo Orion (USA).

2.2. Solvents and Chemicals

Standards of Oxazepam and its impurities were kindly gifted by a supplier along with the declared purity of 99.9% and used as received. Di-potassium hydrogen phosphate (AR grade) and Sodium hydroxide (AR grade) was obtained from Rankem, Acetonitrile (Gradient grade) was obtained from Merck (Darmstadt, Germany) and water was purified with a Elga system from Labindia (Mumbai, India).

2.3. Chromatographic conditions

The column used was Zorbax Extended C-18 column from Agilent (250 x 4.6 mm, 5µ). It has provided baseline separation for the active, its impurities and all the degradation products with gradient conditions given below in the Table 1, at pH 10.5 of the Buffer with a run time of 60 min. The mobile phase consisted of Buffer A and Acetonitrile B, being buffer 0.02M K2HPO4 brought to pH 10.5 with 1N NaOH The oven temperature was kept 25°C, flow rate was kept 1.0 mL.min^{-1} and UV detection was performed at 235 nm.

Table 1: Gradient program

Time (min)	Buffer pH 10.5 (percent v/v)	Acetonitrile (percent v/v)
0	75	25
4	75	25
34	25	75
45	25	75
50	75	25
60	75	25

2.4. Test solution

Test solution was prepared by weighing 40 mg of the substance in a 50 mL of volumetric flask, dissolving and diluting upto the mark with diluent (800 ppm).

2.5. Reference and System suitability solution preparation

In all cases the diluent used for Reference standard and system suitability solution preparation was the mixture of water – acetonitrile, in the ratio 50:50(v/v). The reference solution (a) (diluted) of Oxazepam and all impurities solution was prepared by weighing each

about 40 mg in a separate 50mL standard volumetric flask, dissolving and diluting upto the mark with diluents. Taking 1 mL of each of this solution in a 100 mL volumetric flask and dilute upto the mark with diluent. Further diluting 2mL of this solution to 10 mL with diluent to get final concentration of1.6 ppm each.

3. Result and Discussion

3.1. Chromatography

Different columns and mobile phases were tested but the method from the British Pharmacopoeia [5] was found to be more suitable for separating all impurities and degradation products from the drug substance. The HPLC method was validated for the following parameters.

3.2. Validation

3.2.1. Specificity

Specificity was done in two parts 1) By injecting diluent, Oxazepam and related impurities solution individually and 2) By forced degradation.

By injecting diluent, Oxazepam and related impurities solution of Impurity B [(3RS)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-bezodiazepin-3yl acetate], Impurity D [(2-amino-5-chlorophenyl) phenylmethanone] and Impurity E [7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one4-oxide] (500 ppm each) individually and in combination (1.6 ppm each) into the chromatograph. No peak was observed at retention time of Oxazepam and its related impurities in the diluent and each peak was found well resolved from each other.

The forced degradation study was carried out to know in advance likely degradation products that may be generated during stability study or shelf life i.e to get degradation of the sample up to 10-20% above which might me secondary degradation. Initially while checking the force degradation at lower concentration (i.e. at 0.1N HCl, 0.1N NaOH and 1% H2O2) at Room temperature no degradation was found for longer period of almost 1 week and also Oxazepam is very stable molecule, hence it was exposed to the following dry environmental and harsh wet chemical conditions at different time interavals from Initial, 1hr, 2hr,..till 8hrs at 80°C

A) Dry environmental conditions-

a. White fluorescent light (NLT 1.2 million lux hours)

b. UV Radiation (NLT 200 watt hrs. m^{-2}.)

c. Thermal (at 125^{o}C for 24 hrs.)

B) Wet chemical conditions-

a. Aqueous (water)

b. Basic (Sodium hydroxide, 1N)

c. Acidic (Hydrochloric acid, 1N)

d. Oxidation (Hydrogen peroxide - 3%v/v)

In the forced degradation study it was observed that there was no degradation found in dry conditions, but in wet chemical condition, degradation was found in Basic (3.4%), Acidic (5.2%) and Oxidation (8.9%) respectively, which is shown in Fig 7, Fig 8 and Fig 9. Though degradation was found in the wet chemical condition, peak purity taken on PDA detector showed that the peak of Oxazepam in all condition was found to be pure and well separated

from all the impurities and degradation products. The stability indicating assay was demonstrated by comparing assay of the degraded samples with that of control sample. The assay and purity of the degraded sample is presented in the Table 2.

