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# **To develop and validate a Reverse Phase - High Performance Liquid Chromatography method, using International Conference on Harmonisation (ICH) guidelines, for updating the British Pharmacopoeia (BP) monograph of Loprazolam Mesilate tablets**

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**ABSTRACT:** This laboratory based research was an attempt to develop and validate a Reverse Phase - High Performance Liquid Chromatography method, using International Conference on Harmonisation (ICH) guidelines, for updating the British Pharmacopoeia (BP) monograph of Loprazolam Mesilate tablets. The BP currently requires Thin-Layer Chromatography for analysing the impurity content, an obsolete method with numerous shortcomings. A validated RP-HPLC method improves quantification and detection of impurities, assay, and identification, coupled with making the procedure easier and faster. Loprazolam mesilate 1 mg tablets manufactured by Zentiva were tested. An ultraviolet-scan was used to determine the optimum value of wavelength of light for detecting drug in solution: 330 nm. Mass Spectrometry (MS) confirmed the identity of the eluting substances. An Xterra® MS, C18, 3.5 micrometer, 125Å pore size, 4.6 mm diameter by 100 mm long column was chosen. Different ratios of mobile phases were used in isocratic mode. The best system was found to be a 50:50 ratio of methanol and 0.5 g/l phosphate buffer, at pH 5.1 at 25°C and a flow rate of 1 ml/minute. This ensured impurities elute consistently and fairly rapidly, whilst overlapping of peaks did not occur. The method showed a good selectivity, efficiency, wide concentration range, stability and ruggedness for loprazolam. In regression analysis, an  $R^2$  value of 0.9947 was obtained and a recovery 97.08% was found. The results aid future development of a validated RP-HPLC method for related substances content.

**KEYWORDS:** Loprazolam, Reversed Phase High Performance Liquid Chromatography, Thin-Layer Chromatography, Mass Spectrometry, International Conference on Harmonisation, Mass Spectrometry.

## **I. INTRODUCTION**

Benzodiazepines are one of the most widely used medications for treating anxiety and sleep disorders, especially in the elderly population [1]. They are an important class of drugs since the prevalence of psychological disorders is increasing in addition, the general public is becoming more aware and accepting of these conditions [1]. Patients are now actively seeking treatment for disorders like depression, anxiety, panic attacks, insomnia and anger related issues – all conditions that may be treated with benzodiazepines [1]. Loprazolam is a hypnotic benzodiazepine used for the treatment of insomnia and anxiety for a limited period. It is sold under brand names such as Dormonoc®<sup>®</sup>, Havlane®, Sonin®, and Somnovit®. Loprazolam mesilate BP is the active ingredient in Loprazolam Tablets BP. The 1 mg tablets

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are yellowish in colour, biconvex and about 7mm in diameter. The molecule of lorazepam is polar due to the presence of nitro group; however, the high solubility is largely due to the protonation of an amino group *in vivo*, yielding a positive charge [5]. Lorazepam mesilate (mesylate) is chemically named as (2Z)-6-(2-chlorophenyl)-2-[(4-methylpiperazine-1-yl) methylidene]-8-nitro-4H-imidazo [1,2-a] [1,4]benzodiazepine-1-one; methanesulfonic acid salt. The molecular formula of lorazepam mesilate is  $C_{24}H_{25}ClN_6O_6S$  and the molecular weight is 561.01 g/mol. [7] The molecular formula of lorazepam (the free base) is  $C_{23}H_{21}ClN_6O_3$  and the molecular weight is 464.91 g/mol [7]. The molecular weight of the hydrate (the stable form, as defined in the MHRA report) is 579.1 g/mol and the formula reported in it is  $C_{23}H_{21}ClN_6O_3 \cdot CH_3SO_3 \cdot H_2O$  [13]. Lorazepam is a typical 1,4-benzodiazepine. A seven-membered ring with two nitrogen atoms is named as 1,4-diazepine. When a benzene ring is fused at the 5, 6 bond of the diazepine, it becomes a 1,4-benzodiazepine (the bridgehead carbon atoms being renumbered to 10,11 positions) [8]. Introduction of imidazole moiety onto the benzodiazepine nucleus at the 3, 4 bond seems to increase the hypnotic potency *in vivo* [9]. The aim of the present experiment was to develop and validate an HPLC method to adequately identify, quantify, and determine impurity levels in the tablet.

## II. LITERATURE REVIEW

Mohamed Nasir-uddin et al carried out RP-HPLC of lorazepam from plasma using methanol and acetic acid in 60:40 ratio [33]. They had also reported that salt form of lorazepam was soluble in water and a stock solution of lorazepam was stable up to 6 months if stored at 4°C. In the book titled "Chromatographic Analysis of Pharmaceuticals" the HPLC of lorazepam had been carried out using a C-18 Hypersil, 5 µm column, at a flow rate of 1.5 ml/min and mobile phase consisting of methanol, water and phosphate buffer in 7:1:2 ratio [34]. In a study to test benzodiazepines in the whole blood using LC-MS, the molecular ion of lorazepam was detected with a molecular mass of 465 and parent ion with a molecular mass of 408. They were able to achieve a recovery of 78% with %RSD of 7%. The column that was used was an Xterra MS C-18 column (150 mm × 2.1 mm, 3.5 µm PS) [35]. Another study conducted to test degradation products of lorazepam was able to detect lorazepam at roughly 8.73 minutes. The mobile phase used in this study was methanol-water-phosphate buffer (55:25:20, v/v/v) at a flow rate of 1.5 ml/minute [20].

