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BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TIZANIDINE IN HUMAN PLASMA USING RP-HPLC

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ABSTRACT

A reverse phase HPLC has been developed and validated for estimation of Tizanidine in Human plasma. In this method, a reverse phase column Agilent – C18 Plasma 5 μ m (4.6 $\times$ 250 mm) as a Stationary phase, with a Mobile Phase of Methanol and 0.05% ortho phosphoric acid in water (pH 2.7) in the ratio of (20:80) at 0.7 mL/min flow rate was used with a DAD detection was carried out at 227nm. The volume injected was 20 μ L. The described HPLC method was linear over a concentration range of 10-50 μ g/ml. Clonidine was used as an internal standard. The Tizanidine showed retention time 4.347 min & Clonidine showed retention time 11.459 min respectively. The developed bioanalytical method was found to be linear in concentration range of 10-50 μ g/ml (R^2 = 0.999). The stability of the drug spiked human during three freeze thaw cycles was stable in plasma for about one month when stored at frozen state. Conclusions: It can be concluded that the developed bioanalytical method is capable of quantifying Tizanidine from spiked human plasma samples. The method meets the requirements of the ICH Guidelines and can be applied to Bioavailability or Bioequivalence studies of Tizanidine.

Keywords: Bio-analytical method, RP-HPLC, DAD, Validation, Tizanidine

INTRODUCTION

Bio-analysis is the method used to determine the concentration of drugs, their metabolites and /or endogenous substances in the biological matrices such as blood

plasma, serum, cerebrospinal fluid, urine and saliva ^[6] Bio-analytical methods are widely used to quantitative drugs and their metabolites in the physiological matrices

and the methods could be applied to studies in areas of human clinical pharmacology and non human pharmacology / toxicology. High performance liquid chromatography is one of important techniques in chromatography. HPLC is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems. HPLC is a versatile analytical tool useful in identification and quantitative estimation of low concentration of drugs and metabolites in biological matrices^[7].

Tizanidine hydrochloride is chemically, 5-chloro-4-(2-imidazolin-2-ylamino)-2, 1, 3-benzothiadiazole hydrochloride, is a short acting drug for management of spasticity. It is a 2-adrenergic agonist and centrally acting skeletal muscle relaxant^[8-9] (**Fig 1**).

Figure 1: Chemical structure of Tizanidine

Clonidine is chemically, of 2-[2, 6-dichlorophenyl) imino] imidazoline HCl. Clonidine belongs to a class of medications: alpha-adrenergic central agonists. It acts as a hypotensive agent and is used to treat blood pressure disorders, hyperactivity disorders, addiction migraine

headaches, and to manage opium and alcohol withdrawal symptoms^[10] (**Fig 2**).

Figure 2: Chemical structure of Clonidine

MATERIALS AND METHODS

Chemicals, reagents and Instrumental Conditions

Working Standard of Tizanidine was gifted by Swapnroop drug and pharmaceutical Aurangabad. And Clonidine internal standard was gifted by R.S I T C Jalgaon, Acetonitrile of HPLC grade by Merck, Methanol of HPLC grade by Merck, Orthophosphoric acid (Merck Ltd). All other reagents used were of HPLC grade. The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, (DAD) & Gradient Detector. Equipped with Reverse Phase (Agilent) C₁₈ column (4.6mm x 100mm; 2.5µm), a 20µl injection and UV730D Absorbance detector and running Chemstation 10.1 software.

WENSAR™ High Resolution Balance. Digital, VSI pH meter (VSI 1-B), Micro pipettes Cybero pipettor, Ultra Sonicator were used for investigation.

Chromatographic Conditions:

HPLC conditions stationary phase Agilent-C₁₈ 2.5 µm (4.6× 100 mm). Mobile Phase: Methanol and 0.05% ortho phosphoric acid

in water (pH 2.7) in the ratio of (20:80). Flow rate: 0.7 mL/min. Injection volume: 20 µl using auto sampler injector Detection: at 256 nm for Tizanidine. Mobile phase degassed using ultrasonicator. The experiments were carried out at room temperature of about 25-28 °C.

Preparation of Tizanidine standard stock solution:

Weighed accurately about 10mg of Tiza and transferred into 10mL of volumetric flask, added about 10 mL of diluent, shaken to dissolved and volume was made up to the mark with diluent. (concentration of Tiza is 1000 µg/ml) A-grade bulb pipette into 10 ml volumetric flasks and the solutions were made up to volume with mobile phase to give final concentrations of 10,20,30,40 and 50 µg/ml.

Preparation of plasma sample:

At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. 1.0 mL of sample was pipetted into 10 ml centrifuge tube with this 5mg of internal standard solution (500 µg/ml) and 10 mg of Tizanidine was added. The resulting solution was vortexed for 10 minutes and centrifuged at 5000 r/min for 10 min. 5000 RPM for effective phase separation. organic layer was pipette out into a separate tube and evaporated to dryness. The residue was then reconstituted with 10mL mobile

phase and subjected to chromatographic analysis.

METHOD VALIDATION:

The developed method was validated following ICH guidelines (ICH Q2R1) for accuracy, precision, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness.

Linearity:

Linearity is the ability of the analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical Tiza transformation proportional to the concentration Tizanidine of an analyte in samples within a given span at which the involved response is proportional to the analyte concentration.

Accuracy:

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy designate the digression between the convey merit found and the true merit. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against the standard and blank solutions to ensure that no interference exists.

Precision:

The exactness of a logic method is the level of accord among single test results get

when the method is pragmatic to many sampling of a similar sample. Precision is a measure of the dependability of the whole analytical process.