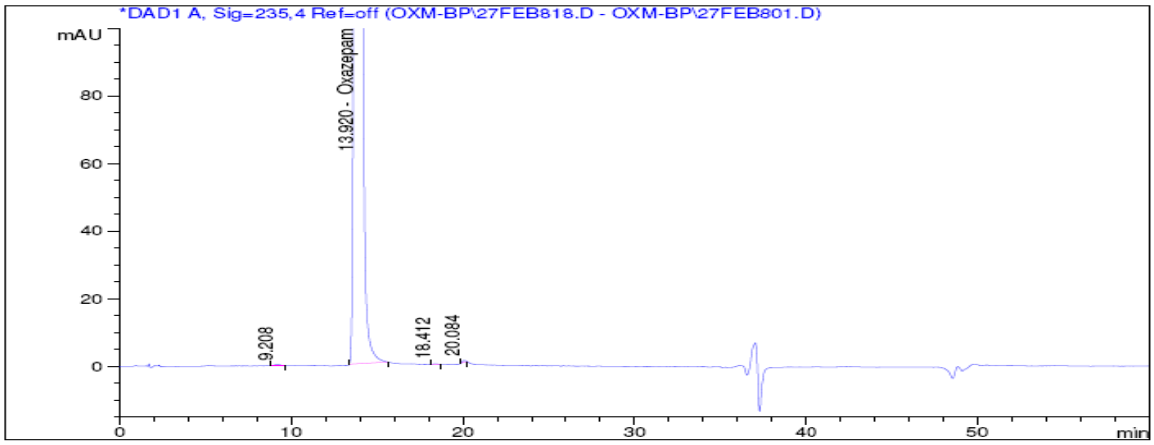


Fig 2. Chromatogram of pure Oxazepam.

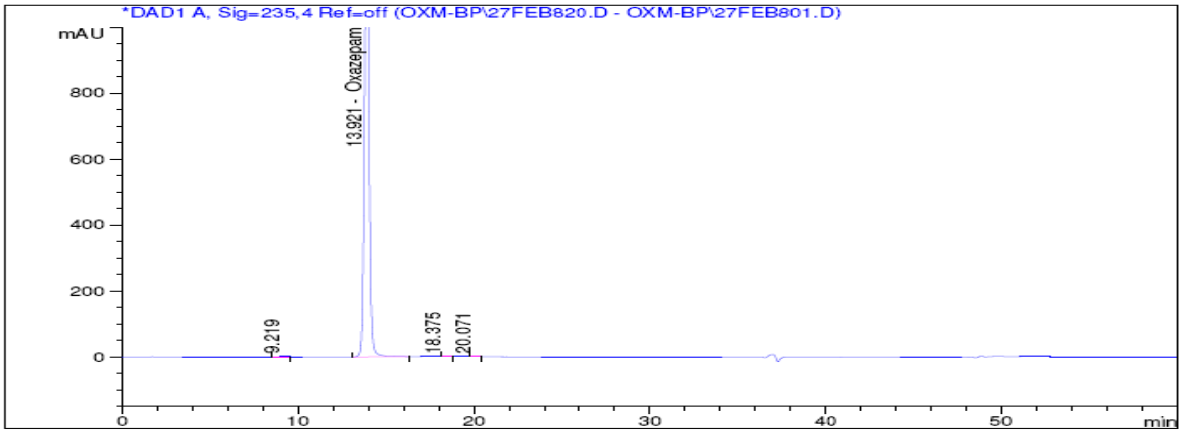


Fig 3: Chromatogram of sample after degradation in White Fluorescent lamp(NLT 1.2 million lux hrs) .