## III. METHOD DEVELOPMENT

**Column Selection:** It was decided to use a C18 column as the lorazepam molecule was a small one and unlike proteins, it would not get tangled in the thick C18 mesh. Three mobile phases were considered methanol, ethanol and acetonitrile. Even though lorazepam had a known solubility in ethanol it was not a major concern as the drug was sufficiently soluble in water. A longer retention time was desirable which was achieved with methanol. Acetonitrile was tested many times but it was extracting the drug early giving shorter retention times. Potassium dihydrogen phosphate (PDP) buffer was used to keep the pH of the mobile phase acidic at a value of around 5. This pH was selected because it was known that within the pH range of 3 to 10 the spectrum of lorazepam solution remains constant. At pH below 3 acid hydrolysis had been reported and at pH above 10, a variation of results with time had been documented [20].

**Sample Preparation and Handling:** Due to the presence of impurities during sample preparation it was quite logical to use standards for comparison. The standard for lorazepam was not available in Europe. Moreover, since commercial tablets were being tested in this study a high standard of purity was expected from them. Utmost care was taken during sample preparation and handling to maintain the purity. Samples were kept in dark and at room temperature. New samples were prepared after every three days as some 1,4-benzodiazepines undergo hydrolysis at room temperature [20]. Before each cycle of HPLC, enough time was given for column purging and washing. The first reading was omitted and the next three readings were taken for the calculations.

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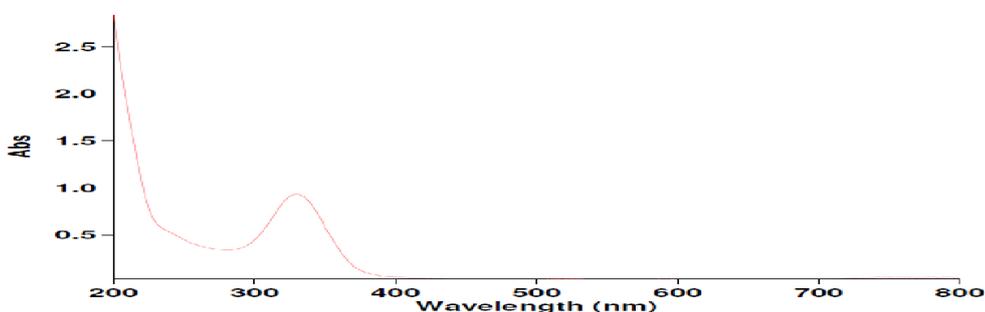
## IV. MATERIALS

**REAGENTS AND CHEMICALS:** Whatman thick filter paper: grade 540, 125mm, 1540-125, hardened ashless circles, Syringe filter paper: category number-6779-0404, 4 mm, 0.45 micrometre PVDF membrane, polypropylene housing, Methanol 100% HPLC grade, De-ionised HPLC grade water, Loprazolam mesylate tablets 1mg (prescription only medicine) manufactured by Zentiva UK, packed in cartons of 10, 28 or 30 tablets; excipients: Povidone, Lactose, Maize Starch, Colloidal silicon dioxide, Microcrystalline Cellulose, Magnesium Stearate, buffer- Potassium dihydrogen phosphate, dibasic (molecular weight: 174.29), 99% analytical grade, Fisher Scientific, UK.

**INSTRUMENTS:** Shimadzu HPLC LC-2010AHT: Pump mode- quaternary, Injector system- Automatic, Software- Shimadzu lab solutions, Column type used- Xterra MS, C18, 3.5micrometer, 125A, 4.6\*100mm column, Part no. W20701K 005, serial no.186000436

**PREPARATION OF STANDARD SOLUTION OF LOPRAZOLAM:** The stock solutions of Loprazolam of different concentrations were prepared in a similar manner. For 1 mg sample, one tablet of Loprazolam which typically contains 1 mg of the active drug was crushed with the help of mortar and pestle. It was diluted with 500 ml of solvent in which methanol, water and PDP buffer solution were present in the ratio of 60, 30 and 10 respectively. A sample of concentration of 0.002mg/ml was obtained. The PDP buffer was prepared by accurately weighing 500 mg of PDP and then diluting it with 1 L of HPLC grade de-ionised water. The pH of this solution was measured and it was recorded to be around 5.1. This same PDP buffer was used along with methanol while carrying out HPLC. Similarly, for preparing 0.004 mg/ml, 0.008 mg/ml and 0.02 mg/ml sample solutions 2, 4 and 10 tablets were crushed and diluted with the same quantity and ratio of the solvent mixture. For obtaining a 0.001 mg/ml sample the 0.002 mg/ml sample was diluted to half. The mixtures were filtered using grade 540 Whatmann filter paper. All the solutions were filtered twice through the filter paper and once through syringe filter paper before putting them in final 1.5 ml HPLC injection vials.

**Measurement of Working Wavelength:** UV/VIS Spectrophotometer, Cary 100 Bio was used to check the wavelengths. Methanol was used as the blank solution. The samples were tested between 200 and 800 nm. A wavelength of 330nm (Figure 1) was obtained for each one of them with increasing intensity corresponding to the increasing drug concentration.



### Scan Analysis Report

Report Time : Fri 31 Aug 01:34:43 PM 2018  
Method:  
Batch:  
Software Version: 3.00(339)  
Operator:

Sample Name: lpz-28th101

Collection Time: 28/06/2018 16:13:41

Peak Table	Peak Area	Peak Threshold	Wavelength (nm)	Abs
1	0.0100	0.0000	330.00	0.931

Figure 1: Representative UV-Spectra of Loprazolam Mesilate.

