World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
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Available online at: http://www.wjpsonline.org/
Original Article

Bazedoxifene acetate quantification in rat serum with the aid of RP-HPLC: Method development and validation

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Received: 10-10-2015 / Revised: 18-11-2015 / Accepted: 25-11-2015

ABSTRACT

Development of simple, sensitive, accurate and reproducible RP-HPLC method for Bazedoxifene acetate estimation in rat serum using ratio of peak area of analyte to internal standard (Raloxifene) for a pharmacokinetic study is the objective of the study. The procedure involves simple liquid-liquid extraction of BAZ and IS from rat serum directly into acetonitrile which is injected onto a Hypersil BDS C_8 reverse phase stainless steel analytical column (4.6 × 150mm, 5µm) at 30^0C and detected using a PDA detector set at 290nm. Mobile phase consisting of buffer (Potassium dihydrogen orthophosphate) and acetonitrile (60:40, v/v) was used at a flow rate of 1.0mL/min. Retention times of BAZ and IS were 5.852 and 4.052 min respectively. The standard curve for BAZ was linear (r^2 >0.99) in the concentration range of 0.1 - 32µg/mL. Absolute recoveries of BAZ and IS were 90 - 110% and >95% respectively from rat serum. The lower limit of detection and quantitation of BAZ was 0.25µg/mL and 0.5µg/mL. The intra, inter–day precisions and accuracy in terms of % error were all found within the acceptable range. Stability studies results indicated that both analyte and IS were stable on bench top, autosampler and freeze-thaw cycles.

Keywords: RP-HPLC, Method development**,** Validation, Bazedoxifene acetate, Pharmacokinetic study

INTRODUCTION

Osteoporosis [1] defined as a skeletal and degenerative bone disease, occurs in postmenopausal women, and prevalence increases with age [2]. Osteoporotic fractures account for substantial healthcare costs, disability and mortality. Drug therapy plays a crucial role in both the prevention and treatment of osteoporosis when detected. Recommended therapies include estrogen supplementation, bisphosphonates, recombinant parathyroid hormone, calcitonin and selective estrogen receptor modulators (SERMs) [1, 2].

SERMs are a heterogeneous class of compounds that exert their pharmacologic effects at estrogen receptors (ERα and ERβ) [3]. Bazedoxifene acetate, which is most recently approved drug in the SERM's class for treating osteoporosis, is currently under development in an effort to maximize potential benefits on bone, lipids and breast tissue while minimizing endometrial hyperplasia and other adverse effects [4]. Such advancement in drug development may expand first-line treatment options for postmenopausal osteoporosis.

Bazedoxifene acetate (1*H*-Indol-5-ol, 1-[[4-[2-(hexahydro-1*H*-azepin-1yl) eZthoxy] phenyl] methyl] 2-(4-hydroxyphenyl)-3methyl, WAY-140424) is a nonsteroidal, indole-based estrogen receptor ligand [5, 6]. Within the class of SERMs, chemical differences in the location and structure of side chains determine tissue selectivity, pharmacologic action, and lead to a mixed functional activity at the estrogen receptors [7]. Bazedoxifene binds to both ERα and ERβ with higher affinity toward ERα. Bazedoxifene exerts

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pharmacologic activity by binding to estrogen receptors in bone tissue as an agonist promoting preservation of bone mineral density (BMD). At breast and uterine tissue, bazedoxifene acts as an antagonist, therefore lacking stimulation and proliferative activity within these tissues [8].

The aqueous solubility of bazedoxifene is poor with systemic exposure and extensive first pass metabolism. It has mean half-life of 28 hrs and maximum concentration is reached in 1-2hrs after oral administration. It is mainly excreted by feces (84.7%) while renal excretion is negligible [9].

Bazedoxifene acetate is not yet official in any pharmacopeia. Extensive literature survey revealed a reported analytical method for estimation in bulk by UV spectroscopy [10] and no analytical methods have been reported for its determination in pharmaceutical formulations and biological fluids. Among the various methods available for the determination of drugs, chromatography continues to be very popular, because of its simplicity, specificity, and low cost.