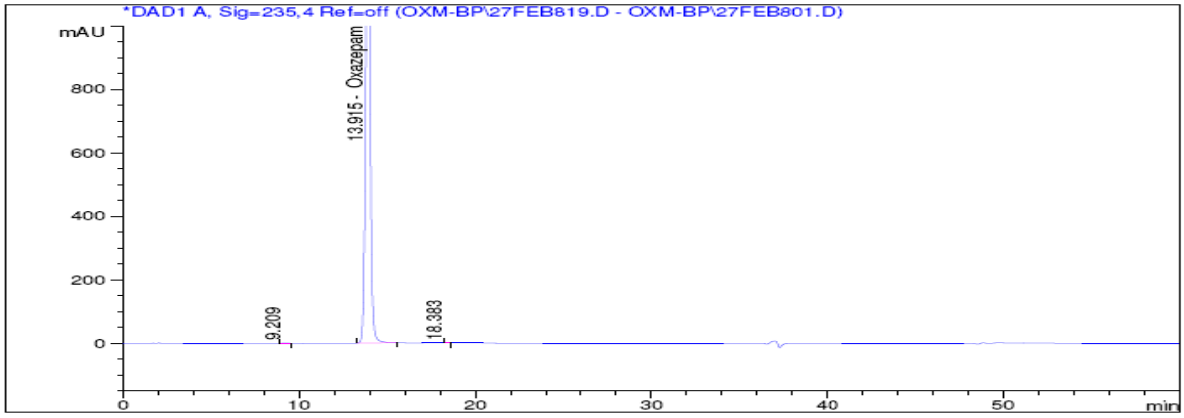


Fig 4. Chromatogram of sample after degradation in UV-radiation (NLT 220 watt hrs/seq.meter).

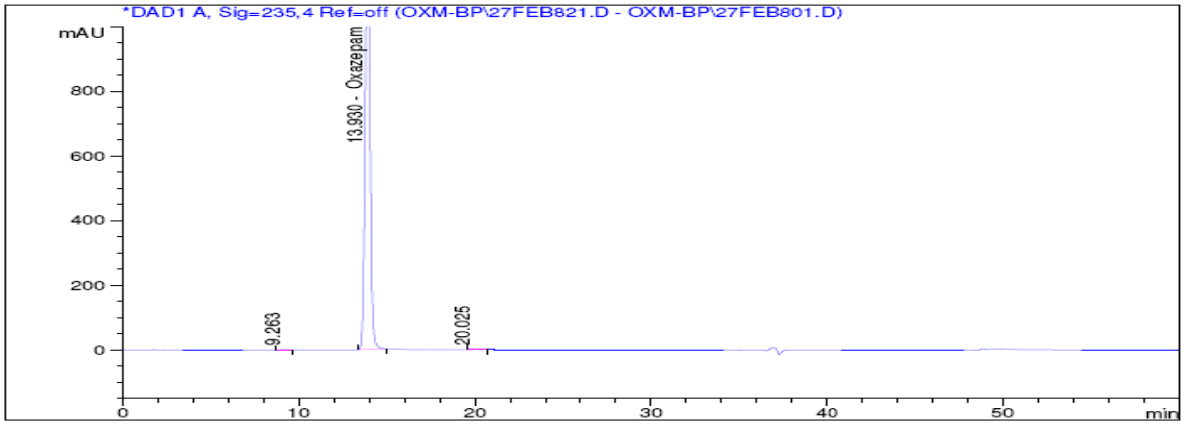


Fig 5: Chromatogram of sample after degradation in thermal heat (125°C) for 24 hrs.