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## V. METHOD

An RP-HPLC with 50% methanol and 50% buffer solution was carried out. A flow rate of 1 ml/min, a wavelength of 330nm, a pH of 5 and a temperature of 25°C were maintained. With this composition, a retention time of around 8 minutes was achieved as shown in Figure 2.

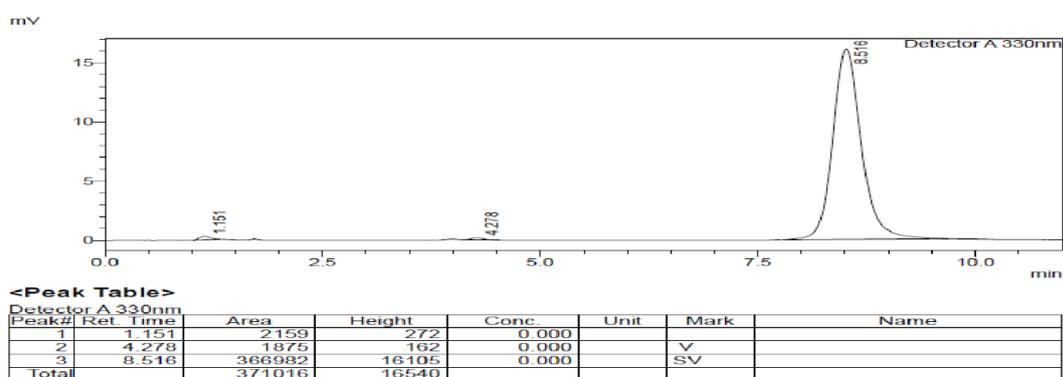


Figure 2: Representative Chromatogram of LPZ 0.008 mg/ml 50/50 Methanol and Buffer.

Observed from these graphs was the presence of minor tailing effect and slightly broad peaks. The peak broadening observed here could be due the presence of dead volume in the HPLC equipment [22]. The overall quality of the peaks was judged as acceptable.

## VI. RESULTS AND DISCUSSION

**LINEARITY TEST:** A plot of mean peak area versus drug concentration showed a linear relationship between the two variables. The representative graph is shown in Figure 3. The solid line represents the trend irrespective of the intercept and the dotted line represents the trend line after adjusted the intercept to zero.

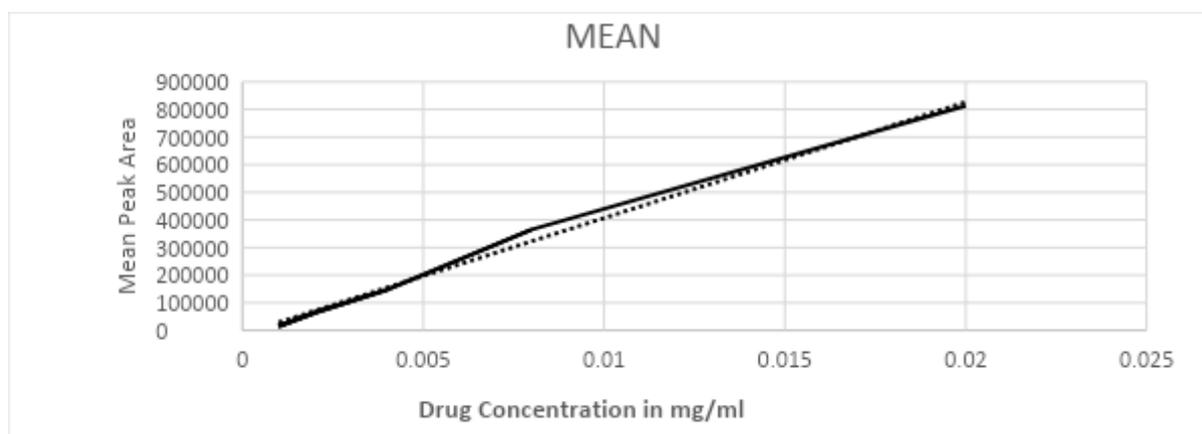


Figure 3: Correlation Graph of Drug Concentration vs. Mean Peak Area.

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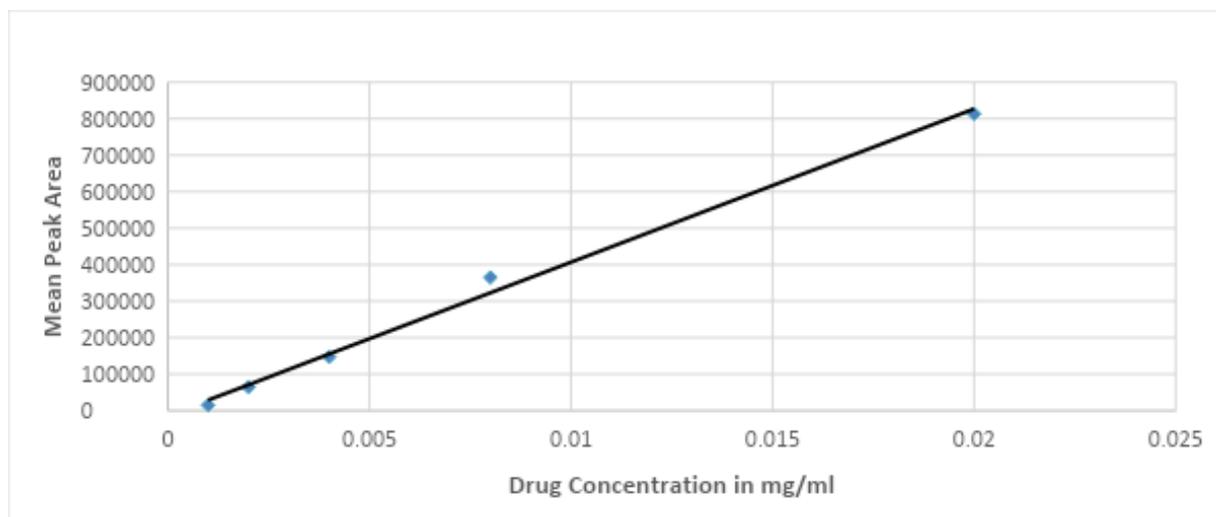
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The samples for each of the five concentrations used were run four times and three peak area values obtained for each sample were used for regression analysis. The values are shown in the Table 1.

