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DEVELOPMENT AND VALIDATION OF RP- HPLC METHOD FOR DETERMINATION OF VENLAFAXINE HCL IN HUMAN PLASMA.

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ABSTRACT

A rapid and sensitive RP-HPLC method has been developed and validated for quantification of Venlafaxine hydrochloride in human plasma. The human plasma samples were extracted by using anhydrous diethyl ether. Alltima^{TM} C_8 column provided chromatographic separation of the analyte. The proposed method has been validated with linear range of 2-25 ng mL for Venlafaxine hcl. The precision and accuracy values are within 10%. Total elution time was 10 min. The all validation parameters lie within the acceptable limits.

KEYWORDS: Venlafaxine hcl, Human plasma.

INTRODUCTION

Venlafaxine, 1-2-(dimethylamino)-1-(4-methoxyphenyl) ethyl -cyclohexanol hydrochloride is a new phenethylamine bicyclic antidepressant, which has a neuropharmacologic profile distinct from that of existing antidepressants including tricyclic compounds. It imparts antidepressant effects by inhibiting the neuronal uptake of nor epinephrine, serotonin and dopamine and lacks the adverse side effect profile of tricyclic antidepressants Venlafaxine is well absorbed in humans and undergoes extensive metabolism in the liver and has several metabolites, one of which is biologically active. Venlafaxine is extensively metabolized to O-desmethyl Venlafaxine (ODV), a major metabolite with an activity profile similar to that of Venlafaxine. The analytical method developed extraction procedure like liquid–liquid extraction, long run time and high quantification limit. It is necessary, therefore, to develop a simple, specific, rapid and sensitive analytical method for the quantification of the Venlafaxine hcl.

This paper describes development and validation of a simple, specific, rapid and sensitive

Bioanalytical RP- HPLC method for the determination of Venlafaxine hcl in human plasma with a Lower limit of quantification (LLOQ) of 2 ng mL for VEN with a run time of 10 min.

METHOD

Chemical and reagent

The reference standards of Venlafaxine hcl (purity: 99.67%) were obtained from Wockhard Pvt Ltd (Aurangabad, India) . HPLC grade methanol and acetonitrile were purchased from E. Merck Ltd. (Mumbai, India). Drug free (blank) heparinized human plasma was obtained from a Synapsis Lab. (Pune, India) and was stored at −20 °C prior to use.

Calibration curves

The Stock solutions of Venlafaxine were prepared in methanol of 1000 μg mL Secondary working standard solutions were prepared from stock solutions by dilution with methanol: O-phosphoric acid 0.1% (50:50, v/v). These diluted working standard solutions were used to prepare the calibration curve and quality control samples. Blank human plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of Venlafaxine. A six point standard curve of Venlafaxine was prepared by spiking the blank plasma with appropriate amount of Venlafaxine. The calibration curve ranged from 2 ng to 25 ng mL Quality control samples were prepared at three concentration levels of 5, 15 and 25 ng mL for Venlafaxine. The samples were vortexes and stored at –20 ± 2 0C until processing.

Sample preparation

A 0.6 ml aliquot of human plasma sample was mixed with 0.2 mL working standard solution 100.0 ng mL of Venlafaxine and 0.6 ml of 0.4M NaOH. The resulting solution was vortexed for 5 minutes and extracted with 9 ml of anhydrous Diethyl-ether, vortexed for 2 min and centrifuged at 3000 rpm for 15 min. Supernatants from the above solutions were separated evaporated at RT and diluted with mobile phase used for the analysis.

Instrumentation

Chromatographic separation was carried out in Acme-9000(Young Lin) HPLC with Alltima^{TM} C_8 (150 x 4.6 mm i.d, 5μ), column. A mobile phase consisting of a mixture of 0.1% O-phosphoric acid: methanol (50:50%v/v) was used with a flow rate of 0.7 mL min. The total run time for each sample analysis was 10 min. The data acquisition was ascertained by Autochrom-2000 software.

Validation

The method was validated for System suitability, linearity, precision, accuracy, and stability. System suitability was determined in terms of LLOQ (Lower Limit of Quantification) where the response of LLOQ was at least three times greater than the response of interference in blank matrix at the retention time of the analyte. For linearity, different concentrations of standard solutions were prepared to contain 2.0 ng mL to 25 ng mL of Venlafaxine hcl. These solutions were analyzed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor vs. concentration of the standard solutions. Standard curve fitting was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests .The precision of the method was determined by intraday precision and interday precision. The intra-assay precision and accuracy was calculated for five replicates at each Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The results are given in Table 3, 4.

