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Original Article

DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL RP-HPLC METHOD FOR AZILSARTAN MEDOXOMIL WITH LIQUID-LIQUID EXTRACTION

SNEHAL KARPE, SANDEEP SONAWANE*, PRIYA RAHADE, SANJAY KSHIRSAGAR

MET's Institute of Pharmacy, MET League of Colleges, Bhujbal Knowledge City, Adgaon, Nashik, Maharashtra, India
Email: sandeeps.iop@gmail.com

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ABSTRACT

Objective: There are many analytical methods available for estimation of Azilsartan medoxomil in biological samples and for pharmaceutical preparations. However, no specific RP-HPLC method with UV detection based on liquid-liquid extraction technique is available for estimation of Azilsartan medoxomil in human plasma.

Methods: A simple, rapid and accurate RP-HPLC with UV detection method was developed and validated as per US-FDA guidelines for the estimation of Azilsartan medoxomil in spiked human plasma using liquid-liquid extraction technique.

Results: Azilsartan medoxomil was well resolved from human plasma interference and internal standard (Aceclofenac) using C 18 (250 × 4.6 mm, 5 µ) column with methanol: 20 mm phosphate buffer (pH 3.0), (70: 30 %, *v/v*) as mobile phase, at a flow rate of 1 ml/min. The detection was performed at 249 nm. The calibration curve was found linear in the range of 500-16000 ng/ml. During calibration experiments, the heteroscedasticity was minimized by using weighted least square regression model with weighing factor $1/x^2$. In accuracy and precision studies, intra-day and inter-day, % relative error was found between±15 and % RSD was less than 15 %. Stability experiments indicated that the drug remained stable after three freeze-thaw cycles.

Conclusion: A simple, rapid and accurate RP-HPLC method with UV detection was developed and validated for estimation of Azilsartan medoxomil based on liquid-liquid extraction technique. The developed method meets the requirements of US-FDA guidelines. Also the developed method does not require expensive chemicals and solvents and does not involve complex instrumentation, hence it is economic.

Keywords: Azilsartan medoxomil, Bioanalytical, Heteroscedasticity, Liquid-liquid extraction, RP-HPLC, Weighted regression.

INTRODUCTION

Azilsartan medoxomil chemically is (5–methyl–2–oxo-1,3–dioxol–4-yl) methyl 2–ethoxy–3-[[4-[2- (5–oxo-2H-1, 2, 4–oxadiazol–3-yl) phenyl] phenyl] methyl] benzimidazole–4-carboxylate [1]. It is Angiotensin II AT_1 receptor blocker [2]. The structure of Azilsartan is given in [fig. 1].

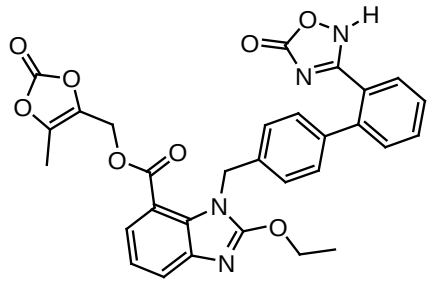

Fig. 1: Structure of Azilsartan medoxomil

Literature survey revealed few methods for quantitative analysis of Azilsartan medoxomil in biological samples and in pharmaceutical formulations alone as well as in combination with other drugs. These include RP-HPLC method with PDA detection in human plasma by solid-phase extraction procedure [3], UPLC with MS/MS in beagle dog plasma with its application to pharmacokinetic studies by protein precipitation method [4], HPTLC method [5], determination of potential impurities in tablets by UPLC with UV detection [6], UV spectrophotometric method for determination of Azilsartan medoxomil in bulk and pharmaceutical dosage form [7]. Also, quantification methods of Azilsartan medoxomil in combination with Chlorthalidone include, LC with electrospray ionization operated in negative multiple reaction monitoring (MRM) mode in rat and human plasma by liquid-liquid extraction method [8], stability-indicating RP-HPLC method with UV detection [9] and spectrophotometric method based on first derivative spectra and spectro fluorometric methods[10].

Most of the earlier methods are based on the use of sophisticated instruments like LC-MS, UPLC-MS, which are not available in routine quality control laboratories and academic institute laboratories.