Concentration (mg/ml)	1st run	2nd run	3rd run	Mean	SD	%RSD
0.001	14101	15464	15261	14942	735.3659	4.921
0.002	66508	64992	62037	64512.33	2273.768	3.524
0.004	144689	148818	147585	147030.7	2119.581	1.442
0.008	366982	366603	361725	365103.3	2931.853	0.803
0.02	839156	786178	814171	813168.3	26503.23	3.259

**Table 1: Mean Values and Relative Standard Deviations at Different Drug Concentrations:**



**Figure 4: Linear Regression Line of Best Fit**

### SUMMARY OUTPUT

<i>Regression Statistics</i>	
Multiple R	0.997335
R Square	0.994676
Adjusted R Square	0.992902
Standard Error	27490.49
Observations	5

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### ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	4.24E+11	4.24E+11	560.534	0.000165
Residual	3	2.27E+09	7.56E+08		
Total	4	4.26E+11			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	-13135.7	17476.84	-0.75161	0.506876	-68754.8	42483.34	-68754.8	42483
X Variable 1	42012439	1774504	23.6756	0.000165	36365176	47659702	36365176	5E+07

### RESIDUAL OUTPUT

<i>Observation</i>	<i>Predicted Y</i>	<i>Residuals</i>
1	28876.69	-13934.7
2	70889.13	-6376.8
3	154914	-7883.31
4	322963.8	42139.53
5	827113	-13944.7

An  $R^2$  of 0.99% was obtained which indicates that the regression line fits the data quite well. In addition, there were no visible trends and the relationship looks almost linear. The null hypothesis was there is no correlation between the increasing concentration and peak area. From the ANOVA table shown above, it could be seen that F value is greater than F critical. The null hypothesis was rejected on this basis and it was concluded that there was a correlation between the two variables and change in one affected the other in a linear manner (visual inspection of the graph in figure 4), values of which could be predicted from the regression equation i.e.  $y = -13933 + 84121x$ . The x in this equation was the concentration and as the equation had a plus sign which meant an increase in 'x' would cause an increase in 'y' which was the peak area of the analyte. Table 2 shows the means of the areas predicted by the equation.

Observation (mg/ml)	Actual Mean	Predicted Mean	Residuals
0.001	14942	28876.69	-13934.7
0.002	62208.33	70889.13	-6376.8
0.004	147030.7	154914	-7883.31
0.008	365103.3	322963.8	42139.53
0.02	813168.3	827113	-13944.7

**Table: 2 Actual vs. Predicted Mean:**

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**SINGLE FACTOR ANOVA:** A p-value of 0.625 was obtained which was greater than the significance level of 0.05 so on this basis null hypothesis could not be rejected. Also, 'F' value of 0.648 was less than the F- critical value of 5.192 which also meant that the null hypothesis could not be rejected. This meant that all five observation were statistically equal and small variations among them could be attributed to the experimental conditions. Again the results could be refined further by increasing the number of HPLCs performed per sample and refinement in sample preparation [23].

**ACCURACY:** Accuracy is a measure of closeness of the experimental values to the actual values present in the reference material or standard compound as certified by the manufacturer [25]. It is one of the parameters of validation specified by the ICH [41]. Loprazolam mesilate 1mg tablets manufactured by Zentiva were used for this analysis and it was expected that each contains 1 mg of the active drug [26].

Under ideal conditions, 100% recovery is expected. By obtaining the values of means from Table 1 and the regression equation from the graph in Figure 4, the drug amount recovered are shown in Table 3.

Drug Amount Expected (in mg/ml)	Mean Peak Area	Drug Amount Recovered	% Recovery
0.001	14942	0.0007	70
0.002	62208.3	0.00088	94
0.004	147030.7	0.0004	100
0.008	365103.3	0.00095	118.2
0.020	813168.3	9.83	103.2
			97.08

**Table 3: Per Cent Recovery:**

For non regulated products a mean recovery of 90% to 110% is acceptable but for regulated substances like pharmaceuticals, a recovery within 100+-2% is recommended [41]. The mean per cent recovery achieved here is 97.08%.

**REPEATABILITY TEST:** The HPLC analyses were repeated on the same sample, which was two days old by then, to check whether an older sample would cause any remarkable change in the results of the chromatogram. The values obtained this time are shown in Table 4 and the linear regression graph is shown in Figure 5.