Limit of Detection (LOD):

The detection limit of an individual analyte procedure is the lowest amount of analyte which can be detected not necessarily quantified as an exact value. LOD was calculated using the following formula.

$$\text{LOD} = 3.3\sigma/S$$

Where σ is the standard deviation calculated from accuracy of the response and S is the slope from linearity

Limit of Quantification (LOQ)

The quantification limit of an individual analytical procedure is the lowest amount of analyte which can be quantitatively determined. LOQ was calculated using the following formula.

$$\text{LOQ} = 10.\sigma/S$$

Where σ is the standard deviation calculated from accuracy of the response and S is the slope from linearity.

System suitability parameter:

System suitability parameter is the evaluation of a composition of an analytical system to show that the performance of the system meets the standard required by the method. This parameter can be calculated experimentally to provide a quantities system suitability test report number of theoretical plates (efficacy) capacity factor,

separation (relative retention), resolution, telling factor relative standard deviation (precision).

RESULTS AND DISCUSSION

UV absorption of 10 $\mu\text{g/mL}$ solution of Tizanidine in METHANOL was generated and absorbance was taken in the range of 200-400 nm. λ_{max} of Tizanidine in Methanol was found to be 227 nm.

HPLC Method Development and Optimization

Initially, pure drugs solution was chromatographed using a mobile phase consisting of a mixture of 0.05% ortho-phosphoric acid in water (pH2.7) and Methanol in the ratio of (80:20) v/v at a flow rate of 0.7 ml/min. gives well-resolved peaks of drugs. Detection was carried out at 227 nm. The retention time under the optimized condition of Tizanidine and IS was found to be 4.347 min. & 11.45 min. respectively. The total run time of the chromatogram was about 15 minutes. A typical chromatograph of a mixture of standard and sample plasma and Tizanidine and internal standard is summarized by Fig. 4 and Fig. 5 respectively.

VALIDATION OF THE METHOD**System Suitability:**

The suitability of the system was demonstrated by assessing various parameters. It was established by

injecting two replicate injections of the standard solution. Theoretical plates were found to be 4142, tailing factor of 0.75, and %RSD of peak area was 0.19 for Tizanidine. All the system suitability parameters were well within the limits, indicating that the system was well suitable for performing the analysis.

Linearity

Linearity was established by the least-squares linear regression analysis of the calibration data. Calibration plots were linear over the concentration range of 10-50 µg/ml for Tizanidine. Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves. The linear curve of Tizanidine was shown in Fig. 4 respectively. The linear regression equation obtained was $Y=39.65x+3.178$ for Tizanidine with correlation coefficient 0.999 respectively. The results of linearity are shown in Table 1.

Accuracy:

Accuracy was computed by recoveries studies. The mean percentage recoveries values for three levels were found to be between 95.39-97.19% for Tizanidine respectively. The percentage of recoveries values within the limits, indicating the method developed was accurate. The results of recovery are shown in Table 2.

Precision

The %RSD of intraday precision and interday precision were 0.21 and 0.90 for Tizanidine. The percentage RSD of system, method, and intermediate precision study was well within the limits (<2%), indicate that the method was precise.

LOD and LOQ:

The LOD was found to be 0.1954 µg/ml For Tizanidine. The LOQ was found to be 0.5923 µg/ml for Tizanidine. The values of LOD and LOQ indicate that the method was greatly sensitive (Table 4).

Robustness

The robustness of the method was designed by changing the optimized condition adequately. To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between Tizanidine was evaluated. On the assessment of the result it can be deduced that the variation in the changing wavelength, the flow rate does not affect the method significantly. %RSD <2% specifies that the developed method was robust. The results of robustness are shown in Table 3.

Analysis of Tizanidine from marketed tablets

The percentage assay of tablet formulation was found to be 98.42 for Tizanidine respectively (Table 4).

Figure 3: UV spectrum of Tizanidine

Fig no.4: Chromatogram of Blank Plasma

Fig no.5: Chromatogram of Tizanidine and internal STD

Figure 6: Linearity curve of Tizanidine

Table 1: Linearity results of Tizanidine

Parameter	Tizanidine
Theoretical Plate	4759
Retention Time (Rt)	4.347
Tailing factor	0.75
%RSD	0.52

Table 2: Recovery Result

Drug	Level	Amount taken	Amount Found	% Recovery
Tiza	80	8	17.63	95.39
	100	10	19.72	97.19
	120	12	21.58	96.53

*Average of three determinations

Table 3: Robustness results

Condition		Tizanidine	
		SD	%RSD
Change in wavelength (210±1 nm)	226nm	1.27	0.16
	228nm	1.75	0.21
Change in flow rate (1.0±0.1 ml/min)	0.6	1.04	0.11
	0.8	1.69	0.24
Change in mobile phase (1.0±0.1 ml/min)	19+81	1.97	0.24
	21+79	2.73	0.34

*Average of three determinations, %RSD: Percentage relative standard deviation

Table 4: Summary of validation parameter

Parameter	Tizanidine
Calibration Range ($\mu\text{g/ml}$)	10-50
Optimized wavelength (nm)	227nm
Retention Time	4.347
Precision (% RSD)	0.21-0.90
% Assay	98.42
LOD ($\mu\text{g/ml}$)	0.1954
LOQ($\mu\text{g/ml}$)	0.5923

Average of five determinations, LOD: Limit of detection, LOQ: Limit of quantification

CONCLUSION

Based on the data presented in this report, it can be included that present method is validated for the estimation of Tizanidine in human plasma over a concentration range of 10-50 $\mu\text{g/ml}$. The precision and accuracy are very much within the prescribed limits in this concentration range. Expected recoveries are observed in the present processing technique for 80%, 100%, and 120%. The drug is found to be very stable to the effect of freeze thaw cycles.

Conflicts of interest

Authors declare that they have no conflict of interest exists in this investigation.

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