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Research Article

To Develop Simple, Accurate and Precise Method for Analysis of Drug in the Blood Plasma and Validate Them for Trazodone Hydrochloride

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Abstract

The pharmaceutical dosage forms are widely present with multiple active components i.e. in combined dosage forms. This has opened new task for analyst for simultaneous estimation of different drugs in such combined dosage forms. In the present study, a successful attempt was made for the HPLC quantitative estimation of drug used for the treatment of hepatitis C in marketed combination. The entire work was performed on waters HPLC with U.V. Vis detector. The result obtained shows the developed method to be precise, simple, rapid and accurate. Thus these can be used for routine analysis of Trazodone HCl in bulk drug and tablet dosage form. The simplicity, rapidity reproducibility and economy of the proposed methods completely fulfill the objective of this research work.

Keywords: Trazodone Hydrochloride, HPLC method, Analysis of drugs.

Introduction

Chromatographical method

Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; a separation is achieved.

Chromatography principles

Retention of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by adsorption, either physical adsorption, in which the binding forces are weak and easily reversible, or chemisorptions, where strong bonding to surface can occur[5-7]. Another important mechanism of retention is partition, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert material or chemically bonded to it.

Classification of Chromatography

Chromatographic methods can be classified according to the nature of the stationary and mobile phases.

The different types of chromatography are

1. Adsorption chromatography
2. Partition chromatography
3. Ion exchange chromatography
4. Size exclusion or gel permeation chromatography.

The modern instrumental techniques of GLC and HPLC provide excellent separation and allow accurate assay of very low concentrations of wide variety of substance in complex mixtures.

High Performance Liquid Chromatography

Liquid chromatography is based upon the phenomenon that, under the same conditions, each component in a mixture interacts with its environment differently from other components. Since HPLC is basically a separating technique, it is always used in conjunction with another analytical tool for quantitative and qualitative analysis.

Modes of Separation:

a. Normal Phase

In normal phase mode, the stationary phase (e.g. silica gel) is polar in nature and the mobile phase is non-polar. In this technique, non-polar compounds travel faster and are eluted first. This is because less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because more affinity towards stationary phase and takes more time to be eluted from the column.

b. Reverse phase

In reverse phase technique, a non-polar stationary phase is used. The mobile phase is polar in nature. Hence polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster.

Table 1: Normal vs. Reversed Phase HPLC

	Normal Phase	Reverse Phase
Packing polarity	High	Low
Solvent polarity	Low	High
Elution order	Non-polar first, then polar	Polar first, then non-polar
Effect of increasing solvent polarity	Decreases retention time	Increases retention time

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DRUG PROFILE:

Trazodone Hydrochloride

A serotonin uptake inhibitor that is used as an antidepressive agent. It has been shown to be effective in patients with major depressive disorders and other subsets of depressive disorders. It is generally more useful in depressive disorders associated with insomnia and anxiety. This drug does not aggravate psychotic

symptoms in patients with schizophrenia or schizoaffective disorders.