The main objective of the present work is to develop and validate a simple, sensitive, accurate, reproducible, improved and fast RP-HPLC method for determination of Bazedoxifene acetate in bulk which can be applied to pharmaceutical formulations and biological fluids as per ICH guidelines [11]. The method describes the application of the assay to determine the pharmacokinetic disposition after a single oral dose to rats.

MATERIALS AND METHODS

Bazedoxifene acetate and Raloxifene hydrochloride were kind gift from MSN Laboratories. Potassium dihydrogen orthophosphate was procured from Rankem and orthophosphoric acid from Rankem. All aqueous solutions for the HPLC mobile phase was prepared by milli Q (Millipore, USA) grade water. All the solvents used were of HPLC grade procured from sd fine–chem. limited.

The HPLC Waters system Model no. 2695 auto injector, with a PDA detector Model no. 2996 was used for analysis.

Preparation of standard solution: Standard solutions of Bazedoxifene acetate (BAZ) and Raloxifene hydrochloride (Internal standard, IS) are made in methanol. Appropriate dilutions of BAZ were made in methanol to produce working stock solution of 32,16,8,4,2,1,0.5,0.25,0.1µg/ml. The prepared solutions were stored at approximately 4ºC. Working stock was used to prepare serum calibration standards. A working IS solution (1µg/ml) was prepared in methanol. Calibration samples were prepared by spiking with the appropriate amount of the analyte and IS on the day of analysis into 100µL of control rat serum. Prior to spiking, methanol which is used to prepare standard solution of Bazedoxifene has to be evaporated by using vacuum oven followed by addition of 100µL of control serum to respective tubes. Samples for the determination of recovery, precision and accuracy were prepared by spiking control rat serum in appropriate concentrations into different tubes, and depending on the nature of the experiments were stored until further analysis.

Preparation of sample solution: To unknown concentration of standard, 100µL of serum sample, 100µL methanolic solution of (IS) was added and mixed on a cyclomixer (Remi instruments, Mumbai, India) for 1min followed by addition of 0.2mL acetonitrile, the mixer was vortex for 10mins followed by centrifugation (Remi instruments, Mumbai, India) for 10mins at 4000rpm. The supernatant was separated and 20µL is injected onto HPLC column.

Calibration curves: Calibration curves were acquired by plotting the peak area ratio of BAZ: IS against the nominal concentration of calibration standards.

Accuracy and precision: The intra-assay precision and accuracy were estimated by analyzing four replicates containing BAZ at four different levels, i.e. 4,2,1,0.5µg/mL. The inter –assay precision were determined by analyzing the four levels repeatedly for four times. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal value and precision within 15% relative standard deviation (RSD) [12, 13].

Stability experiments: The stability of BAZ and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 24hr (in the autosampler at 5ºC) after the initial injection. The peak areas of the analyte and IS obtained at initial cycle were used as the reference to determine the relative stability at subsequent points. Stability of BAZ in the biomatrix during 6hr (bench-top) was determined at ambient temperature (25±3^{0}C) at four different concentrations in quadruplicates. The stability of BAZ in rat serum following repeated freeze-thaw cycles was assessed. The samples were thawed by allowing them to stand at room temperature for approximately 2hr. After drawing out the required volume, the samples were then returned to the freezer. The stability of BAZ was assessed after

three freeze–thaw cycles. Samples were processed. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal value and precision within 15% relative standard deviation (RSD) [12, 14].

Extraction recovery: Two sets of standard containing the analyte and IS at three different concentration were prepared. One set was prepared in rat serum and the other set was prepared in methanol. The recovery was determined by comparing peak areas of spiked serum extracts with those of unextracted neat standards prepared in methanol. The recovery value was calculated at various concentrations of BAZ.

Animal study: The study was conducted at Albino research center, with following Registration No. 1722/RO/Ere/S/13/CPCSEA. Male albino wistar rats (180–200 g) used in the study had free access to food and water. Six animals were kept for overnight fasting prior to dosing and were administered with oral suspension of BAZ in sodium carboxymethyl cellulose (0.25%). At predetermined time intervals, blood samples (250 µL) were collected from retro orbital plexus into microcentrifuge tubes. The blood was allowed to clot, and the serum was separated by centrifugation at 10,000 rpm for 10 min in a microcentrifuge tube and used for further analysis.

