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Research Article

Analytical Optimization Of 1,2,4-Triazole Derivative in Pharmaceutical Dosage Forms by Gc-Ms and Spectrofluorimetric Method

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ABSTRACT

This article mainly emphasizes on simple, effective and selective analytical procedures especially two methods adopted for quantification of Letrozole in pharmaceutical dosage forms by using gas chromatography with mass spectrometry and spectrofluorimetric techniques. Method 1: GC-MS involves separation using column with temperature programming. The EI mass spectrum was characterized by [M]⁺ at 416 and a base peak at m/z 73 for Letrazole respectively. Quantification of the analytes were based upon estimating peak areas. Method 2: The suggested method is depended upon measuring the relative fluorescence intensity in distilled water and methanol (40%v/v:60%v/v) at an excitation and emission wavelength of 364nm and 407nm respectively. The reliability and analytical performance of the mentioned methods includes linearity, ranges, precision, accuracy, robustness, ruggedness and quantification limits were validated statistically. The proposed methods were successfully applied for the determination of Letrazole in commercially available tablet formulations.

INTRODUCTION

Triazoles is titled as one of the most potent nitrogen-based heterocycles with predominant pharmacological applications extending across a

wide range. Because of the structural specificities, both 1,2,3-triazoles and 1,2,4-triazoles can adopt a broad series of substituents around the core molecule for the generation of diverse bioactive

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molecule. [1] The unique structure of triazoles aids to make bonds with a variety of enzymes and receptors and induces extended spectrum of pharmacological activities. [2] Gas chromatography as well as gas chromatography-mass spectroscopy have proven to be the most powerful analytical technique with wide range of application background. GC-MS coupling can be used in characterization and recognition of unknown components within a mixture. The foremost coupling of GC-MS was reported in late 1950's. Mass spectrometry detector are appropriate in both full scan mode and SIM mode. In full scan mode, scanning the mass range recorded over m/z 50 to m/z 500 is scanned where in SIM mode, only the m/z of the preferred components are analysed. Since Helium is inert in both GC system and MS detector, it has been demonstrated to be a suitable carrier gas for GC. [3]

Letrazole (LTZ), 4,4'-(1H-1,2,4-triazole-1-ylmethylene)bisbenzotrile, (**Figure 1**) belongs to the category of reversible nonsteroidal aromatase enzyme inhibitor. Letrazole induces its mechanism of action by competitively obstructing the aromatase enzyme by conjugating with the heme portion of the microsomal enzyme cytochrome P450 subunit and reduces estrogen biosynthesis.[4]

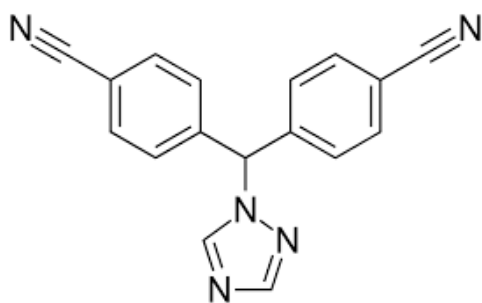


Figure 1: Chemical structure of Letrazole

With respect to literature evidences, we came to a conclusion that there is no reported works on gas chromatographic and fluorometric method for the estimation LTZ in pharmaceutical as well as bulk formulations till this date but the studies regarding

quantification of Letrazole in human plasma[5], rat plasma[6-8], pharmaceutical formulations using UV[9], HPLC[10-13,] LC-MS/MS. [14] For furnishing a novel impact, through this article, we aimed to develop a fast and user-friendly methodology without time-wasting sample preparation steps for routine analysis for the estimation of LTZ using GC method with MS detection and spectrofluorimetry. The described methods were entirely validated and flawlessly employed for the assay of the stated drug in its tablet dosage form.

MATERIALS AND METHODS

Drugs and Reagents

The investigated drug, Letrazole (Amneal Pharmaceuticals, India) demanded 99.5 % purity. Chromatographic grade methanol, ethanol and water was procured from Sigma Aldrich, India. The commercial Letroz tablets (Sun pharmaceuticals Ind. Ltd, India) claimed to include 2.5mg letrazole per tablet.