Accuracy of the developed method was determined by relative standard deviation and % mean accuracy experiments. The relative recovery of the drug was calculated by comparing the amount of the drug obtained from the drug supplemented plasma with the actually added amount. Studies were carried out for three levels at six times and the percentage recovery, mean, standard deviation and coefficients of variation were calculated.

As part of the method validation, stability studying was carried out. Room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and bench top stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 hours by injecting four replicates of prepared stock dilutions of Venlafaxine hcl equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of Venlafaxine hcl and internal standard at 3 and 8 hours was carried out against the zero hour value. Refrigerated stock solution stability was determined at 2, 4 and 6 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. The stability studies of plasma samples spiked with

Venlafaxine hcl were subjected to three freeze - thaw cycles, short term stability at room temperature for 3 h and short term stability at – 20° C over six days. In addition, stability of standard solutions was performed at room temperature for 6 h and freeze condition for four weeks. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with Venlafaxine hcl was kept at room temperature for 1.00 to 3.00 h before extraction. The plasma samples for short term stability were stored in the freezer at -20^0C until the time of analysis.

RESULT & DISCUSSION

Method development

The objective of this work was to develop and validate a simple, rapid and sensitive Bioanalytical method for the quantification of Venlafaxine hcl. To achieve the objective, different options was evaluated to optimize sample extraction, detection parameters and chromatography during method development. The standard solutions of Venlafaxine hcl were analyzed by RP-HPLC.

Optimization of the chromatographic condition

Optimization of the chromatographic condition are intended to take into account the various goal of method development and to achieve each goal (resolution, runtime, sensitivity, peak symmetry, etc) accurately, according to the requirement of RP-HPLC methods being used for the estimation of drugs in the biological fluids. Different mobile phases, namely, acetonitrile, methanol and 0.1%OPA in aqueous phase were used at a flow rate of 1.0 or 0.7 mL min. For the initial separation conditions, acetonitrile was used because of its favorable UV transmittance, low viscosity and better solubility. When acetonitrile was substituted by other solvents, the solvents to buffer ratios were calculated using solvent strength. The resulting ratios of the mobile phase were prepared and the drugs chromatographed. These mobile phases gave well retained and symmetrical peaks. Methanol or 0.1% OPA was used as the mobile phase for further studies. The standard solution was chromatographed with mobile phases with different ratios of organic and aqueous phases at a flow rate of 1 mL/min or 0.7 mL min.0.1% OPA: methanol in the ratio of 50: 50 was selected as the mobile phase. Different reversed phase stationary phases (C_8, and C_{18}) were used and the chromatograms were recorded. Based on the retention and peak shape, Alltima ^{TM} C_8 column was selected for Venlafaxine hcl. Typical chromatograms of Venlafaxine hcl are given in Fig. 1.

Validation[7]

Estimation of Venlafaxine hcl in plasma was carried out using optimized chromatographic conditions. The validation parameters such as accuracy, precision (repeatability and reproducibility), linearity and range, sensitivity (limit of detection and limit of Quantitation), robustness/ruggedness, stability, selectivity/specificity and system suitability were evaluated. The validation results are given in Table 2 and 5.

Accuracy

The accuracy of the assay was calculated as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values, expressed as percentage. Accuracy should be measured using minimum five determinations per three concentrations (5, 15,25µg/ml).The accuracy of the optimised methods was determined by standard deviation and % coefficient of variation experiments. The percentage mean accuracy values for Venlafaxine hcl ranged from 94.00 to 96.52%. The coefficient of variation (%) of these values was less than 10.00%. It is indicative that the developed methods are accurate and reliable.(Table 3)

Precision

The optimized method for the estimation of Venlafaxine hcl was found to be precise. This was evident from the coefficient of variation values, which were less than 10.00% at all concentrations. The validation results are given in Table 4.

Linearity

It was observed that the optimised methods were linear within a specific concentration range for Venlafaxine hcl. The calibration curves were plotted between response factor and concentration of the standard solutions. The linearity ranges were found to be 2.00 to 25.00 ng mL. (Table 1)

Limit of Detection

The limit of detection (LOD) values was found to be 2.00 ng/mL for Venlafaxine hcl & their limit of Quantitation (LOQ) value was 5.00 ng/mL.