Therefore, the present work was undertaken with the objective of developing and validating a simple and rapid RP-HPLC with UV detection method for determination of Azilsartan medoxomil in human plasma based on liquid-liquid extraction technique which is cost effective and an efficient extraction technique. Also, the second objective of work was to suggest an approach in the selection of optimum calibration model.

MATERIALS AND METHODS

Materials and instrumentation

Pharmaceutical grade Azilsartan medoxomil and Aceclofenac used as an internal standard were provided as *ex-gratis* from Glenmark Pharmaceuticals, Sinnar, Nashik, India and Blue Cross Laboratories Ltd., Ambad, Nashik, India, respectively. Blank human plasma was provided as a gift sample from Dr. Vasantrao Pawar Medical College, Hospital and Research Centre, Nashik, India. Blank plasma was pooled by thoroughly mixing the plasma obtained from six different sources. Methanol used in the analysis was of HPLC grade, and all other chemicals were of analytical reagent grade, purchased from SD Fine Chemicals, Mumbai, India. The Durapore, 0.45 µ × 47 mm, membrane filter papers were purchased from Millipore (India) Pvt. Ltd., Bengaluru, India. Freshly prepared double distilled water used in the analysis was prepared from Borosil All Glass Double Distillation Assembly, purchased from Borosil, Mumbai, India.

Chromatographic analysis was carried out using an HPLC system consisting of pump PU-2080 Plus (JASCO Corporation, Tokyo, Japan)

equipped with 100 µl Rheodyne loop injector (7725*i*) and detection was carried out on UV-2075 detector (JASCO Corporation, Tokyo, Japan) using Borwin Chromatography software (Version 1.50).

Preparation of standard stock solution and working standard solution for azilsartan medoxomil and aceclofenac

The stock solution of (1 mg/ml) of Azilsartan medoxomil was prepared in methanol and was further appropriately diluted with methanol to get six different working standard solutions with concentration 5, 10, 20, 40, 80 and 160µg/ml. Similarly, the stock solution (10 mg/ml) of Aceclofenac was prepared in methanol and appropriately diluted with methanol to get working standard solution of 100 µg/ml.

Preparation of calibration curve (CC) standards and quality control (QC) samples

Aliquots of 200 µl of pooled blank plasma were taken in stoppered glass tubes of capacity 20 ml. To this, 25 µl of 25 µg/ml methanolic standard stock solution of Azilsartan medoxomil (2500 ng) was added and to each tube, 25 µl of working standard solution of Aceclofenac (as internal standard) was added. The resulting solutions were vortex mixed for 1 min to get CC standards containing 500, 1000, 2000, 4000, 8000 and 16000 ng/ml of Azilsartan, respectively. The QC samples were similarly prepared to contain three concentrations [1500 ng/ml Low-Quality Control (LQC), 8000 ng/ml Middle-Quality Control (MQC) and 16000ng/ml High-Quality Control (HQC)].

Liquid-liquid extraction (LLE) experiments

Aliquots of pooled human plasma (200 µl) were taken in 20 ml stoppered glass test tubes. To each of these tubes 25 µl of 25 µg/ml methanolic solution of Azilsartan medoxomil and 25 µl of 100 µg/ml of working standard solution of Aceclofenac was added. To each tube, 3 ml of organic solvent was added, and the tubes were shaken in an inclined position on a reciprocating shaker at 100 strokes/min for 3 min. Further, these tubes were centrifuged at 3000 rpm for 10 min. The separated organic layer from each tube was transferred to separate glass tube and evaporated to dryness under a stream of nitrogen. The residue obtained upon evaporation to dryness was reconstituted with 250 µl of mobile phase and 100 µl was injected into HPLC system under optimal chromatographic conditions.

Chromatographic conditions

Chromatographic analysis was carried out on a C 18Phenomenex Hyperclone column (250 × 4.6 mm, 5 µm) with mobile phase consisting of methanol: 20 mm potassium phosphate buffer (pH 3.0), (70:30 %, *v/v*) at a flow rate of 1 ml/min. The detection was carried out at 249 nm.

Calibration runs

In the calibration experiments, 200 µl aliquots of all CC standards were analyzed in six replicates using optimized LLE method and appropriate chromatographic conditions. All calibration curves (CC) were analyzed in six replicates. Prior to analysis, each CC standard was mixed with 25 µl of 100 µg/ml methanolic solution of Aceclofenac (as internal standard). At the end of the calibration runs, the chromatograms of CC standards were processed to get the peak areas for Azilsartan medoxomil and Aceclofenac. For each CC standard, the area ratio of Azilsartan medoxomil and Aceclofenac was calculated.

