

Research Article

Reversed-Phase Liquid Chromatographic Internal Standard Method Using Losartan Potassium for Quantitative Estimation of Ivabradine Hydrochloride in Pharmaceutical Tablet Dosage Form and Plasma

Makhdoom HS^{1*}, Saeed A¹, Saleem F¹, Sultana K¹ and Mazhar M²

¹Department of Pharmacy, University of Lahore, Pakistan

²Obsons Pharmaceuticals, Quaid-e-Azam Industrial Estate, Lahore, Pakistan

***Corresponding author:** Makhdoom HS, Department of Pharmaceutical Chemistry, University of Lahore, Pakistan

Received: November 16, 2021; **Accepted:** December 07, 2021; **Published:** December 14, 2021

Abstract

A reversed-phase liquid chromatographic method for ivabradine hydrochloride using Diode Array Detector (DAD) and internal standard technique was developed and validated according to ICH and SWGTOX guidelines. The prime objective of this study was to develop a precise and accurate method that can be equally applicable to biological (plasma) as well as non-biological (active pharmaceutical ingredient and pharmaceutical tablets) matrices. Losartan potassium was used as an internal standard due to its easy availability. After liquid-liquid extraction using acetonitrile, the ivabradine hydrochloride and internal standard were chromatographed on Agilent 1200 series HPLC system equipped with DAD detector, auto-sampler and chemstation software. Analytical separation was achieved on Agilent C-18 (5 μ m, 25cm x 4.6mm) reversed-phase column at 30°C column oven temperature, 10 μ L injection volume and 286nm wavelength. Isocratic mobile phase system comprised of 60:40 v/v ratio of HPLC grade methanol and water adjusted to pH 6.8 using orthophosphoric acid was employed with 1mL/min flow rate. The method linear range was 0.025-3 μ g/mL (25-3000ng/mL) for pharmaceutical tablets and plasma with the coefficient of linearity ranged 0.997-0.999. Results for precision, accuracy, recovery, stability and matrix effect studies were within acceptable limits for both plasma and tablets. Method was successfully applied to the commercial tablet products and patient plasma samples to estimate the amount of ivabradine hydrochloride.

Keywords: Ivabradine; Losartan; Plasma; Tablets; Matrix factor; Liquid chromatography

Introduction

The chemical name for ivabradine hydrochloride [1] is 3-((3-(((7S)-3,4-Dimethoxybicyclo[4.2.0]octa-1,3,5-trien-7-yl)methyl) methyl amino) propyl)-1,3,4,5-tetrahydro-7,8-dimethoxy-2H-3-benzazepin-2-one, hydrochloride (Figure 1). Its empirical formula is C₂₇H₃₇N₂O₅Cl which corresponds to a molecular weight of 504.24g/mol.

Ivabradine hydrochloride is the first selective bradycardic agent which directly affects the pacemaker If current [2] of sinoatrial node. The U.S. Food and Drug Administration approved Corlanor (ivabradine) to reduce hospitalization from worsening heart failure on April 15, 2015. It blocks the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel responsible for the cardiac pacemaker If current, which regulates heart rate. In clinical electrophysiology studies, the cardiac effects were most pronounced in the Sino-Atrial (SA) node and there were no effects on ventricular repolarization and myocardial contractility [3]. It is whitish to pale yellow crystalline solid which is very much soluble in methanol as compared to ethanol, soluble in DMSO and slightly soluble in 0.1M HCL solution. Melting point of Ivabradine is 190-193°C [4].

Losartan potassium is an angiotensin II receptor antagonist which

is used for the treatment of hypertension and diabetic nephropathy [5]. Its chemical name is potassium;[2-butyl-5-chloro-3-[[4-[2-(1,2,4-triazol-3-azanidacyclopenta-1,4-dien-5-yl)phenyl] phenyl] methyl] imidazol-4-yl] methanol (Figure 2) and empirical formula is C₂₂H₂₂ClKN₆O which corresponds to a molecular weight of 460.12g/mol [6]. It is soluble in methanol.

Literature survey [7-19] reveals that limited analytical methods for determination of ivabradine hydrochloride employing internal standard in pharmaceutical preparations. The reported methods include few UV-Spectrophotometric and liquid chromatographic determinations using Diode array detector or tandem mass spectrometry. Most of these methods are either applicable to pharmaceutical preparations or biological samples. The one that was applicable to both type of samples utilized internal standard which is expensive and not easily available [20]. This study was aimed to develop and validate a simple, inexpensive and reliable method for determination of ivabradine that can be applicable on both biological and non-biological samples (Active pharmaceutical ingredient or pharmaceutical dosage forms). Validated method was applied to the commercially available pharmaceutical dosage forms and patient plasma. The use of internal standard rendered this method least affected by the potential matrix interferences.