Ruggedness & Robustness

The ruggedness and robustness of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (operators, instruments, source of reagents and column of similar type) and optimised conditions (pH, mobile phase ratio and flow rate).

Stability studies[1, 7, 9]

The stability studies of plasma samples spiked with selected drugs were subjected to freeze-thaw cycles, short term stability at room temperature for 2, 4, 6 days. In addition, stability of standard solutions was performed at room temperature for 6 h and freeze condition for four days. The mean concentrations of the stability samples were compared to the theoretical concentrations. The results indicate that selected drugs in plasma samples can be stored for a week without degradation in frozen state. The results of short term storage at room temperature stability and freeze thaw cycles indicate no degradation of the selected drugs in plasma as well as in sample solution and hence plasma samples could be handled without special precautions. Stability data are given table 5.

Linearity

Table 1: Liniarity of Venlafaxine hcl

Conc.(ng/ml)	Area	R^2
2	125	0.998
5	300	
10	694	
15	1078	
20	1514	
25	1881	

System suitability

Table 2: System suitability of Venlafaxine hcl

Conc.(ng/ml)	Area	Mean	S.D	% C.V	% Mean Accuracy
LLQC (2 ng/ml)	2.01	2.104	0.0541	2.5	105
	2.12				
	2.13				
	2.12				
	2.14				

S.D.-standard deviation,

CV-coefficient of variance,

N-total number of observations for each concentration.

LLOQ- Lower Limit of Quantification

LQC - Lower Quality Control

MQC - Middle Quality Control

HQC - High Quality Control

Accuracy

Table 3: Accuracy of Venlafaxine hcl

Conc.(ng/ml)	Area	Mean	S.D	%C.V	%Mean accuracy
LQC 5ng/ml	4.72 4.76 4.86 4.89 4.70	4.78	0.0847	1.7	94
MQC 5ng/ml (n=5)	14.9 14.7 14.1 14.8 14.3	14.54	0.343	2.3	96.66
HQC 25ng/ml	24.7 25.3 24.3 24.2 25.9	24.88	0.715	2.8	99.52

Precision

Table 4: Prcission of Venlafaxine hcl

Conc. (ng/ml)		SD		%CV
		Interday	Intraday	
LQC 5ng/ml (N=3)	Day 1 2	0.492 0.489	0.494 0.485	9.6 9.4
MQC 15ng/ml (N=3)	Day 1 2	0.133 0.291	0.115 0.259	0.91 2
HQC 25ng/ml (N=3)	Day 1 2	1.21 0.512	1.22 0.650	4.7 2.01

Stability

Table 5: Stability studies of Venlafaxine hcl

Stability	Conc. (ng/ml)	Mean	SD	% CV	%Mean stability
Freez, Thaw & Auto sampler stability	LQC MQC HQC	5 14.9 25.2	0.106 0.081 0.057	2.1 0.543 0.22	86.2 100.6 99.2
Bench top stability	LQC MQC HQC	4.8 14.5 25.4	0.02 0.65 0.06	0.41 4.3 0.23	82.75 96.6 101
Short term stability	LQC MQC HQC	5.8 15 25.8	0.057 0.650 0.694	0.99 4.32 2.6	100 96.6 98.4