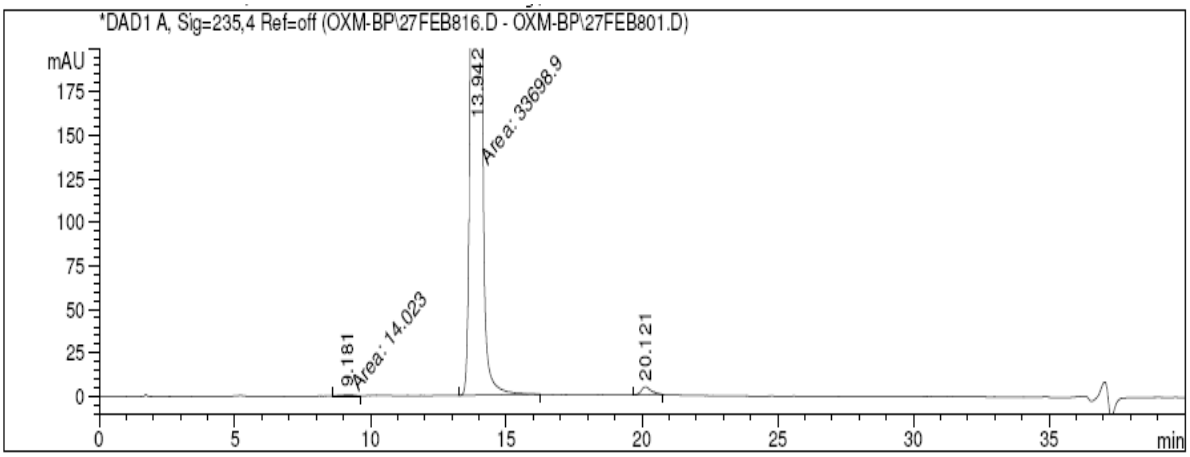


Fig 6. Chromatogram of drug in water.

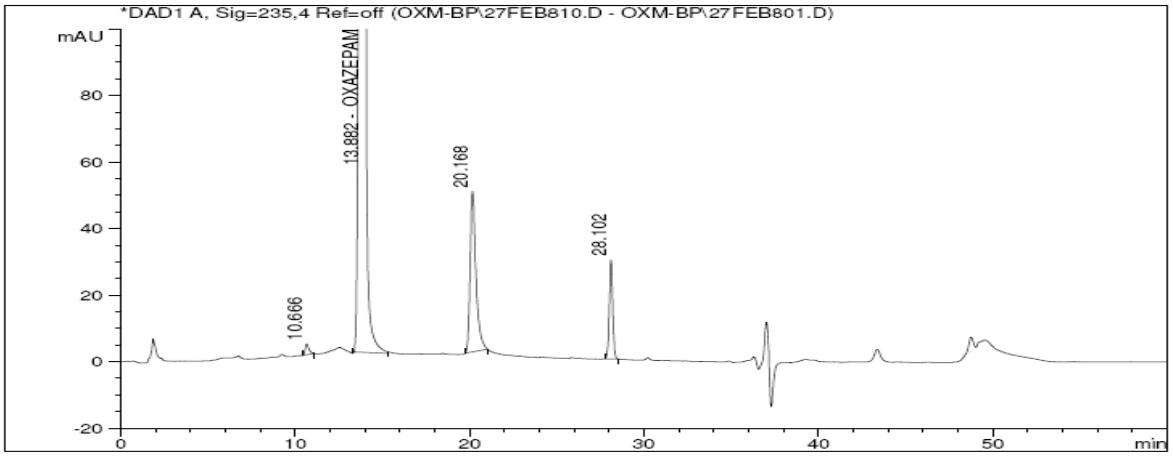


Fig 7. Chromatogram of sample after degradation in 1N hydrochloric acid at 80°C for 1 hr.