Pharmacokinetic parameters: The peak concentration (C_{max}) and its time (T_{max}) were obtained directly from the serum concentration vs. time profile. The area under the curve AUC_{0-t} was calculated by using trapezoidal rule method. The $AUC_{t-\infty}$ was determined by dividing the serum concentration at last time point with elimination rate constant (K_{el}).

RESULTS AND DISCUSSION

Chromatography: For better resolution and sensitivity the chromatographic conditions including mobile phase composition and column type were optimized. Several trials were carried out to achieve good resolution and increase the signal of the analyte. The feasibility of mixtures using acetonitrile and buffer (Potassium dihydrogen orthophosphate) in varying ratios along with altered flows (in the range of 0.4–1.0mL/min) was tested for complete chromatographic resolution of Bazedoxifene and Raloxifene (IS) from interfering biological matrix. The presence of a small amount of orthophosphoric acid to adjust the pH to 3.0 in mobile phase improved the detection and recovery of the analyte. The versatility, suitability and robustness of the method were checked with several C_{18} and C_8 columns. The Hypersil BDS C_8 reverse phase stainless steel analytical column 4.6 × 150mm, 5µm particle size provided very good selectivity, sensitivity and peak shape with acceptable system suitability parameters for Bazedoxifene and IS. Isocratic mobile phase consisting of buffer (Potassium dihydrogen orthophosphate pH adjusted to 3.0 with orthophosphoric acid) and acetonitrile (60:40, v/v) run at a flow rate of 1.0mL/min equipped with LC-10 AT solvent delivery unit was found to be suitable during LC optimization. The eluate was monitored by a PDA detector with sensitivity of 0.005 AUFS at ambient temperature set at 290nm, at which the maximum absorption was observed both for Bazedoxifene and adequate for Internal standard. The data acquired was processed using EMPOWER 2.0 software.

Specificity: Specificity is defined as the absence of any endogenous interference at retention times of peaks as evaluated by chromatograms of blank rat serum and serum spiked with BAZ and IS. Both the analyte and IS were separated with retention time of 5.852 and 4.052 min, respectively with good selectivity. The system suitability parameters for the method were as follows: a theoretical plate for BAZ is 8393 and for IS it is 4447. USP tailing is 0.94 and 1.44 for BAZ and IS respectively. Chromatograph shown in Fig. 2 illustrate that the developed method is specific to analyte and internal standard.

Calibration curve: Peak area ratios of BAZ to the IS were measured and calibration graph of peak area ratio (BAZ to IS) vs. BAZ concentration in the range resulted in the regression equation $y = 5.0983x + 0.2304$. The standard curve for BAZ was linear with $R^2>0.9923$ value in the concentration range of 0.1 - 32µg/mL. The calibration curve is represented in Fig. 3 and the data is used for quantification of BAZ.

Accuracy and precision: Accuracy and precision data for intra- and inter - day plasma test samples are represented in Table 1. The intra-day accuracy in terms of % error is 0.6, 0.8, 0.8, and 1.0 and for inter-day is 1.9, 1.0, 0.9 and 1.6 for 0.5, 1, 2, 4µg/mL respectively. The intra-day precision in terms of %RSD is 0.343, 0.675, 0.252 and 1.401 for inter-day is 0.790, 0.555, 0.639 and 1.251 for 0.5, 1, 2, 4µg/mL respectively.

Stability: The results for bench top, autosampler and 3 freeze-thaw cycles were all within the nominal concentrations and are found to be within the specified variability. BAZ was found to be stable for studied concentrations that are 0.5 - 4µg/mL within the variability of ±15%. The data of which is given in the Table 2.

Extraction recovery: The percentage recovery indicates that the extraction method is effective and can be extended for studying drug release profile or in pharmacokinetic study using pharmaceutical formulations. The absolute recoveries ranged from 90 to 110% across the concentration range of 4, 2, 1, 0.5µg/mL. The absolute recovery of internal standard at 1ug/mL was close to 100%. The portrayed results indicate that the developed method can be extended to pharmacokinetic study.