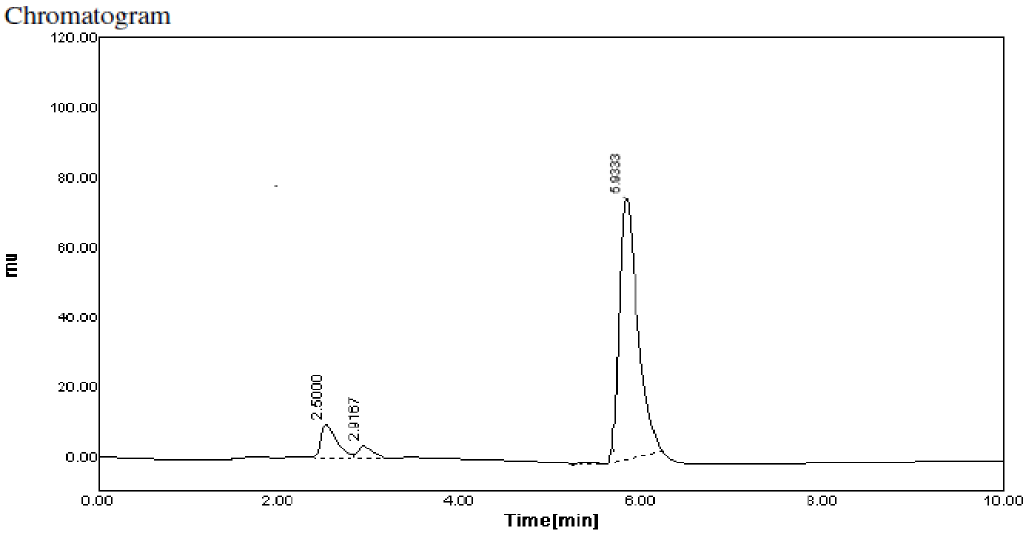

Figure 1: Chromatogram of Venlafaxine hcl.

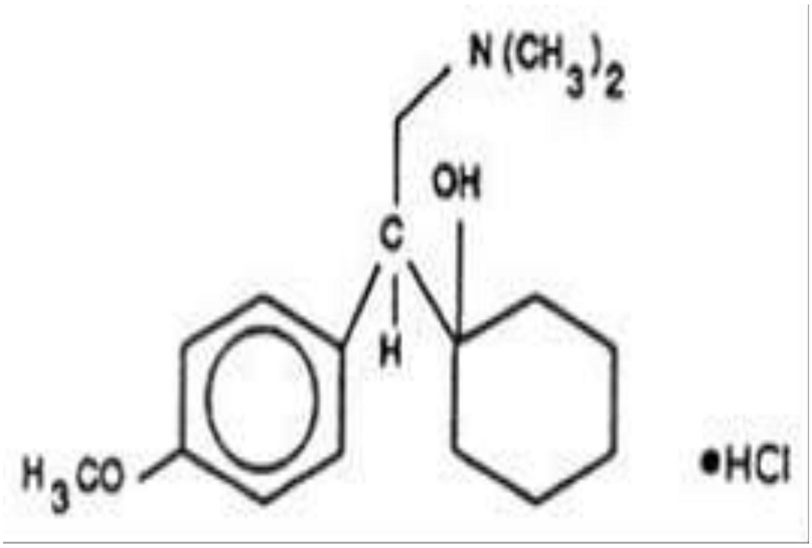

Figure 2: Chemical structure of Venlafaxine hcl

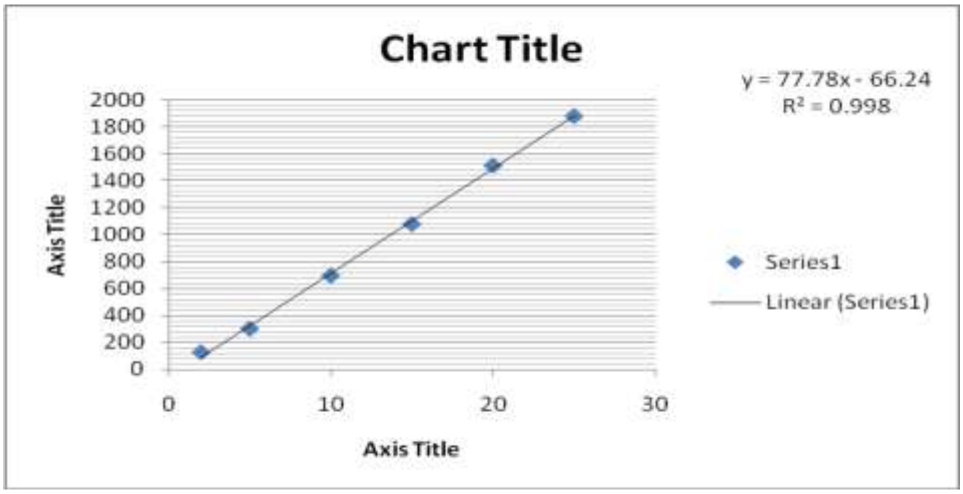

Figure 3: Liniarity of Venlafaxine hcl

CONCLUSION

A simple, specific, rapid and sensitive Bioanalytical method for the determination of Venlafaxine hcl in human plasma has been developed. The method provided excellent specificity and linearity for Venlafaxine hcl. The developed Bioanalytical RP-HPLC method was successfully & the validated parameters were found to be satisfactory and therefore the methods may be used for routine analysis.

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