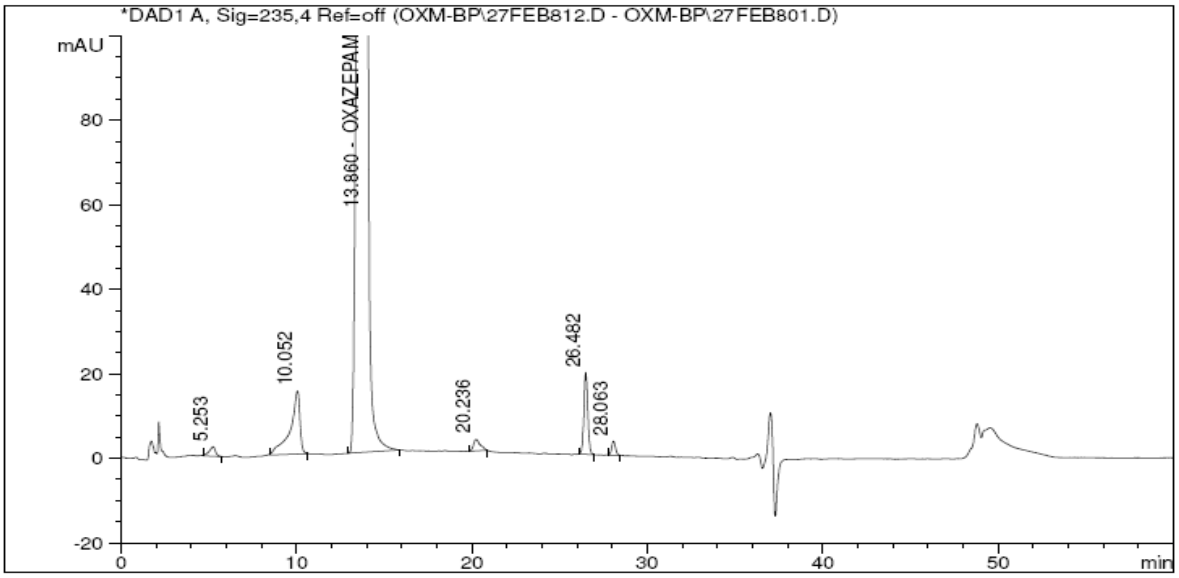


Fig 8. Chromatogram of sample after degradation in 1N sodium hydroxide at 80°C for 2 hrs.

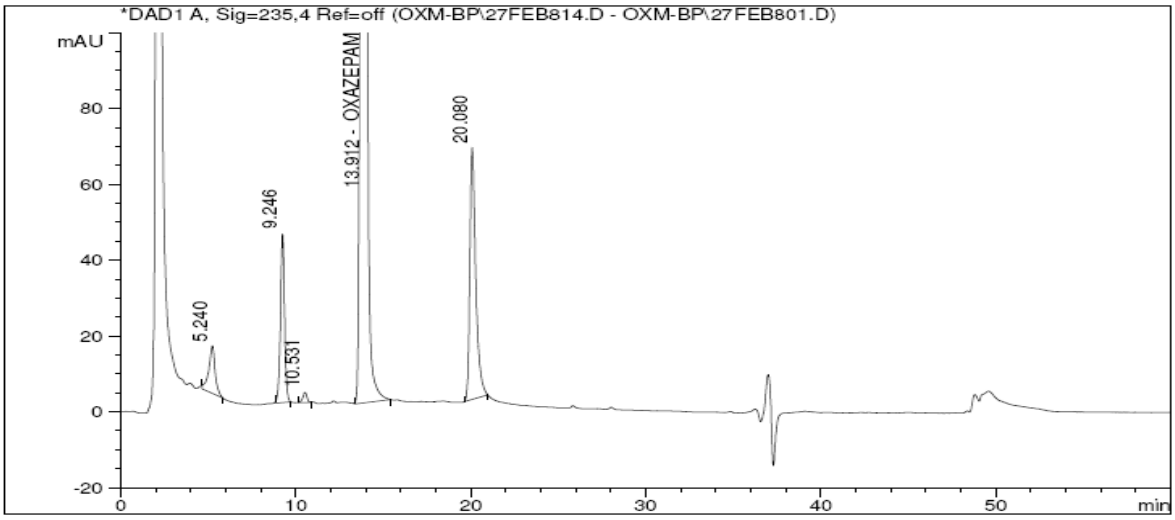


Fig 9. Chromatogram of sample after degradation in hydrogen peroxide at 80°C for 2 hrs.

Table 2. Assay and Purity of the degraded Oxazepam samples

Degradation condition	Purity (Area%)	Assay with respect to control sample, %	RRT of the major degraded peaks
Control sample (No degradation)	99.94	99.78	-
White fluorescent light (NLT 1.2 million lux hours)	99.94	99.16	No major degradation peak observed
UV Radiation (NLT 200 watt hrs. m^{-2}.)	99.97	99.91	No major degradation peak observed
Thermal (at 125^{o}C for 24 hrs.)	99.89	99.61	No major degradation peak observed
Aqueous (water) (at 80°C for 8hrs.)	99.61	99.05	1.44
Basic (1N NaOH) (at 80°C for 8hrs.)	96.59	95.26	0.38,0.73, 1.44,1.91
Acidic (1N HCl) (at 80°C for 8hrs.)	94.82	93.15	0.73,1.44,2.02
Oxidation (3% v/v Hydrogen peroxide) (at 80°C for 8hrs.)	91.13	90.22	0.38,0.66,0.73,1.44