Chromatographic System

The Shimadzu (Model QP2020) Auto sampler AOI 20i was adopted to perform a GC-MS study of the ethanolic extract of LTZ. The apparatus characterizes a capillary column of 30 m x 0.25 mm ID x 0.25 μ m utilized with helium and pumps at an average of 1.2 mL/min. The temperature of oven was automated as follows, with the injector running at 290 $^{\circ}$ C: 70 $^{\circ}$ C for two minutes, accompanied by a slow climb to 250 $^{\circ}$ C after ten minutes. One microlitre of the sample was instilled in the split mode (1:10) with the help of a syringe. The spectrometer was automatized at an ionizing voltage of 70 eV having a mass range of m/z 35-550. NIST libraries and a comparison of their retention indices act as the basis for component determination. The components were identified by comparing them to those found in the NIST computer library, which is connected to the GC-MS device. The outcomes acquired were then tallied.

Spectrofluorimetric System

The study was conducted with a Shimadzu (RF-5301) fluorimeter to estimate the fluorescence ranges in pharmaceutical formulations. The light source employed for the measurement was 15 watt Xenon lamp and quartz cells having a dimension of 48mm height, 10mm in path length and 0.5mm slit width were accommodated for measurement. A preliminary investigation was done to determine the excitation and emission wavelength. Initially, LTZ solutions made of 50ng/mL concentration was prepared using suitable solvent and the prepared solutions were scanned fluorometrically to get the corresponding excitation and emission wavelengths. The scanning was executed over 200nm to 600nm and excitation as well as emission wavelength were noticed as 364nm and 407nm respectively.

Preparation of Stock and Standard Solutions

Letrazole stock and standard solutions were made in distilled water at the concentration of 10 µg/mL. Later practical solutions of LTZ were prepared by progressive dilutions of the already made stock solution in distilled water. All the solutions were readied on a daily basis.

Accurately weighed 5mg of LTZ reference standard were transferred into 10ml volumetric flask and made upto mark with methanol to obtain 1000 microgram per ml solution (stock solution 1). The prepared stock solution 1 was further diluted to acquire calibration standards.

Procedure for Pharmaceutical Preparation

A total of 10 LTZ tablets were properly weighed and ground to fine powder. For GC-MS method, a quantity of the tablet powder equal to 0.1 mg was weighed and added 50mL of methanol in a 100 mL calibrated flask and then the flask was sonicated to 10 minutes at room temperature to ensure complete dissolution of drugs. Methanol was added to the flask until it reached the desired volume and the solutions were filtered through Whatman filter paper no. 42 and suitably diluted

to get a final concentration (100, 200, 300, 400 500 ng/mL) within limits of linearity for the respective proposed method. For spectrofluorimetric method, sample equivalent to 0.5 mg was weighed and diluted to obtain final concentration (50, 150, 250, 350, 450 ng per ml) using methanol as solvent system.

Validation of Analytical Results

The results obtained were thoroughly validated statistically and evaluated as per International Conference on Harmonization (ICH) guidelines Q2 (R1)¹¹. The validation protocols includes linearity, precision, accuracy, limit of detection, quantitation limit, accuracy, range, specificity, system suitability, and robustness etc.

Specificity of the test was validated on comparison with the retention time of each peak of standard solution to those in sample solutions. The blank solution should not compromise with analysis in both methods. The specificity was evaluated by the standard edition method.

To evaluate the linearity of analytical technique, five working standard solutions at varying concentration ranges (100-500ng/ml for GC-MS, 50-450ng/mL for fluorimetry) were introduced into the system in three individual replicates. A calibration curve was constructed by graphing concentration of QC samples on X-axis and average peak area on Y-axis to demonstrate that the instrumental response was directly proportional to the analyte concentration. Regression equation and value of co-relation coefficient was computed.

A signal/noise ratio of at least 10:1 was considered as LOQ while a ratio of 3:1 was considered as Limit of detection or LOD.

To conclude the accuracy of the proposed method, various quality control solutions were made separately from stock solutions and analyzed at level of 100%, 120 % and 180% (GC/MS) and 80%, 100% and 120% (Fluorimetry) of its



predefined concentrations by standard addition method.

The precision of the developed method was evaluated in respect to repeatability and intermediate precision. For determination of repeatability, triplicate evaluations were done on the same day at three strengths. In similar fashion, the constructed solutions were analyzed over the course of on three consecutive days in order to determine intermediate precision,.