Drug Concentration(mg/ml)	Peak Area 1st Run	Peak Area 2nd Run	Peak Area 3rd Run	Mean Peak Area	Standard Deviation	%RSD
0.002	15767	16980	16378	16375	606.5056	3.703
0.004	122264	130447	135777	129496	6806.511	5.256
0.008	215511	300008	301135	272218	49112.94	18
0.02	508616	581674	571433	553907.7	39556.55	7

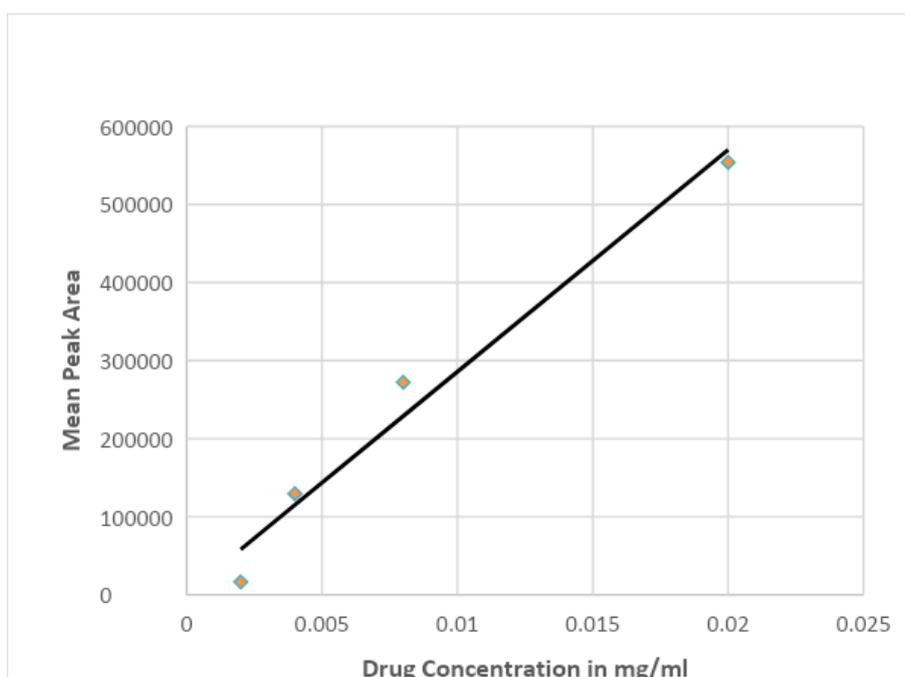
**Table 4: Mean and RSD on Repeat Readings:**

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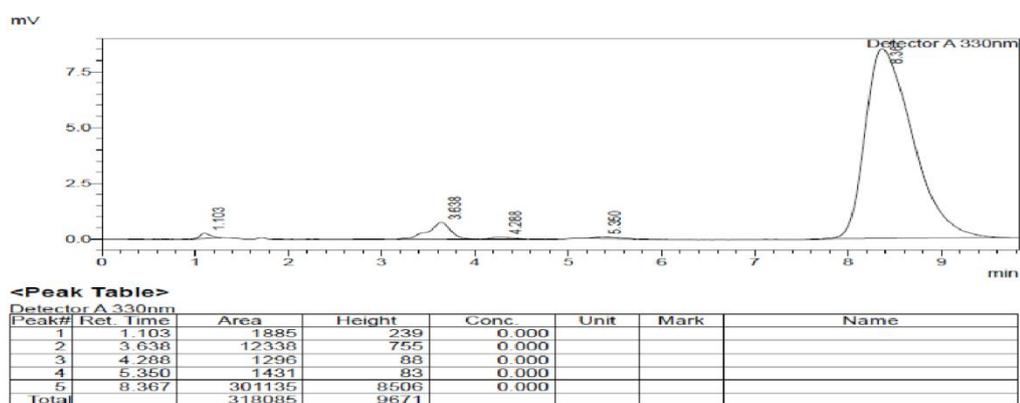
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**Figure 5: Linear Regression Line of Best Fit for Repeatability Study:**

The representative graph for a reading of a 0.004 mg/ml sample is shown in Figure 6. The presence of an extra peak signified that loprazolam had disintegrated over time giving rise to more degradation products. The assays i.e. unaltered loprazolam content calculated for these three days old samples had also dropped from 99% to around 95%.



**Figure 6: Representative Chromatogram of LPZ 4 mg for Repeatability Test**

**ROBUSTNESS:** The RP-HPLC was carried out with a different mobile phase concentration. This time 60% of methanol and 40% of 0.5g/l phosphate buffer were used. The readings were taken from four sample solutions of loprazolam mesilate. All the other parameters were kept the same as before. An  $R^2$  value of 0.975 was obtained and the