Sample analysis: The serum samples were processed by protein precipitation method. Briefly, 100 µL of serum sample was treated with 100µL of internal standard (1 µg/mL of Raloxifene in methanol) and vortexed for 1 min. The drug was extracted with 0.3mL of acetonitrile, vortexed for 15mins followed by centrifugation at 4000rpm for 15mins and the separated organic layer was injected onto the HPLC (20µL). The limit of detection and quantification were 25 and 50 ng/mL, respectively. The concentration vs. peak area ratio plot was linear ($r2 > 0.9923$) over the concentration range of interest (standard preparation), and the Bazedoxifene acetate content was quantified using this plot.

Application of the method: After a single oral administration of 10mg/kg BAZ to rats, the concentrations were determined by the described method. The mean serum concentration vs. time profiles for BAZ is depicted in fig 4. Inspection of fig 4 reveals that the newly developed method has the required sensitivity to characterize the absorption, distribution and elimination phases of BAZ following oral dosing. The pharmacokinetic parameters were calculated. Maximum concentration (C_{max} 0.639 ± 0.016 µg/mL) was achieved at 2.0 ± 0.00 hr (T_{max}). The half-life ($t_{1/2}$) of BAZ was 31.735 ± 0.663 hr, while the AUC ($_{0-\infty}$) was 19.346 ± 0.242 µg.h.mL^{-1}.

CONCLUSION

The developed method is a simple, sensitive, accurate and reproducible for the analysis of BAZ in rat serum. The method can be easily extended to quantitate BAZ in rat serum for routine monitoring of levels of BAZ.

ACKNOWLEDGEMENTS

The authors are thankful to the Management, Sri Venkateshwara College of Pharmacy, Madhapur, Hyderabad, Telangana, India for providing the required facilities to carry out this research work.

TABLE-1: INTRA AND INTER – DAY PRECISION OF DETERMINATION OF BAZEDOXIFENE ACETATE IN RAT SERUM.

Theoretical concentration (µg/mL)	**Measured Concentration (µg/mL)**			
	Mean	**SD**	**RSD**	**% error**
Intra-day variation (four replicates at each concentration)				
0.5	0.497	0.002	0.343	0.6
1	0.992	0.007	0.675	0.8
2	1.984	0.005	0.252	0.8
4	3.961	0.056	1.401	1.0
Inter-day variation (four replicates at each concentration)				
0.5	0.491	0.004	0.790	1.9
1	0.990	0.006	0.555	1.0
2	1.981	0.013	0.639	0.9
4	3.937	0.049	1.251	1.6

% Accuracy is expressed in terms of % Error

%Error= (Measured Value-Accepted value/Accepted Value) ×100

RSD= (SD/Mean) ×100

TABLE-2: STABILITY DATA OF BAZEDOXIFENE ACETATE IN RAT SERUM.

Spiked Concentration(µg/mL)	**Stability**	**Mean*± SD (µg/mL) n=4**	**% Error**	**Precision (%CV)**
0.5	0 h(for all)	0.497±0.002	0.6	0.34
	6h(BT)	0.493±0.007	1.4	1.49
	6h(Injector)	0.494±0.003	1.2	0.64
	3 F/T	0.493±0.005	1.5	1.03
1	0 h(for all)	0.992±0.007	0.8	0.68
	6h(BT)	1.005±0.015	0.5	1.54
	6h(Injector)	0.994±0.018	0.6	1.84
	3 F/T	0.998±0.020	0.2	1.97
2	0 h(for all)	1.984±0.005	0.8	0.25
	6h(BT)	1.983±0.031	0.9	1.58
	6h(Injector)	1.99±0.015	0.5	0.74
	3 F/T	1.996±0.012	0.2	0.62
4	0 h(for all)	3.961±0.056	1.0	1.4
	6h(BT)	4.021±0.055	0.5	1.38
	6h(Injector)	4.025±0.064	0.6	1.59
	3 F/T	4.015±0.058	0.4	1.45

* Mean of 4 replicates. % CV=Coefficient of Variance; F/T= Freeze-Thaw cycles; BT=Bench top
% Accuracy is expressed in terms of % Error.
%Error= (Measured Value-Accepted value/Accepted Value) × 100.