Materials and Methods

Chemicals

Certified reference materials of Ivabradine hydrochloride and losartan potassium were provided by Obsons Pharmaceuticals (Lahore, Pakistan). Three commercial brands of Ivabradine marketed under brand names Coralan (5mg and 7.5mg tablets, Servier Laboratories, Pakistan), Ivaset (5mg and 7.5mg tablets, Highnoon Laboratories, Pakistan) and Ivatab (5mg tablets, Nabi Qasim Industries, Pakistan) were purchased from local market of Lahore-Pakistan. High-Performance Liquid Chromatography (HPLC) grade methanol and water were purchased from Honeywell (Seelze, Germany). Blank plasma was purchased from Biorad.

Instrumentation

Reversed Phase Liquid Chromatographic analysis of Ivabradine hydrochloride was carried out using an Agilent 1200 series HPLC system equipped with automatic injector, Chemstation software and diode array detector. Chromatographic separation was achieved on Ocatadecyl Silane (ODS) C-18 column (5 μ m, 25cm x 4.6mm). Other equipments used include Micro-analytical balance (Shimadzu), pH meter (Innotech), ultrasonic bath (Elmasonic), centrifuge (Eppendorf), microanalytical pipettes (Eppendorf), Nitrogen evaporator (PCi Analytics) and hot air oven (Pol-eko).

Chromatographic conditions

Isocratic mobile phase system comprised of 60:40 v/v ratio of HPLC grade methanol and water adjusted to pH 6.8 using ortho-phosphoric acid. 1mL/min mobile phase flow rate, 30°C column oven temperature, 10 μ L injection volume and 286nm wavelength were selected. The chromatographic data obtained thus was processed using Chemstation software.

Methods

Preparation of standard stock solution of Ivabradine hydrochloride (100 μ g/mL): Stock solution was prepared by dissolving 2.5mg of certified reference material of ivabradine hydrochloride in methanol quantity sufficient to 25mL, filtered it through 0.45 μ m syringe filter and stored in labeled storage flask. Solution was protected from light. The working standard solutions were prepared by appropriate dilution of this stock solution.

Preparation of internal standard stock solution of Losartan potassium (100 μ g/mL): Stock solution of certified standard of losartan potassium was prepared by dissolving 10mg in methanol q.s.100mL, filtered it through 0.45 μ m syringe filter and stored in labeled storage flask.

Preparation of mobile phase and diluent: Simple isocratic Mobile phase consisted of 600mL HPLC grade Methanol and 200mL HPLC grade water. The pH of buffer was adjusted to 6.0 with phosphoric acid. This mixture was filtered through 0.2 μ m cellulose acetate filter, transferred to solvent reservoir and degassed in ultrasonic bath prior to use. Mobile phase was used as diluent wherever required.

Preparation of blank sample solution of drug-free placebo tablets: 5mg powdered drug-free placebo tablets were weighed and transferred to a 100ml volumetric flask. Contents were dissolved in small amount of diluent using ultrasonic bath. Final volumes were made up to the mark using diluents, filtered through 0.45 μ m syringe

filter and stored in labeled storage flask. This solution was used as blank placebo sample for the preparation of calibration levels, positive and negative quality control samples used in this study to evaluate matrix effect.

Preparation of calibration levels and positive Quality Control (QC) standards in drug-free placebo tablets solution: These samples were prepared by spiking appropriate volumes of stock standard solution of ivabradine hydrochloride (100 μ g/mL) in blank placebo tablets solution.

Preparation of test sample solution of pharmaceutical tablets: Powdered tablets containing 5mg and 7.5mg of ivabradine hydrochloride were separately weighed and transferred to a 100ml volumetric flask. Contents were dissolved in small amount of diluent using ultrasonic bath. Final volumes were made up to the mark using diluents, filtered through 0.45 μ m syringe filter and stored in labeled storage flasks.

Patient's plasma sample collection: The patient's peripheral blood samples were collected into Ethylene Diamine Tetra Acetate (EDTA) purple-top collection vials. Plasma is separated from blood samples by centrifugation at 11,000 revolutions per minute (rpm) for 10 minutes. After centrifugation, plasma layers were separated, collected in labeled glass tubes and stored at -20°C for further use.