Robustness is one of the focal parameters as a tiny accidental change in the method like solvent composition, pH, buffer strength etc. may arise during normal use and negatively impact the method's effectiveness. It is anticipated that such a tiny change has no effect on performance.

Robustness was performed by changing the solvent composition for both methods.

Ruggedness refers to the degree of reproducibility of the test results produced by analysis of the samples under a variety of instrumental conditions.

RESULTS

Gas chromatography of the drug was estimated by scanning the range of the peak area by using Shimadzu LC solution method with temperature setting.

The mass spectrum of LTZ (**Figure 2**) was characterized by $[M]^+$ at 416 and a base peak at m/z 73 with analogues peaks at m/z 207, 281 and additional ions of low relative abundance.

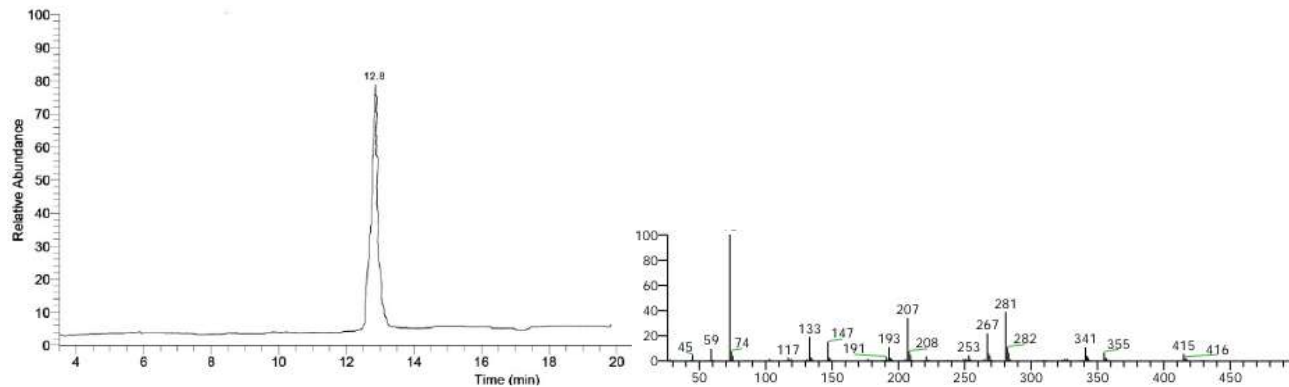


Figure 2. Gas Chromatogram and Mass Spectra for Letrazole

The spectrum of letrazole solution in the range of 200-400nm exhibited absorption maxima at 518nm. The result also demonstrated no

interference of the excipients used in formulation at the absorption maxima (**Figure 3**).

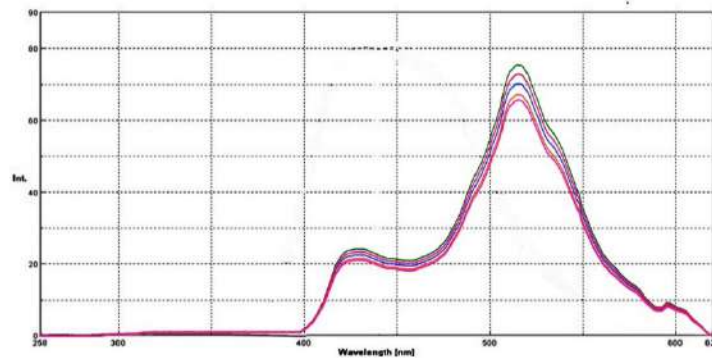


Figure 3. Flourimetric Overlain Spectra of Letrazole

The proposed methods were efficaciously applied to quantify letrazole in commercial tablets. The

amount of LTZ in the marketed formulation was found to be 2.4721 ± 0.2328 mg and

2.4536±0.1903mg with %assay of 98.88% and 98.14% (Table 1) for gas chromatographic and spectrofluorimetric method.