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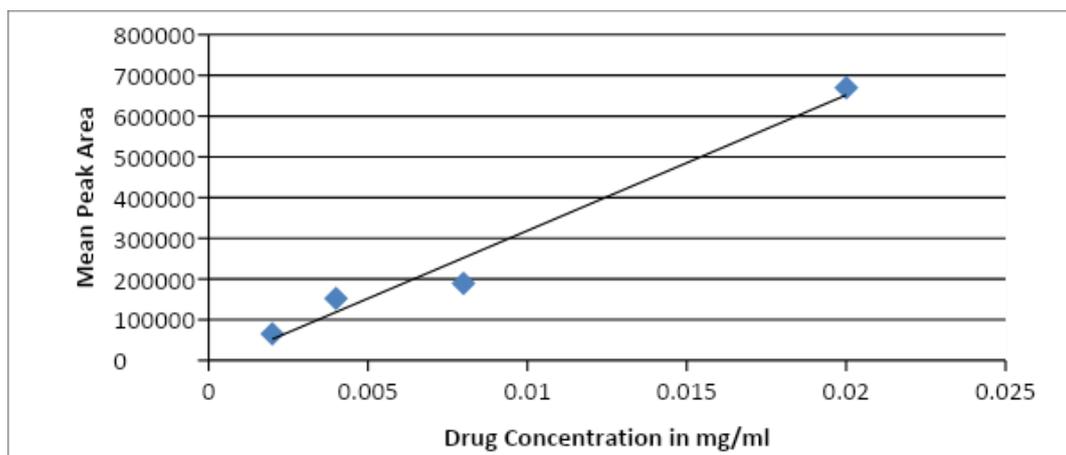
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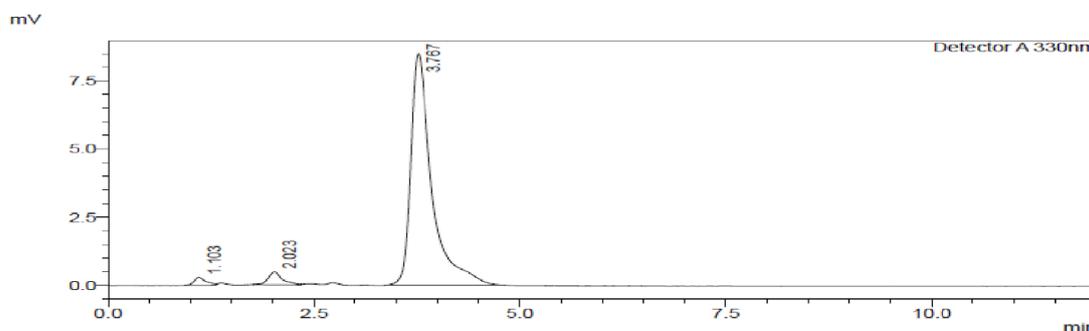
% RSD obtained this time were again higher than the specified value of 2%. The values obtained in this experiment are shown in Table 5. The graph depicting line of best fit is shown in figure 7. A representative chromatogram is show in Figure 8.

Concentration (mg/ml)	1st run	2nd run	3rd run	Mean	Standard Deviation	%RSD
0.002	67699	63745	63982	65142	2217.595	3.4
0.004	150739	152472	152243	151818	941.4303	0.62
0.008	175418	202345	189206	188989.7	13464.8	7.12
0.02	635736	675345	697507	669529.3	31293.46	4.673

**Table 5: Values of Mean and RSD from 60/40 Methanol and Buffer Mobile Phase:**



**Figure 7: Linear Regression Line of Best Fit at 60/40 Methanol and Buffer:**



**<Peak Table>**  
Detector A 330nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	1.103	2825	291	0.000			
2	2.023	5519	470	0.000			
3	3.767	152472	8499	0.000			
Total		160816	9260				

**Figure 8: Representative Chromatogram of a 0.004 mg/ml LPZ at 60/40 Methanol and buffer Mobile Phase:**

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**LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS):** The LC-MS was carried out using Hewlett Packard/Agilent Mass Spectrometer model PGSCIEX API 3000. The machine had a Turbo-ionspray interface. A 200 ng/ml of loprazolam mesilate was prepared by crushing a 1 mg loprazolam mesilate tablet and diluting it with 500 ml of solvent which gave a concentration of 0.002 mg/ml. 10 ml of this solution was further diluted by adding 100 ml of solvent to it which gave a final solution of 200 ng/ml concentration. The solution was directly injected into the mass spectrometer. The flow was kept continuous and total scan time was around 10 seconds. A sharp and distinct peak was obtained at 465.2 which corresponds to the parent molecule of loprazolam.

**RESULTS:** The reading was taken for a molecular mass of 464.91 Da, which was the mass of loprazolam, with a width range of 25 Da. Sharp and distinct peak was obtained at 465.2 Da which represented the parent molecule of Loprazolam present in the solution without the base mesilate. The other peaks adjacent to the prominent peak could be due to the presence of various carbon isotopes, predominant among which were carbon 13 isotopes which amount to about 1% of the total carbon and carbon 14 isotopes which were also seen but in trace amounts [29]. The representative graph is depicted in Figure 9.

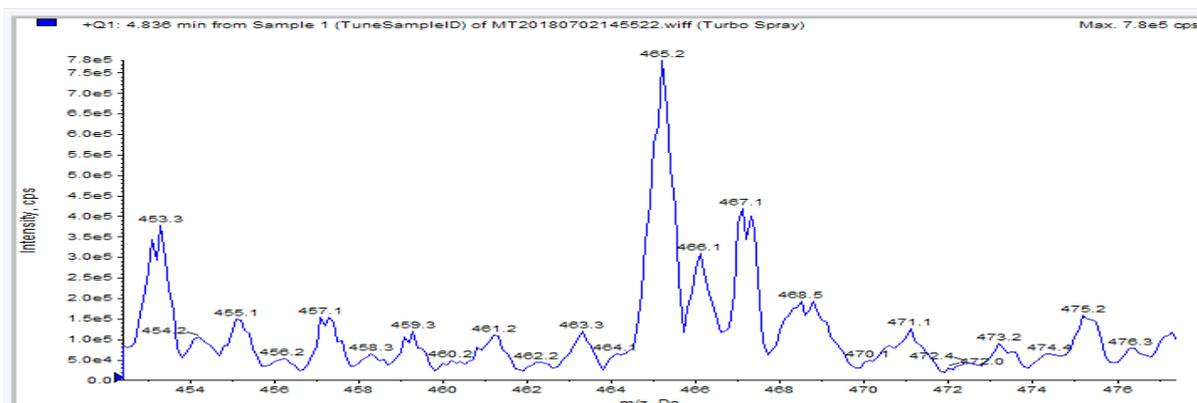


Figure 9: Mass spectra LPZ:

The peaks for various degradation products were also detected by the spectrometer.