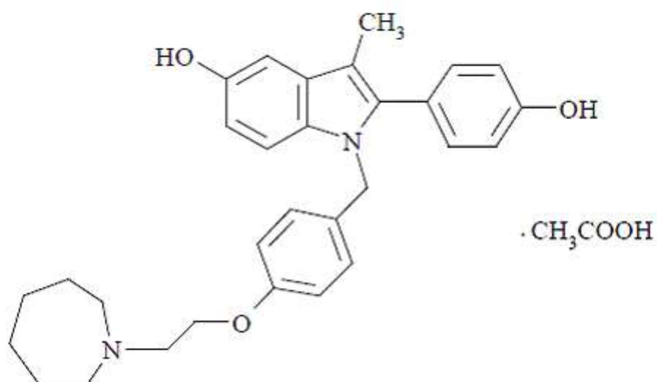

Fig. 1: Chemical structure of Bazedoxifene acetate

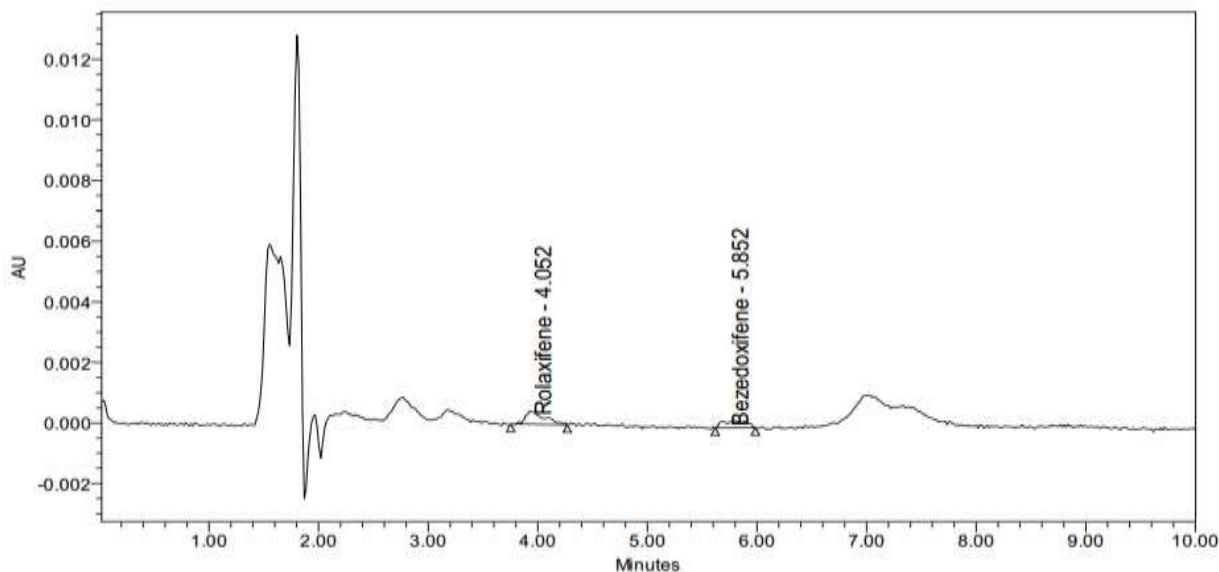

Fig. 2: Chromatograph with drug and internal standard eluted at their respective retention times.

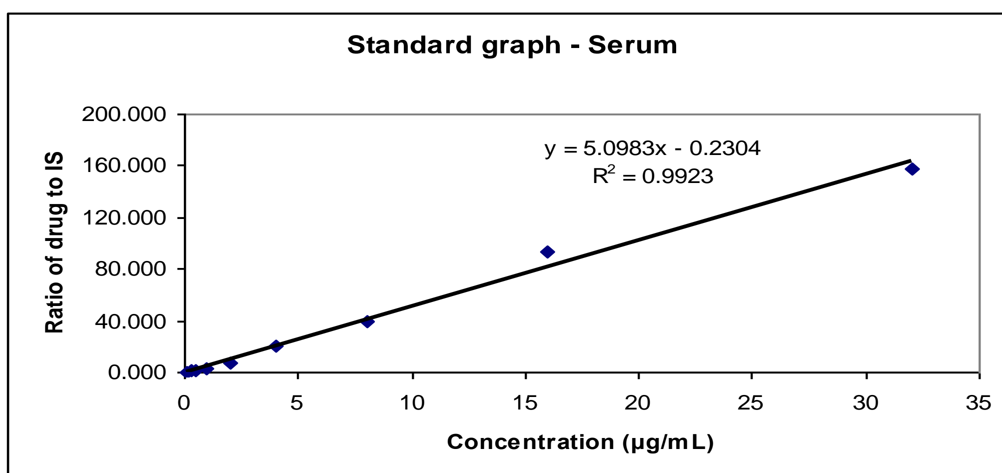

Fig. 3: Calibration curve was plotted against peak area ratio of BAZ & IS on Y-axis and Concentration on X-axis.