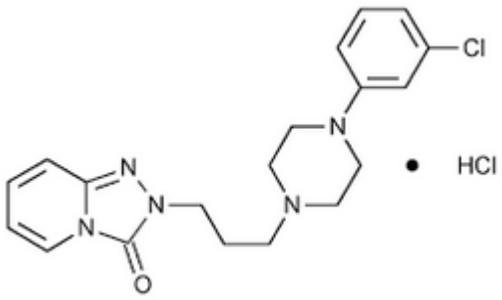

Figure 1: Structure of Trazodone Hydrochloride
Mol. Weight: 408.325
Chemical Formula: $C_{19}H_{23}Cl_2N_5O$
IUPAC Name: 2-{3-[4-(3-chlorophenyl)piperazin-1-yl]propyl}-2H,3H-[1,2,4]triazolo[4,3-a]pyridin-3-one hydrochloride
Pharmacology
Indication: For the treatment of depression.
Pharmacodynamics: Trazodone is an antidepressant and hypnotic chemically unrelated to tricyclic, tetracyclic, or other known antidepressant agents. The mechanism of trazodone's antidepressant action in man is not fully understood. In man, trazodone is well absorbed after oral administration without selective localization in any tissue. Since the clearance of trazodone from the body is sufficiently variable, in some patients trazodone may accumulate in the plasma.
Mechanism of action: Trazodone binds at 5-HT2 receptor, it acts as a serotonin agonist at high doses and a serotonin antagonist at low doses. Like fluoxetine, trazodone's antidepressant activity likely results from blockage of serotonin reuptake by inhibiting serotonin reuptake pump at the presynaptic neuronal membrane. Absorption: Rapidly and almost completely absorbed following oral administration. Food may decrease the rate and extent of absorption.
Protein binding: 89-95% bound to plasma proteins in vitro.
Uses
This medication is used to treat depression. It may help to improve your mood, appetite, and energy level as well as decrease anxiety andinsomnia related
to depression. Trazodone works by helping to restore the balance of a certain natural chemical (serotonin) in the brain.

Materials and Methods

Characterization and identification of Trazodone HCl
Physiochemical characteristics
Description- Solid, white to off white powder.
Melting point- M.P. of the drug was 222-227ºC found through Melting point apparatus.
Solubility- Solubility of Trazodone HCl was established by I.P. method. Results of solubility shown in Table: 5
Identification
FTIR spectrum- IR absorption spectra of Trazodone HCl was obtained by KBr pellet method.
Spectra of pure Trazodone HCl shown in the Figure: 2
Chemicals and solvents

Table No. 2 : Chemicals and Solvents Used

S. No.	Chemicals	Manufacturer
1	Trazodone HCl	Working standard , Aurobindo Pharma Limited
2	Methanol (AR Grade)	Merck Ltd., India
3	Acetonitrile (HPLC)	Merck Ltd., India
4	Methanol (HPLC)	Merck Ltd., India
5	Water (HPLC)	Merck Ltd., India

Determination of solubility
Solubility of Trazodone HCl was performed in different solvents and result was shown in table 5.

Analytical method development by HPLC:-
Mobile Phase Selection
Initially to estimate Trazodone HCl number of mobile phase in different ratio were tried. Results were shown in Table 6.
Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Acetonitrile: Methanol, 50:50 v/v. The mobile phase was filtered through 0.45µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min.
Selection of wavelength
10 mg of Trazodone HCl was weighed accurately and transferred to a 100ml volumetric flask, and the volume was adjusted to the mark with the mobile phase. From above solutions of 0.1 ml was transferred to 10 ml volumetric flasks, and make up the volume up to mark. Resulting solution was scanned over UV range (200-400nm), maximum absorbance was found at Lambda max 264.0 nm.
Selection of Separation Variable
Standard drug solution of Trazodone HCl was prepared in different mobile phase and chromatograph was recorded by using different column (5 and 10 µm) at different chromatographic condition like different flow rate and temperature. Considering the theoretical facts and after several trials separation variables were selected which were constant during whole experiment.
System Suitability Parameters
Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min. After complete saturation of column, three replicates of working standard of Trazodone HCl 10 µg/ml was injected separately. Peak report and column performance report were recorded for all chromatogram.
Preparation of Standard Stock Solution
10mg of Trazodone HCl was weighed accurately and transferred to separate 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000ppm.
Preparation of Working Standard Solution
From stock solutions of Trazodone HCl, 1 ml was taken and diluted up to 10 ml. from this solution 0.5, 1.0, 1.5, 2.0, 2.5 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 100 ml with methanol, gives standard drug solution of 5, 10, 15, 20, 25 µg/ ml concentration.

Preparation of the Calibration Curves of the Drug

Standard drug solutions were injected 3 times and the mean peak area of drug was calculated and plotted against the concentration of the drug. The regression equation was found out by using this curve. A typical chromatogram and the calibration curve are shown in figure 7.6.