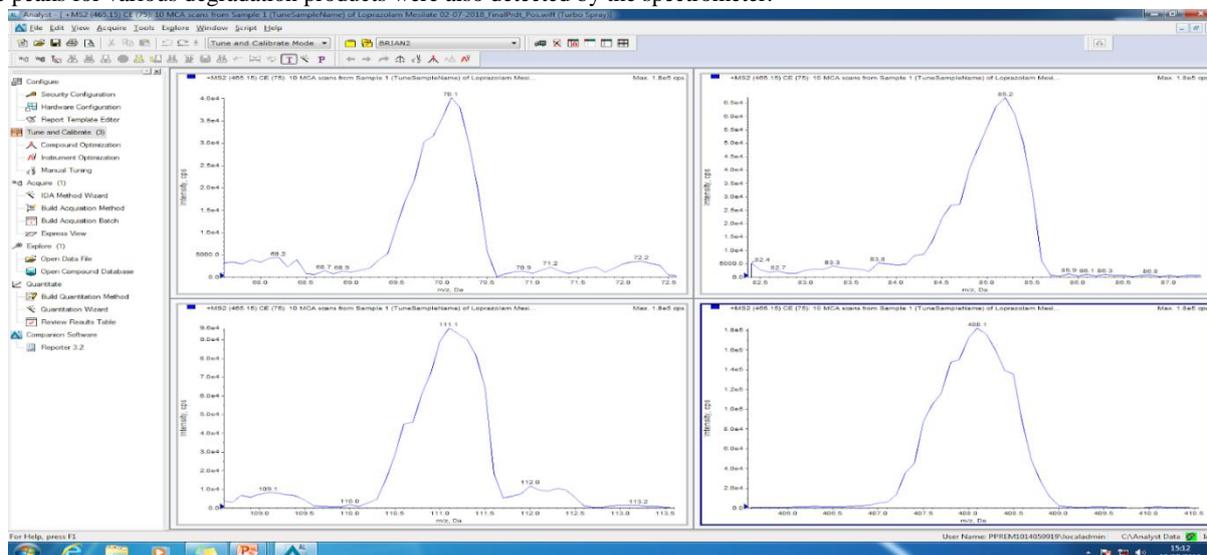


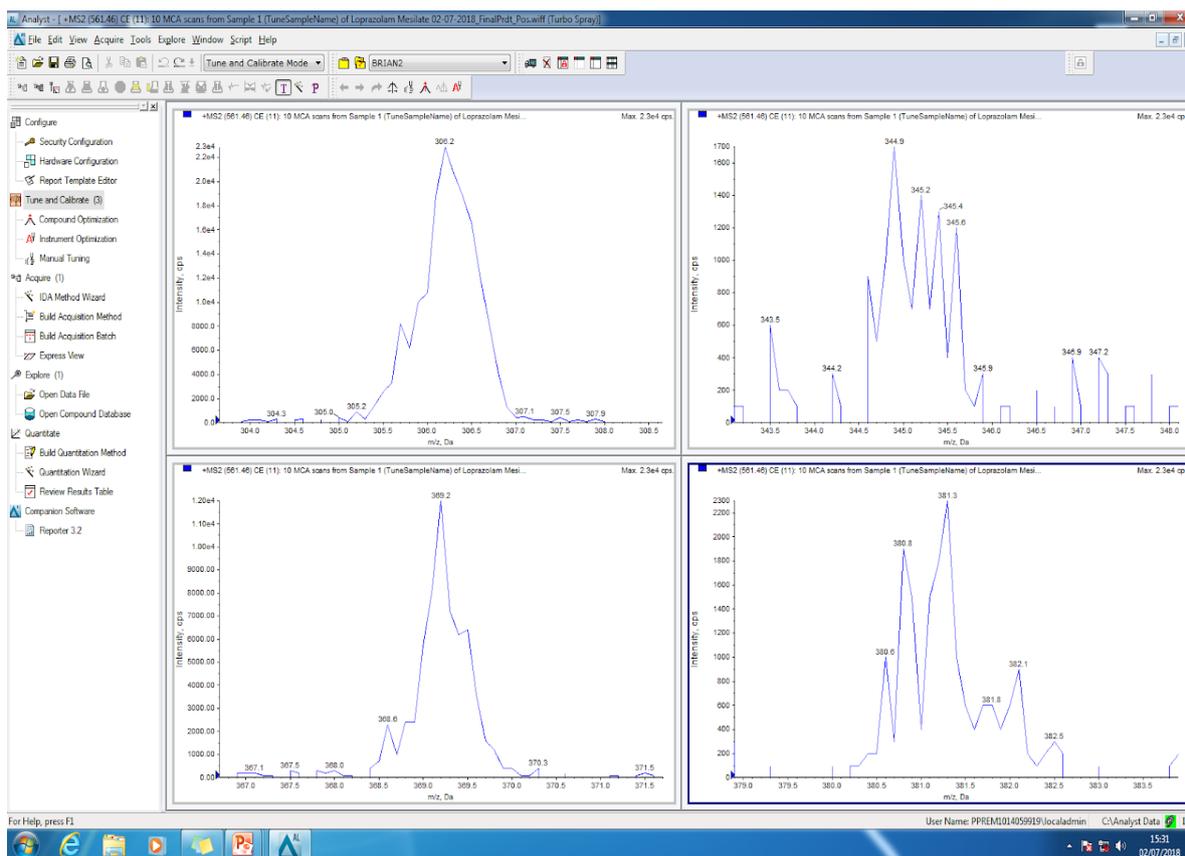
Figure 10: Degradation Products of LPZ detected on Mass Spectrometry:

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**Figure 11: Degradation Products of LPZ detected on Mass Spectrometry:**

The LC-MS results showed that the loprazolam mesilate had dissolved in the solution and some of it had managed to stay stable in the solution kept at room temperature. The presence of various degradation products (Figure 10,11) showed that the molecule had a tendency to degrade or most likely mass spectrometer could have caused the molecule to disintegrate during the process. These assumptions need further verifications and were beyond the scope of this experiment.