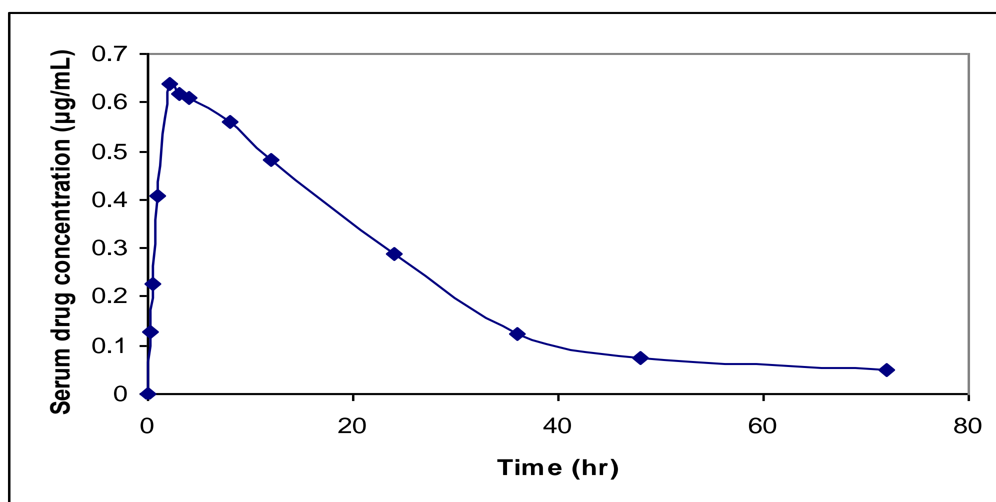

Fig. 4: Pharmacokinetic profiles of Bazedoxifene acetate in serum following oral administration (mean ± SD, n=6)

REFERENCES

1. National Osteoporosis Foundation. Clinician's Guide to Prevention and Treatment of Osteoporosis. Washington, DC: National Osteoporosis Foundation; 2010.
2. The North American Menopause Society. Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society. Menopause 2010; 17: 25–54.
3. Komm BS, Lyttle CR. Developing a SERM: stringent preclinical selection criteria leading to an acceptable candidate (WAY-140424) for clinical evaluation. Ann NY Acad Sci 2001; 949: 317–326.
4. Pinkerton JV et al. Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. Menopause 2009; 16: 1102–1108.
5. Miller CP et al. Bazedoxifene acetate: selective estrogen receptor modulator treatment and prevention of osteoporosis. Drugs Fut 2002; 27: 117–121.
6. Gruber C, Gruber D. Bazedoxifene (Wyeth). Curr Opin Investig Drugs 2004; 5: 1086–1093.
7. Miller CP et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. J Med Chem 2001; 44: 1654–1657.
8. Komm BS et al. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. Endocrinology 2005; 146: 3999–4008.
9. Chandra sekaran A et al. Bazedoxifene acetate metabolic disposition in healthy post menopausal women. J Clin Pharmacol Ther 2003; 37-47.
10. G.Smitha. Determination of Bazedoxifene Acetate in Bulk with the Aid of Uv-Spectroscopy: Development and Validation. Int J PharmTech Res 2014-2015; 7(4): 641-647.
11. ICH-Guidelines Q2 (R1), Validation of Analytical Procedures: Text and Methodology 2005.
12. The United States Pharmacopoeia. United States pharmacopoeial convention: Rockville MD, 1995, 1982.
13. Shah VP et al. Analytical methods validation: Bioavailability, bioequivalence & pharmacokinetic studies. J pharm sci 1992; 81:309-312.
14. T.R. Kumar et al. Determination of rosuvastatin in rat plasma by HPLC: validation and its application to pharmacokinetic studies. Biomed Chromatogr 2006; 20(9):881-87.