Analysis of tablet formulation

Assay of tablet formulation

For analysis of the tablet formulation, weight equivalent to weight 10 mg of Trazodone HCl was transferred to 10 ml volumetric flask and dissolved in mobile phase. The solution was shaking vigorously for 20 mins and filtered through Whattman filter paper no.41, then volume was made up to mark with mobile phase. From the above solution 1 ml of solution was taken and diluted to 10 ml with mobile phase to get a solution containing 100µg/ml. From the above solution 1 ml of solution was taken and diluted to 10 ml with mobile phase to get a solution containing 10 µg/ml of Trazodone HCl . The amounts of Trazodone HCl in tablet formulation were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. Result is shown in Table 9.

Validation

Linearity

Linearity of analytical procedure is its ability (within a given range) to obtain test, which are directly proportional to area of analyte in the sample. The calibration plot was contracted after analysis of five different (from 5 to 25 µg/ ml) concentrations and areas for each concentration were recorded three times, and mean area was calculated. The regression equation and correlation coefficient of curve are given and the standard calibration curve of the drug is shown in figure. From the mean of AUC observed and respective concentration value, the response ratio (response factor) was found by dividing the AUC with respective concentration.

Accuracy

Recovery studies were performed to validate the accuracy of developed method. To preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed.

Precision

(A)Repeatability

Standard dilutions were prepared and three replicates of each dilution were analyzed in same day for repeatability and results were subjected to statistical analysis. Standard dilutions were prepared and three replicates of each dilution were analyzed in different days and by different analysts. Statistical analysis was carried out.

(B) Intermediate Precision

(a) Day to Day

(b) Analyst to Analyst

The intermediate precision expresses with in laboratories variation: different days, different analysts, different equipment etc. The standard dilution was prepared and three replicate of each dilution were analyzed by different analysts for all the developed methods. The statistical analysis method was carried out and the data is presented in Table 14.

Robustness

As per ICH norms, small, but deliberate variations, by altering the pH and / or concentration of the mobile phase were made to check the method capacity to remain unaffected. The effect of change in pH of mobile phase, flow rate, mobile phase ratio on the retention time, theoretical plates, area under curve and percentage content of Trazodone HCl was studied.

Detection limit and quantitation limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

Forced degradation studies

In order to determine whether the method is stability indicating, forced degradation studies were conducted on Trazodone HCl powder and the analysis was carried out by HPLC with a U.V. detector. 20µl of each of forced degradation samples were injected.

1. Acid degradation:

50 mg of Trazodone HCl sample was taken into a 50 ml round bottom flask, 50 ml of 0.1 M HCl solution was added and contents were mixed well and kept for constant stirring for 8 h at 80ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of Trazodone HCl.

2. Alkaline hydrolysis:

50 mg of Trazodone HCl sample was taken into a 50 ml round bottom flask, 50 ml of 0.1 M NaOH solution was added and contents were mixed well and kept for constant stirring for 8 h at 80ºC. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of Trazodone HCl.

3. Oxidative degradation:

50 mg of Trazodone HCl sample was taken into a 50 ml round bottom flask, 50 ml of 3% hydrogen peroxide solution was added, and contents were mixed well and kept for constant stirring for 24 hr at room temperature. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of Trazodone HCl.

4. Thermal degradation:

50 mg of Trazodone HCl sample was taken in to a petri dish and kept in oven at 50ºC for 4 weeks. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of Trazodone HCl.