**DISCUSSION:** In a previous project done on the same topic in 2015 [44] a  $R^2$  value of 0.9424 was obtained and a satisfactory retention time was not achieved. In that study a C8 column was used and mobile phase was 52% methanol and 48% of 0.02M phosphate buffer. The pH was 8 [44]. In this study a C18 column was used and the mobile phase was 50% methanol. The rest of the mobile phase was 50% phosphate buffer of 0.5g/L concentration and the pH was kept close to 5. The  $R^2$  value obtained with this method was 0.9947. Relative standard deviations of peak areas had shown improvement but were still more than the 2% limit specified by ICH. The retention times obtained with current method were highly precise. On keeping the experimental conditions constant the resolution and selectivity of the chromatograms was good. This could be verified from the study of retention times, which were similar, around 8 minutes, even when old samples were used. On changing the concentration of the mobile phase the high efficiency and selectivity of the chromatograms was retained. The  $R^2$  value obtained at 60/40 methanol/buffer concentration was 0.9735 and an  $R^2$  value of 0.9746 was obtained on repeatability testing. The discrepancy in percent peak areas in the readings taken on repeatability testing could be attributed to the increasing hydrolysis of the loprazolam molecule in the sample which were kept at room temperature [20]. Still the loprazolam peaks constituted more than 90% of the peak

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areas in all the chromatograms and in all the different conditions which were used in this project. In order to get better results for % RSD of peak areas the number of readings taken should be increased. Fresh sample contain less disintegrated products and would provide larger values for peak areas. Storing the sample at 4°C might help as it has been suggested by previous research work done on benzodiazepines [33]. The change in mobile phase concentration affected the retention time which was expected but as discussed before the subsequent change in percent peak areas was very less. The method developed here provided a percent recovery of 97.08%. The ICH recommends a recovery of 98-102% for pharmaceuticals, assessed for a minimum of 9 determinants for at least three concentration levels [41]. Given the conditions the percent recovery value obtained seems plausible. It was clear from the result of mass spectrometry that loprazolam had dissolved successfully and was present in predominant concentration in the sample solution. It was detected with a molecular mass of 465g/mol along with various degradation products which were also detected successfully. Even in old samples kept at room temperature the loprazolam molecule was stable to a great extent. The minor peaks observed in these graphs could also be dissolved gases which was a common finding even when the sample and mobile phase used were same, which in this case were methanol/phosphate buffer [39]. Or as determined by the LC-MS and statistical calculation they could belong to the degradation products of the loprazolam. A 6-10% decline in percent peak areas in two days old samples hints at some sort of degradation happening. The mass spectrometer used had a turbo ion spray interface which could cause molecule to disintegrate while carrying out LC-MS but the spontaneous formation of these products in the sample could be verified from the previous research work and the literature [39][44]. The exact molecular determination of the nature of these substances was beyond the scope of this experiment. The tailing of the peaks can be reduced by further dropping the pH to a more acidic value and by choosing a new column which is highly deactivated due to proper end-capping [40]. The slight broadening of peaks can be minimised by checking all the connections of the HPLC system for proper fit. If possible reducing the lengths and diameter of the tubings will help in obtaining sharper and much better gaussian peaks. The main limitations of the study were the small sample size used and small number of observation taken due to time constraints. A great deal of time was spent on finding the right column, a suitable mobile phase and buffer for the study. Finding an optimum mobile phase concentration and pH for adequate resolution and retention time required multiple HPLC readings. The most commonly available column, equipment and chemicals, which were easily available in most laboratories, were used for this study. Use of more sophisticated column or chemicals might result in better outcomes. All these limitations are guiding factors which can be utilized in further studies to achieve better results.

## VII. CONCLUSION

The aim of this experiment was to develop and validate a RP-HPLC technique to accurately quantify loprazolam mesilate tablets. The method was developed after careful study of the existing available literature. A thorough understanding of the techniques and principle of RP-HPLC was developed before and during the project. The selection of column, pH and the mobile phase was based on theory as well as numerous failed attempt to finally obtain valid chromatograms. The graphs obtained showed adequate separation of the active drug molecule and showed consistency in retention time on repeated readings. The method developed was quite selective and efficient but statistical calculations carried out on the peak areas of different concentrations of drug gave percent RSD values of over 2% which are higher than the limit set by ICH guidelines for pharmaceuticals, which states that % RSD should be equal to or less than 2%. The per cent peak area for the loprazolam was between 90 to 99% in both repeatability and robustness testing which made other minor peaks insignificant in comparison.

This method has the potential for further improvement as due to time constraints and limited availability of the HPLC equipment only three to four readings were taken per sample. More number of readings can give better statistical results. Adequately shaped peaks were achieved though there is still room for improvement in reduction of width and tailing effect. More care during sample preparation and storage will definitely yield better results. Nevertheless, the method provides important insight into the properties of loprazolam mesilate and its behaviour in the sample solution.

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