Table 1: Assay of Letrazole in Tablet Formulations

	Labelled claim (mg)	Amount estimated (mg) AM±SD	%Purity	%RSD
Method 1	2.5 mg	2.4721±0.2328	98.88%	0.2333
Method 2	2.5 mg	2.4536±0.1903	98.14%	0.1910

Method Validation

Linearity and Range

Letrazole stock solution was diluted consequently to obtain a concentration range of 100-500ng/mL (for GC/MS) and 50-450ng/ml (for fluorimetry), calibration curves were constructed using concentration verses peak area response and FI reading (Figure 4). The reported data were analyzed by least squares and the regression equation which relate peak area and FI to its

corresponding concentration was computed. The calibration coefficients or R² of all the calibration curves were reliably beyond 0.99, which fulfills analytical method criteria. Thus the linearity was upheld over the concentration range. The regression equations were computed from the calibration graphs, along with standard deviations of the slope and intercept on the ordinate. Table 2 provided more specifics on the linearity parameters.

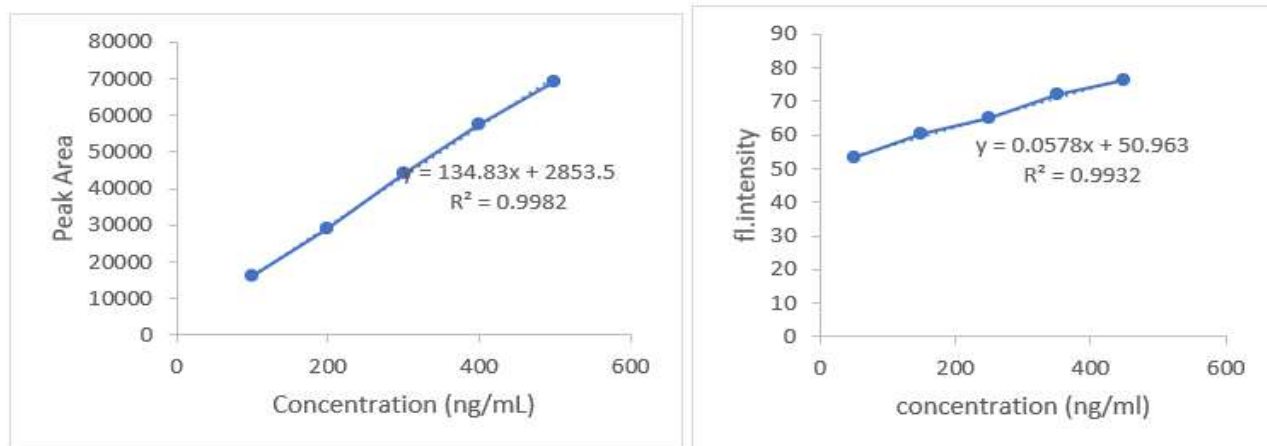


Figure 4. Linearity Curve for Gas Chromatographic Estimation of Letrazole , Linearity Curve for Spectrofluorimetric Estimation of LTZ

Limit of Detection and Quantification

using signal noise ratio approach, the LOD and LOQ of the method were computed based on the

standard deviation of lowermost concentrations and the slope got from the calibration curve, and the findings are displayed in Table 2.

Table 2: Validation Parameter of the Proposed Method

Linearity parameters	Method 1	Method 2
Linearity (ng/ml)	100-500	50-450
Slope	134.83	0.0578 x
Intercept	2853.5	50.963
R ²	0.9982	0.9932
Linearity equation	Y=134.83 x+ 285.5	Y= 0.0578 x + 50.963
LOD (ng/ml)	0.1099	0.8621
LOQ (ng/ml)	0.3332	3.7615

Accuracy and Precision

The test's reproducibility was assessed by repeating the same LTZ concentration at different time intervals on the same day for three consecutive days. The accuracy can be established at 10µg/ml concentration, and at any stage during determination, the results produced would be accurate. In GC-MS, at 100%, 120%, and 180 % mean recovery of the formulations were found to

be in between 98.11-100.24%. In spectrofluorometer, mean recovery obtained was between 97.86 to 100.11 % at 80%, 100% and 120%. % RSD was found to be less than 2 in both techniques. **Table 2** demonstrated that there is a high degree of confidence between the obtained and actual values, indicating ideal accuracy of the designed procedure.