3.2.2. Limit of Detection

For limit of detection, a series of injections of impurity solution having concentration close to theoretical limit of detection is made and signal to noise ratio was calculated. For limit of detection signal to noise ratio must be at least 3: 1 as given in ICH guidelines.

Limit of detection for Impurity B as obtained from Signal to noise ratio is 0.13 ppm. That means as low as 0.13 ppm of Impurity B can be detected by analytical method. The sample solution injected has concentration of Oxazepam as 800 ppm. Hence, limit of detection calculated with respect to Oxazepam is:

$$LOD\ of\ \ Impurity\ B = \frac{0.13}{800} x100\% = 0.016\%$$

Similarly LOD of Impurity D and Impurity E, as obtained from signal to noise ratio is 0.13 ppm and calculated as above is 0.016%.

Presuming that any unknown impurity has response similar to Oxazepam. Therefore limit of detection for any unknown impurity is 0.13 ppm and calculated with respect to sample concentration is 0.016%. Limit of detection of all components in summarized in Table 3.

Table 3: Limit of detection of all components.

Sr. no.	Component	Conc. (ppm)	Conc. (%)	S/N Ratio
1.	Impurity B	0.13	0.016	6.50
2.	Impurity D	0.13	0.016	6.40
3.	Impurity E	0.13	0.016	4.67
4.	Oxazepam	0.13	0.016	6.50

3.2.3. Limit of Quantitation

For limit of quantitation, a series of injections of impurity solution having concentration close to theoretical limit of quantitation is made and signal to noise ratio was calculated. For limit of quantitation signal to noise ratio must be at least 10: 1 as given in ICH guidelines.

Limit of quantitation for Impurity B as obtained from signal to noise ratio is 0.36 ppm. That means as low as 0.36 ppm of Impurity B can be quantified by analytical method. The sample solution injected has concentration of Oxazepam 800 ppm. Hence, limit of quantitation calculated with respect to Sample concentration is:

$$LOD\ of\ \ Impurity\ B = \frac{0.36}{800} x100\% = 0.045\%$$

Similarly LOQ of Impurity D and Impurity E as obtained from signal to noise ratio is 0.36 ppm and calculated as above is 0.045%.

Presuming that any unknown impurity has response similar to Oxazepam. Therefore limit of quantitation for any unknown impurity is 0.36 ppm and calculated with respect to Oxazepam is 0.045%. Limit of quantitation of all components is summarized in the Table 4.

Table 4: Limit of quantitation of all components.

Sr. no.	Component	Conc. (ppm)	Conc. (%)	S/N Ratio
1.	Impurity B	0.36	0.045	11.2
2.	Impurity D	0.36	0.045	12.3
3.	Impurity E	0.36	0.045	10.5
4.	Oxazepam	0.36	0.045	12.0

3.2.4. Linearity

Linearity is determined by serial dilution of all the components to a 7 different concentration (prepared in combination) in the range of LOQ to 150% of impurity limit. Each solution injected into the chromatograph in duplicate and average area of two determinations is recorded.

A graph of Concentration (on X axis) vs Area (on Y axis) is plotted. A correlation coefficient, slope and intercept was calculated. For Imp B, Imp D, Imp E and Oxazepam the correlation coefficient was obtained as 0.997, 0.999, 0.999 and 0.999 respectively showing that response is linear.

3.2.5. Precision

3.2.5.1. System Precision

To ensure that analytical system is working satisfactory and giving precise results, a 1.6 ppm (0.2%) diluted solution of Oxazepam was injected into the chromatograph 6 times. The retention time and area is noted. Relative standard deviation (RSD) for retention time and area was found to be 0.27 and 0.82 respectively for Oxazepam peak. A resolution solution consisting of Oxazepam & impurity B was also injected to ensure system suitability. Resolution was found to be 13.64 between Oxazepam and impurity B.