Preparation of calibration levels and positive quality control (QC) standards in plasma: These samples were prepared by spiking appropriate volumes of stock standard solution of ivabradine hydrochloride (100 μ g/mL) in blank plasma to compensate matrix effect.

Extraction procedure

500 μ L of spiked calibration levels, positive quality control sample, negative quality control sample in matrices and test samples (pharmaceutical test sample or plasma) were taken in labeled centrifuge tubes. 100 μ L of internal standard solution and 2mL of acetonitrile were added in all tubes. The tubes were vortexed and then centrifuged at 11000rpm for 10 minutes. The clear supernatants were transferred to clean labeled glass tubes and evaporated using nitrogen evaporator at about 50°C. The residues were reconstituted using 200 μ L diluents, vortexed, filtered using 0.45 μ m micro-syringe filters and then transferred to labeled HPLC glass vials with inserts.

Results and Discussion

The developed RP-HPLC internal standard method for determination of ivabradine hydrochloride in biological (plasma) and non-biological (pharmaceutical tablets) matrices was validated according to the ICH guidelines [21].

Analytical method validation for Pharmaceutical tablets

Concentration levels used in the validation study were prepared in blank placebo tablets and analyzed after extraction to compensate for matrix effect. The summarized validation study results for estimation of ivabradine hydrochloride in pharmaceutical tablets using proposed RP-HPLC internal standard method is presented in Table 1.

System suitability tests

It is the criterion to be considered in analytical method developments according to the pharmacopoeias. System suitability

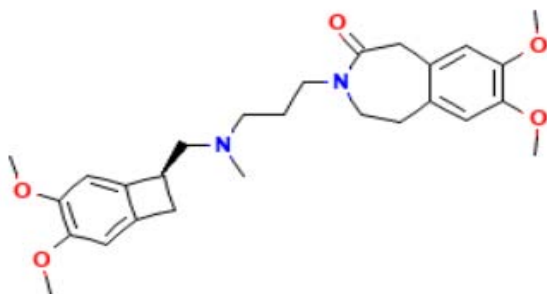


Figure 1: Two-dimensional structure of Ivabradine hydrochloride.

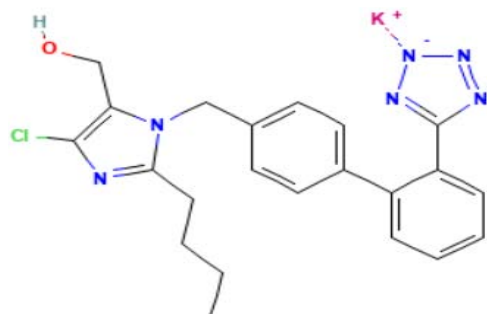


Figure 2: Two-dimensional structure of losartan potassium.

testing is an integral part of many analytical procedures. System suitability tests result are presented in Table 2.

Linearity study

Concentration level range 0.025-3.00 µg/mL for ivabradine hydrochloride was used to conduct linearity study. Each concentration level was run in triplicate per day for five days. Linear response of ratio of ivabradine hydrochloride and losartan potassium (internal standard) was observed in this linearity range with the coefficient of correlation (r^2) value ranged 0.997. The overlay chromatograms of five calibration levels and the linearity curve are shown in Figures 3 and 4 respectively. Acceptance value for coefficient of correlation should not be less than 0.985 therefore an acceptance criterion has been met.

Specificity study

The mobile phase was injected as reagent blank followed by the

injection of standard solution to check interference of mobile phase solvents at retention time of drug. Negative quality control sample was injected followed by the standard solution in placebo tablet matrix to access the matrix interference at retention time of drug. Absence of interfering peaks suggested that solvents and tablet matrix do not interfere in the estimation of ivabradine hydrochloride.

Analyte solution stability study

Pure drug standard solution (1 µg/mL) and drug solution (1 µg/mL) in tablet matrix were subjected to stability study. Solutions were analyzed immediately after preparation and then at different time intervals (1-24 hours at room temperature). The results are shown in Table 1. The precision and accuracy/recovery were found within acceptance criteria. Therefore, drug solution as well as drug solution in tablet matrix is stable for the period of 24 hours at room temperature. Results are presented in Table 1.