Results and Discussions

Result of FTIR of Trazodone HCl of Trazodone HCl

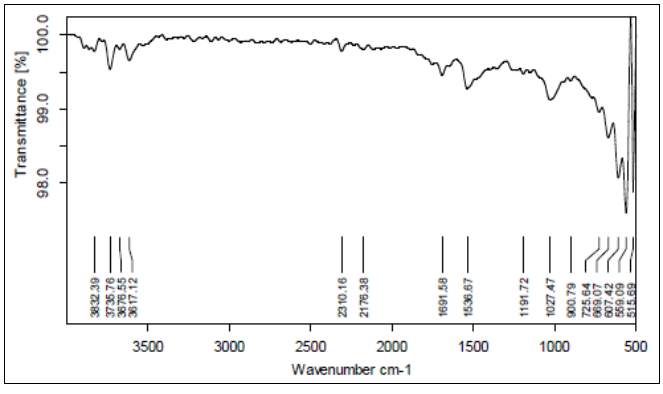

Figure 2: FTIR spectra of Trazodone HCl

Method- RP-HPLC

Table 3: Chemical Reagent used method development

Chemicals/Reagents	Grade	Company
Acetonitrile	HPLC	Merck
Methanol	HPLC	Merck
Potassium dihydrogen phosphate	AR	Rankem
Water	HPLC	Milli-Q
Triethanolamine	AR	Thomas Baker
Orthophosphoric acid	AR	Hi Media

Table 3: Chemical Reagent used method development

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Triethanolamine	AR	Thomas Baker
Orthophosphoric acid	AR	Hi Media

Results of Solubility

Solubility of drug was observed by dissolving them in different solvents.

Table 5 : Solubility of drug in different solvents

Solvent	Solubility
	Trazodone HCl
Water	Slightly Soluble
0.1N HCl	Slightly Soluble
0.1N NaoH	Slightly soluble
Methanol	Soluble
Acetonitrile	Soluble
Acetate Buffer	Slightly soluble
Phosphate Buffer	Soluble

Chromatograms of mobile phase trial

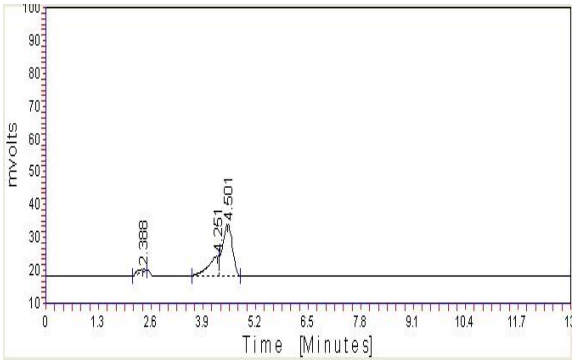

Figure 4: Mobile phase selection (Acetonitrile: water, 50:50 v/v)

Results of HPLC method development

Determination of λ_{max}

Accurately weighed 10 mg of drug was transferred into 10 ml volumetric flasks and dissolved in 10 ml of methanol and vortex it to get complete dissolution. From that 0.1 ml of stock solution dissolve in 10 ml of methanol which gives 10µg/ml of standard solution of Trazodone HCl.

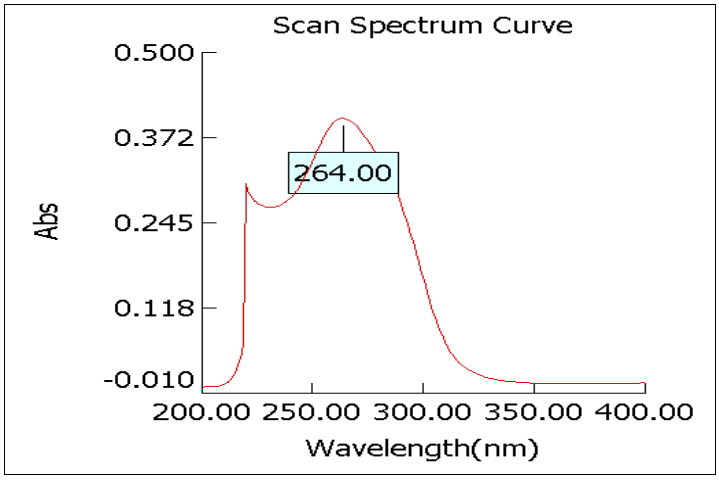

Figure 3: Determination of λmax of drug

Selection of Mobile Phase

Initially to estimate Trazodone HCl in fix dosage form number of mobile phase in different ratio were tried. A result was shown in Table 6.