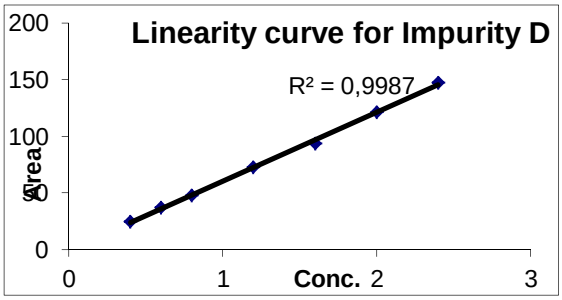


Fig 10. Linearity curve of Impurity B

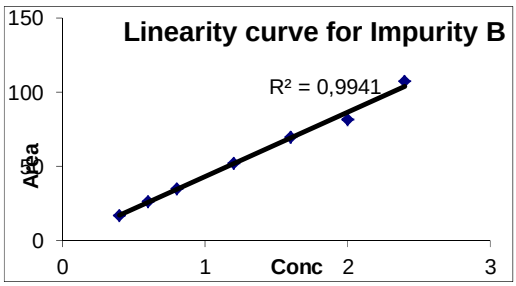
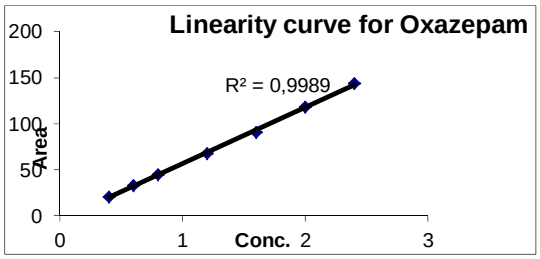


Fig 11. Linearity curve of Impurity D



Linearity curve of Impurity E

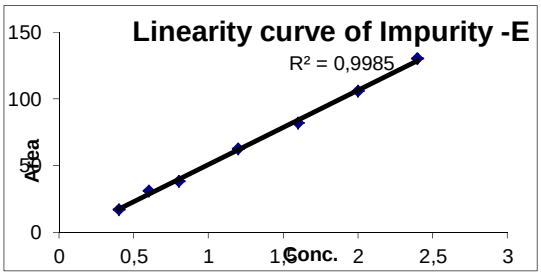


Fig 12. **Fig 13.** Linearity curve of Unknown impurity (Oxazepam)

3.2.5.2. Method precision

Method precision was determined by using six separate sample preparation which are prepared by using pooled sample at working concentration (800 ppm) and spiked with 0.2 % impurities (i.e. impurities B, D & E) and were analyzed by HPLC system. %RSD for Imp E, Imp B, unknown Imp and Imp D was found to be 0.00, 0.00, 3.03 and 0.00 respectively. The RSD values obtained for all known impurities are well within the limit of 10% indicating analytical method is precise.

3.5.6. Accuracy

The accuracy was carried out from 50% to 150% of the impurity limit. Each impurity was spiked into the sample at three levels, 50%, 100% and 150% of impurity limit. Three samples of each level are prepared and injected into the chromatograph. From the data obtained, % recovery for each sample and at each level is calculated against added amount. The recovery for Impurity B was found to be in the range of 94.48 % to 103.94% with %RSD 2.87. Similarly the recovery for Impurity D and Impurity E was found to be in the range of 97.75% to 103.03% and 98.21% to 104.07% respectively with %RSD 1.89 and 2.03 respectively. The mean recovery obtained for all components was within 80.0% to 120.0% and RSD calculated for all levels was less than 10% indicating accuracy of analytical method.

4. Conclusion

The method was found to be specific stability indicating as proved by injecting known components into the chromatograph and by forced degradation study. Limit of detection and limit of quantitation for known related impurities and unknown related impurities has been established. The Analytical method was found to be linear in the specified range and also found to be Accurate and Precise. From the above data, it is concluded that, the analytical method can be used for analysis of related substances of Oxazepam and is capable of giving accurate and precise results.

References

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