Limit of detection (LOD) & Limit of quantitation (LOQ)

Three replicate injections per day for five days of different concentrations of ivabradine hydrochloride in lowest concentration ranges were evaluated to determine LOD and LOQ of proposed method. The response of the drug that can be precisely detected using proposed method was 0.01 µg/mL. Signal-to-Noise (S/N) ratio at this concentration was 3.1:10 which is acceptable for estimation of LOD. The lowest concentration in the linearity curve that showed precise and accurate result selected as LOQ for the proposed method is the least concentration in the calibration curve, which is 0.025 µg/mL. The S/N ratio at this concentration was 10.3:1 which is acceptable for estimation of LOQ.

Precision and accuracy

Precision and Accuracy of this method were determined at three different concentration levels of drug. Results are presented in Table 1. Both accuracy and precision were in acceptable ranges which reflected that method is accurate and precise.

Bio-Analytical method validation in plasma

The simplest method for extraction of ivabradine from plasma using acetonitrile was developed and validated using SWGTOX guidelines [22].

Linearity

Linearity was accessed in the range of 0.025-3.0 µg/mL (25-3000 ng/

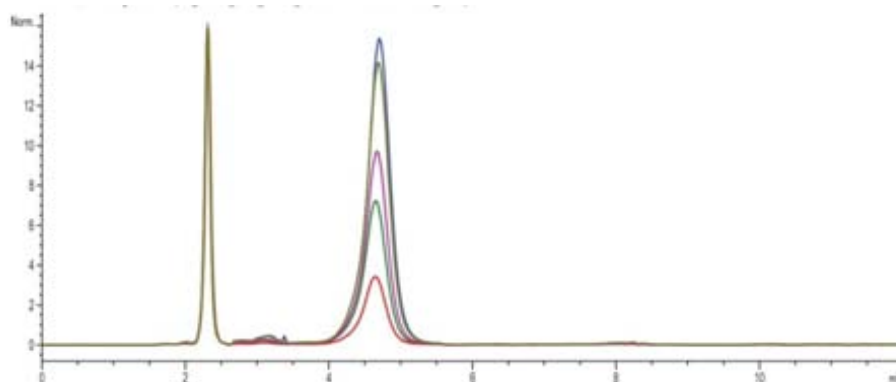


Figure 3: Overlay chromatograms of five calibration levels of Ivabradine hydrochloride.

Table 1: Results of Analytical Method Validation for Tablets.

Sr. no.	Parameter	Results
1	Limit of Detection (µg/mL)	0.01
2	Limit of Quantitation (µg/mL)	0.025
3	Linear range (µg/mL)	0.025-3.00
4	Coefficient of linearity (r^2)	0.997
5	Inter-day Precision (%CV)	0.32-0.45
6	Intra-day Precision (%CV)	0.61-0.87
7	Accuracy (%recovery)	92.81-98.97
8	Stability (%CV)	0.178

Table 2: Results of system suitability parameter.

Sr. no.	Parameter	Ivabradine hydrochloride
1	Retention time (min)	4.56
2	Theoretical plates (N)	2150
3	Tailing factor	1.1
4	Precision (%RSD)	0.3
5	Resolution (R)	20

Table 3: Method Validation results for Estimation of Ivabradine in plasma.

Sr. no.	Parameter	Results
1	Limit of Detection (ng/mL)	10
2	Limit of Quantitation (ng/mL)	25
3	Linear range (ng/mL)	25-3000
4	Coefficient of linearity (r^2)	0.999
5	Inter-day Precision (%CV)	5.21-10.60
6	Intra-day Precision (%CV)	1.14-6.32
7	Accuracy (%recovery)	93.2-99.5
8	Stability at room temp. (%CV)	5.34-11.82
9	Stability at 2-8°C (%CV)	3.61-12.13
10	Freeze and Thaw Stability (%CV)	2.98-10.17
11	Matrix Effect (Matrix Factor)	0.95

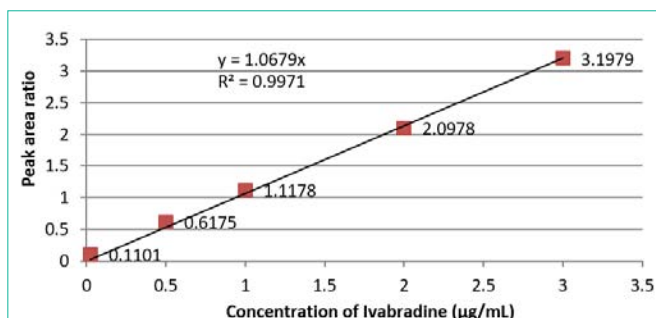
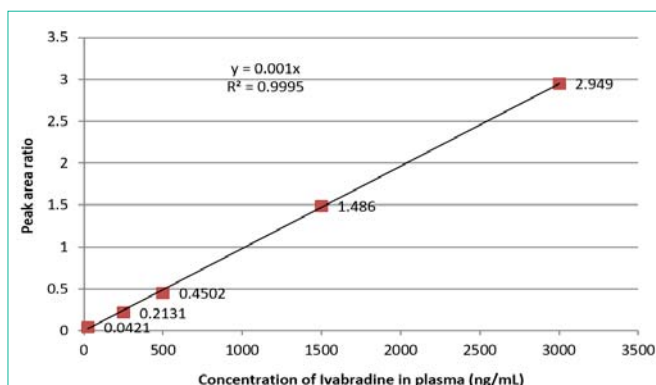
mL) of ivabradine. Matrix effect was compensated by preparing drug concentrations in blank plasma as well as use of internal standard. The response was found linear over the entire concentration range used. The coefficient of linearity was 0.997.