Table 6: Mobile phase selection

Mobile Phase	Ratio	Retention Time
		Remark
Methanol : Water	50 : 50 v/v	Tailing in peak
Acetonitrile : Methanol	50 : 50 v/v	Most suitable

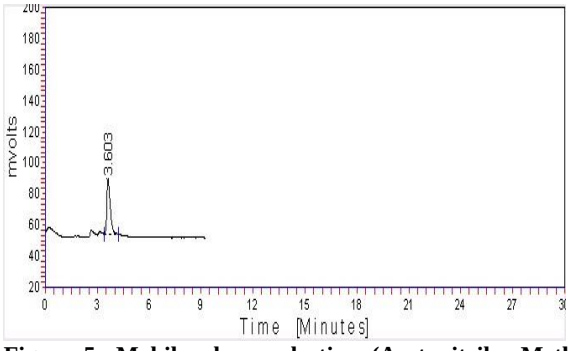

Figure 5: Mobile phase selection (Acetonitrile: Methanol, 50:50 v/v)

Selection of Diluent

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials mobile phase was used as diluents.

Selection of separation variable

Table 7.: Selection of separation variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5µ
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	
Methanol	50%
Acetonitrile	50%
Diluent	Acetonitrile
Flow rate	1.0 ml/min
Temperature	Ambient
Sample Size	20 µl
Detection wavelength	264 mm
Retention time	
Trazodone HCl	3.603 ± 0.3 min.

Preparation of the calibration curves of the drug

Each of the working standard solutions were injected 6 times and the mean peak area ratio of each drug to that of internal standard were calculated and plotted against the concentration of the drug. The regression of the concentration of each drug over the mean peak area ratio was obtained and these regression equations were used for the assay of tablets containing these drugs. A typical chromatogram and the calibration curves are shown later in figure 6.

Table 8: Linearity of Trazodone HCl

Standard Concentration µg/ml	Area under Curve (AUC)						Mean
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
0	0	0	0	0	0	0	0
5	452.236	461.875	456.698	472.321	469.985	462.623	452.236
10	895.569	889.458	885.478	892.125	888.745	890.275	895.569
15	1256.458	1250.248	1265.478	1260.365	1274.458	1261.401	1256.458
20	1680.256	1720.145	1715.589	1715.458	1730.478	1712.385	1680.256
25	2145.569	2155.478	2145.741	2165.478	2170.245	2156.502	2145.569