Table 2: Accuracy Result of GC-MS and Spectrofluorimetric Method

	% Recovery	Amount added ng/mL	Amount recovered ng/mL	% Recovered	%RSD
Method 1	100	0.1	0.9919±0.5963	99.19%	0.6012
	120	0.1	1.185±0.1658	99.03%	0.1675
	180	0.1	1.450±0.1086	99.53%	0.0915
Method 2	80	0.5	0.3929±0.6628	98.23%	0.6628
	100	0.5	0.4928±0.2265	98.55%	0.2298
	120	0.5	0.5930±0.2613	98.84%	0.2644

*Minimum 3 determinant's

Robustness and Ruggedness

Robustness for the specified GC-MS and spectrofluorimetric methods were performed by altering the solvent composition and proved that it does not affect the conductance of the method. The percentage RSD values lies in the middle 0.125 and 0.6284. Ruggedness was carried out by analyzing the concentration at different experimental conditions. Results of sample analysis and data processing unveils % relative standard deviation values in between 0.169-0.639.

DISCUSSION

GC-MS and fluorimetry is titled as the most sophisticated and widely used analytical technique for both qualitative and quantitative analysis. GC-MS generally holds high-resolution and high-sensitivity with exceptional precision and accuracy. Wide range of meta-analysis relating to this topic established that there are no cases of experiments with GC-MS and fluorimetry on LTZ were reported. This current research is pointing on a sensitive technique for the determination of drugs bearing triazole nucleus in its core structure.

The main intention of the validation is to get accurate, reliable and consistent data. The results collected from the validation procedures prove the quality, quantity and accuracy of the API within pharmaceutical formulations. The amount of LTZ in the tablet formulation was found to be 2.4721±0.2328mg and 2.4536±0.1903mg. The percentage assay of LTZ was 98.88% and 98.14%, which is in the acceptable range of 98.0-101.0% as per ICH guidelines. The percentage RSD manifested the reliability of the designed methods. The GC-MS method is validated by conducting linearity, range, accuracy, precision, LOD and LOQ analysis. From the analysis, the method illustrate high linearity ($r \geq 0.998$) over the concentration range of 100-500ng/mL for all the selected formulations. The validity was demonstrated by recovery studies using typical addition method. The results were found to be reproducible with extremely minima SD and RSD. There were no signs that common excipients were interfering. The RSD for inter-day and intra-day variations were beneath 0.1616 % for all solutions, which falls below the acceptance criteria. The

LOD and LOQ values were noticed to be 0.15 ng/ml and 0.35 ng/ml respectively. The lowest values of LOD and LOQ is more suitable for the evaluation. The obtained precision results also hold within the acceptance criteria as well. The accuracy of the results proved that the mentioned approach does not require any internal standard. The study revealed a linear correlation between relative fluorescence intensity and LTZ serial standard solutions within 50-450ng/mL when the reaction conditions found optimal. It is clear that the curve followed linear regression line, $y=0.0578x + 15.966$. The goodness of fit (R^2) was 0.9932, shows maximum linearity. The results of the recovery of LTZ yielded a good accuracy finding (98.23-98.84). LOD and LOQ were found to be 0.8621 and 3.7615 respectively. Lower LOQ portraits that the method is highly sensitive enough to measure Letrazole content of samples at its lower level. The proposed method was confirmed to be precise; the %RSD was in the acceptable range and the P value of statistical results was >5 . Obtained RSD values for robustness found to be below 2 and it is depicted that the method is robust and rugged in nature. Therefore, when compared to the previously stated methodologies, the proposed approach is more accurate and precise and user friendly.

CONCLUSION

In a nutshell, the two proposed method were sensitive, simple, straightforward, accurate, precise and reasonably priced for routinely analysing Letrazole in bulk and pharmaceutical formulations. The methods are found to be direct, requiring minimal sample preparations, sample recoveries in all the formulations and in promising agreement with their respective label claims. Ultimately, the proposed methods were reliable for those drugs having a triazole nucleus, and making them convenient for quality control purposes. Additionally, the two methods were implemented

for content symmetry test for the stated drug in its tablet formulation.

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ABBREVIATIONS

GC-MS- Gas chromatography with mass spectrometry, LTZ- Letrazole, QC- Quality control, FI- Fluorescence Intensity, LOD- Limit of detection, LOQ- Limit of quantification, AM- Arithmetic Mean, SD- Standard Deviation, % RSD-Percent Relative Standard Deviation, API- Active Pharmaceutical Ingredient, ICH- International Conference for Harmonization.

CONFLICT OF INTEREST

We do not have any conflict of interest.

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