Matrix effect

Matrix factor was used to determine matrix effect. Matrix factor is the ratio of response in extracted plasma to that of standard solution. Matrix factor less than one represents suppression, greater than one signifies enhancement whereas equal to one depicts that there is no matrix effect in the analytical method [23]. Matrix effect was studied in the concentration range of 10-3000ng/mL. The matrix factor determined was 0.95 which shows that there is least matrix effect causing response suppression.

Accuracy and precision

Accuracy and precision studies at three different concentrations of ivabradine (30, 1000 and 2500ng/mL) were evaluated. The precision and accuracy were found within acceptable ranges of %CV≤10 and

**Figure 4:** Linearity Curve of Ivabradine hydrochloride in Pharmaceutical Tablet Dosage Form.**Figure 5:** Linearity Curve of Ivabradine hydrochloride in plasma.**Table 4:** Application of proposed method on commercial products and patient's plasma.

Sr. no.	Sample identity	Amount of Ivabradine obtained*
1	Coralan tablets, 5mg	4.82mg
2	Coralan tablets, 7.5mg	7.39mg
3	Ivaset tablets, 5mg	4.96mg
4	Ivaset tablets, 7.5mg	7.43mg
5	Ivatab tablets, 5mg	4.95mg
6	Plasma A	55.6ng/mL
7	Plasma B	28.7ng/mL
8	Plasma C	63.9ng/mL

*Average of three replicates.

±20%, respectively as shown in Table 3.

Recovery

Different volumes of acetonitrile were used to evaluate recovery of drug from plasma but 2mL of acetonitrile showed the best recovery. Recovery was within ±20% of the target concentration of ivabradine as shown in Table 3.

Stability study

Stability study of ivabradine in plasma was conducted at three storage conditions (room temperature, refrigeration temperature, freeze and thaw). The proposed method was found reproducible, accurate and precise. The linearity curve is shown in Figure 5 and bio-analytical method validation results are summarized in Table 3.

Application of proposed method on commercially available pharmaceutical tablets and patient plasma samples

The proposed validated method was applied for quantitative estimation of ivabradine in five commercial drug products and three patient's plasma samples. Patient's samples were collected and assayed on their consent. The results are presented in Table 4.

Conclusion

The proposed RP-HPLC internal standard method for the quantitative estimation of ivabradine in biological (plasma) and non-biological (pharmaceutical tablet dosage form) samples had qualified the acceptance criteria for all studied validation parameters according to ICH and SWGTOX guidelines. Simplest analytical HPLC method and extraction techniques had been employed to save the precious time of analysts as well as to efficiently cope up the work load of testing laboratories. The method was linear over the wide range of 0.025-3 μ g/mL (25-3000ng/mL) in pharmaceutical tablets and plasma. The method was precise and sensitive at concentration of 0.01 μ g/mL ivabradine hydrochloride. Therefore, the proposed method is reproducible, accurate and precise without significant matrix interferences that can be employed for bioequivalence studies of newly formulated pharmaceutical dosage forms.