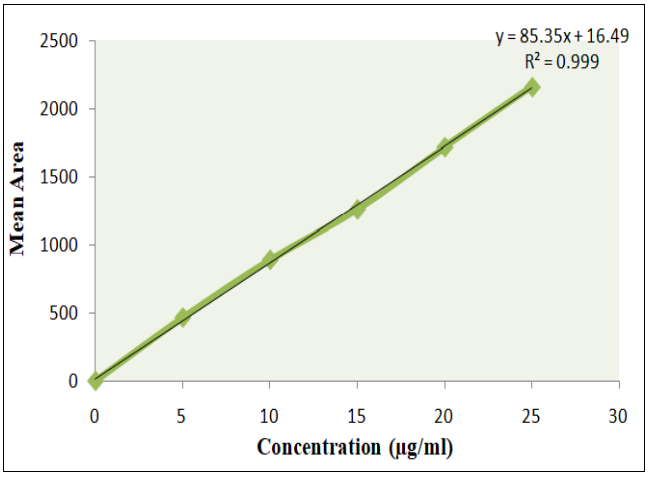

Figure 6: Calibration Curve of Trazodone HCl Regression Equation

Y= **mx +c,** AUC = 85.35 conc. + 16.49

Y= AUC

m= slope = 85.35

X= Conc. in µg/ml

c= Intercept 16.49

r^2= 0.999

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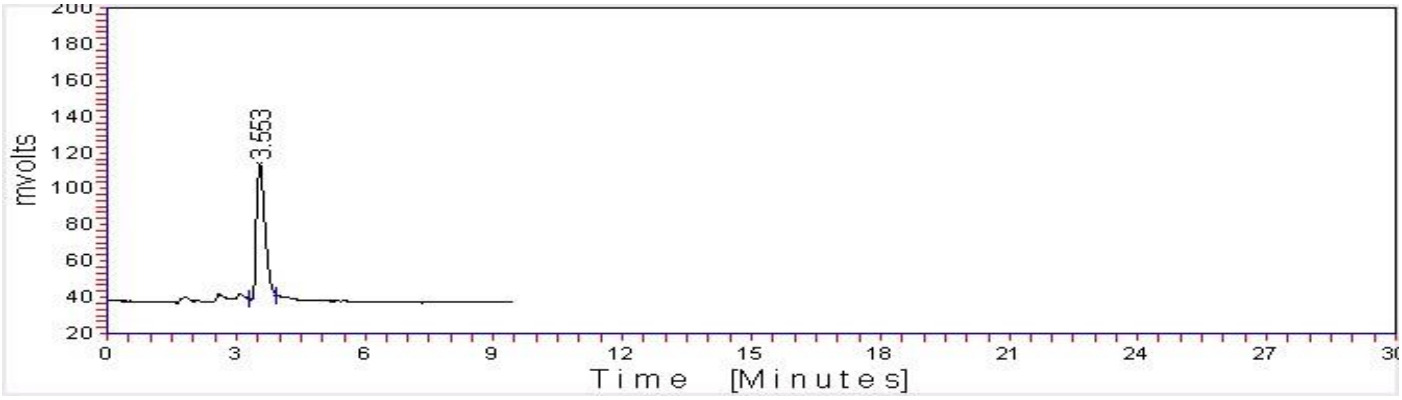

Figure 7: Chromatogram of Trazodone HCl

Analysis of Tablet Sample

Twenty tablets were taken and their average weight was determined. They are crushed to fine powder; amount equal to 10 mg of Trazodone HCl was taken in 100 ml volumetric flask. The volume is made up to the mark by methanol and filtered by whatmann filter paper (no.41) and the filtrate was used to prepare samples of 10µg/ml concentration.

Table 9: Analysis of tablet sample

Validation of developed method

Linearity

From the mean of AUC observed and respective concentration value, the response ratio (response factor) was found by dividing the AUC with respective concentration. The Curve was plotted between response ratio Vs. Concentration.

S. No		Drug
		Trazodone HCl
1.	Mean	99.25
2.	S.D.	0.125
3.	% RSD	0.365

Table 10: Response Ration Data for Linearity of Trazodone HCl

Replicates	Concentration (µg/ml)	Mean AUC	Response Ratio
Rep-1	5	466.4462	93.289
Rep-2	10	891.1573	89.116
Rep-3	15	1262.089	84.139
Rep-4	20	1719.763	85.988
Rep-5	25	2161.328	86.453
Mean		87.794	
S.D.		3.174	
R.S.D.		3.615	