References

1. National Center for Biotechnology Information. PubChem Compound Summary for CID 3045381. Ivabradine hydrochloride. 2021.
2. Sulfi S, Timmis AD. Ivabradine-The first selective sinus node I(f) channel inhibitor in the treatment of stable angina. *Int J Clin Pract*. 2006; 60: 222-228.
3. O'Neil MJ. The Merck Index: An Encyclopedia of Chemicals, Drugs and Biologicals. 14th ed. Whitehouse Station: Merck Research Laboratories, Division of Merck and Co. 2006: 907.
4. European Medicines Agency Science Medicines Health. Procoralan Epar Product Information: Summary of Product Characteristics. (Chairman: Harald Enzmann). Suresnes, France: Les Laboratoires Servier. 2006.
5. Timmermans PB, Duncia J, Carini D, Chiu A, Wong P, et al. Discovery of losartan the first angiotensin II receptor antagonist. *Journal of human hypertension*. 1995; 9: S3-18.
6. National Center for Biotechnology Information. PubChem Compound Summary for CID 11476710. Losartan potassium. 2021.
7. Korgaonkar G, Vaishali B, Jadhav, Jain A. Analytical method development and validation of ivabradine HCl in bulk and formulation. *Int J Thesis Projects Diss (IJTPD)*. 2018; 6: 8-13.
8. Gopinath Thete P, Bhanudas Saudagar R. Quantitative determination and validation of novel derivative spectrophotometric method for estimation of ivabradine hydrochloride in bulk and marketed formulation. *Asian J Pharm Pharm*. 2018; 4: 697-701.
9. Nowakowska J, Pikul P, Marcin, Ciura K. Application and validation of simple isocratic HPLC-UV-DAD method with dual wavelength detection for ivabradine determination and its application in the study of stress degradation. *Hindawi Journal of Chemistry Volume*. 2017: Article ID 2069571.
10. Maheshwari S, Khandhar AP, Jain A. Quantitative determination and validation of ivabradine HCL by stability indicating RP-HPLC method and spectrophotometric method in solid dosage form. *Eurasian J Anal Chem*. 2010; 5: 53-62.
11. Muzafar-ur-Rehman MO, Nagamallika G. Validated RP-HPLC method for the determination of ivabradine hydrochloride in pharmaceutical formulation. *Int J Pharm Sci Drug Res*. 2017; 9: 228-233.
12. Patil PA, Raj HA, Sonara GB. Q-absorbance ratio spectrophotometric method for simultaneous determination of atenolol and ivabradine hydrochloride in synthetic mixture. *Pharm Biol Evaluat*. 2016; 3: 27-33.
13. Patel H, Jivani N. Development of validated RP-HPLC method for simultaneous estimation of carvedilol and ivabradine. *World J Pharm Pharm Sci*. 2015; 4: 630-639.
14. Panda S, Patra S. Rapid and selective UV spectrophotometric and RP-HPLC methods for dissolution studies of ivabradine controlled release formulations. *Pharmatutor*. 2014; 2: 201-213.
15. Juanjuan J, Tian L, Huang Y, Li Y. Development and validation of a sensitive LC-MS/MS-ESI method for the determination of ivabradine in human plasma: Application to a pharmacokinetic study. *Biomed Chromatogr*. 2013; 27: 1603.
16. Motisariya MH, Govindbhai Patel K, Shah PA. Validated stability-indicating high performance thin layer chromatographic method for determination of ivabradine hydrochloride in bulk and marketed formulation: An application to kinetic study. *Science Direct*. 2013; 51: 233-241.
17. Rezowanur Rahman Md, Asaduzzaman Md, Ashrafur Islam SM. Development and validation of RP-HPLC method for analysis of ivabradine hydrochloride in tablet dosage forms. *Res J Pharm Biol Chem Sci*. 2012; 3: 1032-1043.
18. Seerapu S, Srinivasan BP. Development and validation of RP-HPLC method for estimation of ivabradine hydrochloride in tablet. *Indian J Pharm Sci*. 2010; 72: 667-671.
19. Klippert P, Jeannot JP, Polvé S, Lefevre C, Merdjan H. Determination of ivabradine and its N-demethylated metabolite in human plasma and urine, and in rat and dog plasma by a validated high-performance liquid chromatographic method with fluorescence detection. *J Chromatogr B Biomed Sci Appl*. 1998; 719: 125-133.
20. Polker A, Padmavathi Y, Kumar R, Babu NR. Quantitative bioanalytical and analytical methods for estimation of ivabradine hydrochloride in pure and pharmaceutical dosage form. *J Rep Pharm Sci*. 2020; 9: 1-10.
21. Validation of Analytical Procedures: Text and Methodology (ICH Q2(R1)) published by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human (ICH) is a tripartite harmonized guideline. ICH Secretariat, c/o IFPMA, Geneva, Switzerland.
22. Scientific working group for forensic toxicology (SWGTOX). Standard practices for method validation in forensic toxicology. SWGTOX Doc 003, Revision 1. 2013.
23. Patel GV. Review article on matrix effect in bioanalytical method development. *Int J Med Pharm Res*. 2015; 1: 159-165.