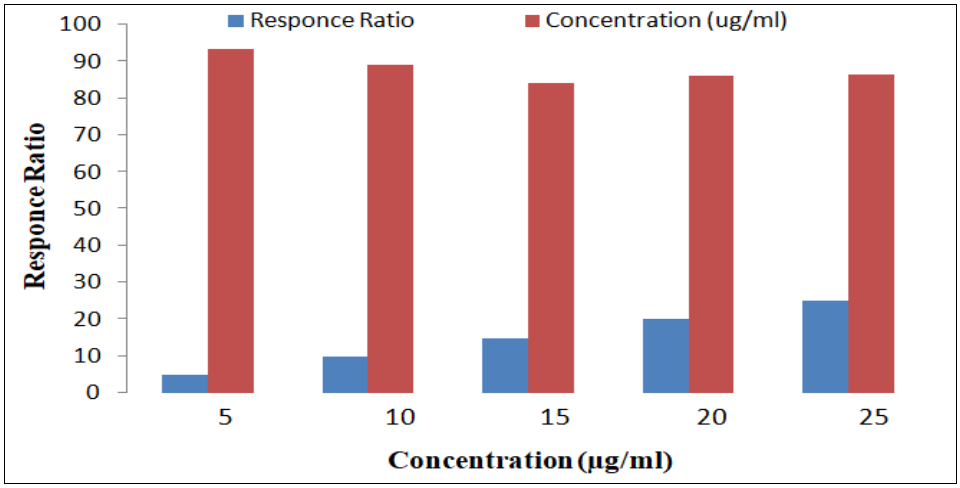

Figure 8: Response Ration graph for linearity of Trazodone HCl

Accuracy

Recovery studies were performed to validate the accuracy of developed method. To preanalysed sample solution, a definite concentration of standard drug was added and then its recovery was

Conc. of drug in sample (mg)	**10**	**10**	**10**
Std drug added (mg)	**8**	**10**	**12**
Replicate 1	7.9	9.95	12.05
Replicate 2	7.99	10.02	11.93
Replicate 3	7.85	9.86	11.95
Mean	7.913	9.943	11.977
SD	0.058	0.065	0.052
%RSD	0.732	0.659	0.438
Mean % Recovery	98.750	99.500	100.417

Table 11: Recovery study for accuracy of Trazodone HCl

Precision Repeatability: Standard dilutions were prepared and three replicates of each dilution were analyzed in same day for repeatability and results were subjecte to statistical analysis.

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Table 12: Result of repeatability for Trazodone HCl

Conc. of drug in sample µg/ml	**5**	**10**	**15**	**20**	**25**
Replicate 1	4.98	9.95	14.85	20.12	24.56
Replicate 2	5.01	9.99	14.99	19.95	24.52
Replicate 3	4.99	9.98	15.02	19.85	24.85
Mean	4.995	9.98	14.965	19.98	24.7325
SD	0.0125	0.017	0.074	0.112	0.154
%RSD	0.250	0.174	0.497	0.558	0.621

Standard dilutions were prepared and three replicates of each dilution were analyzed in different days and by different analysts. Statistical analysis was carried out.

Intermediate Precision: (A) Day to Day

Table 13: Result of intermediate precision for Trazodone HCl

Conc. of Drug in sample µg/ml Replicate	**5**	**10**	**15**	**20**	**25**
Replicate 1	4.85	9.95	14.23	19.98	24.56
Replicate 2	4.75	9.68	14.85	19.65	24.23
Replicate 3	4.65	9.89	14.69	19.85	24.15
Mean	4.8125	9.88	14.6925	19.87	24.485
SD	0.087	0.117	0.268	0.137	0.197
%RSD	1.817	1.189	1.822	0.692	0.805

Intermediate Precision: (B) Analyst to Analyst

Table 14: Result of intermediate precision for Trazodone HCl

Conc. of Drug in sample µg/ml Replicate	**5**	**10**	**15**	**20**	**25**
Replicate 1	5.02	10.02	14.65	19.98	24.56
Replicate 2	5.06	10.05	14.85	19.95	24.85
Replicate 3	4.98	9.98	14.78	19.62	24.89
Mean	5.015	10.0125	14.82	19.8875	24.825
SD	0.0328	0.029	0.088	0.164	0.150
%RSD	0.653	0.287	0.595	0.825	0.604

Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

Table 15: LOD and LOQ of Trazodone HCl

Name	LOD (µg/ml)	LOQ (µg/ml)
Trazodone HCl	0.16	1.48

Results of Forced Degradation studies

Table 16 : Results of Forced degradation studies of Trazodone HCl

Stress conditions	Drug recovered (%)	Drug decomposed (%)
Standard drug	99.85	0
Acidic hydrolysis	83.26	16.74
Alkaline hydrolysis	82.23	17.77
Oxidative degradation	90.23	9.77
Photolytic degradation	94.45	5.55

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