Selection of calibration model and range

Data obtained from the run calibration experiments was subjected to unweighted and weighted least square regression analysis to generate the respective calibration equations [11]. In weighted regression, weighting factors (w) of $1/x$ and $1/x^2$ were used, where x is the concentration of the CC standards of Azilsartan medoxomil.

In order to select the best calibration model, each calibration model and equation was evaluated with respect to % Relative error (%RE), Residual plot and homogeneity of variance (homoscedasticity) in the linear range [12].

The area ratios for the CC standards were referred to the calibration equation to get the back-calculated concentrations (interpolated concentrations) for the each CC standards. The total % RE was calculated as the sum of % RE for all CC standards.

The predicted area ratios for CC standards were calculated by entering the nominal concentration of each CC standard into the calibration equation. A plot of residuals was constructed by plotting the differences between measured and predicted area ratios against nominal log concentration, and the scatter of residual was evaluated.

To evaluate homoscedasticity in the linear range, the variance of residuals at highest CC standard to the lowest CC standard was evaluated by means of one-way ANOVA.

The calibration model with minimum % RE, random scatter of points in the plot of residuals and no significant difference in one-way ANOVA was selected.

Validation studies

The developed method was validated as per US-FDA Guidance for Industry: Bioanalytical Method Validation (September 2013)[13]. Selectivity was studied at the lower limit of quantification (LLOQ) at 500ng/ml by comparing blank responses of plasma from six different sources with peak areas afforded by LLOQ samples. The Calibration curve standards were evaluated by preparing and analyzing CC standard solutions spiked with an internal standard for five days. The concentrations of each CC standard were back calculated using suggested calibration model and the deviation of the back-calculated concentrations from nominal values was studied and expressed as % nominal.

Precision and accuracy were studied by analyzing five bioanalytical batches over five days. Each batch consisted of one blank, all CC standards and five replicates of LQC, MQC and HQC samples. The calibration equation was determined for each batch from analysis of CC standards and was used to calculate the concentration of Azilsartan medoxomil in LQC, MQC and HQC samples. The *within batch* and *between batch* accuracy and precision was determined in terms of % RE and % RSD, respectively.

Stability of Azilsartan medoxomil in plasma was evaluated under various conditions *viz.* freeze-thaw cycles, stability at–20 °C for 30 d and stability at room temperature for 6 h. The amount of Azilsartan medoxomil in stability samples was found out and the % nominal and % RSD of the determinations were calculated.

RESULTS

When Azilsartan medoxomil and Aceclofenac were subjected to chromatographic analysis in mobile phases of different strengths and compositions, it was found that mobile phase consisting of methanol: 20 mm potassium phosphate buffer (pH 3.0), (70:30 %, *v/v*) gave adequate retention at a flow rate of 1 ml/min. The wavelength at which detection was carried out was 249 nm. The retention time for Azilsartan medoxomil was 5.53 min and for Aceclofenac, it was 9.61 min. various organic solvents like n-hexane, dichloromethane, diethyl ether and tert-Butyl methyl ether were tried in which good recovery was obtained with tert-Butyl methyl ether. Also, when aliquots of blank plasma were extracted with tert-Butyl methyl ether and chromatographed under mentioned chromatographic conditions, it was found that there were no significant interfering peaks at the retention times of Azilsartan medoxomil and Aceclofenac. Thus, it was concluded that tert-Butyl methyl ether could be further used as LLE solvent for Azilsartan medoxomil and Aceclofenac. The chromatogram of blank plasma extracted in tert-Butyl methyl ether is shown in [fig. 2(a)]and the chromatogram of Azilsartan medoxomil and Aceclofenac extracted in tert-Butyl methyl ether is shown in [fig. 2(b)]. The extraction recovery obtained for Azilsartan medoxomil and Aceclofenac was 58.25 % and 60.25 %, respectively.

During calibration experiments, when data obtained from [table 1]was subjected to unweighted and weighted linear regression with weighting factors $1/x$ and $1/x^2$;unweighted regression resulted in the equation $Y = 0.0002x + 0.01099$ and with $1/x$ and $1/x^2$weights,

resulted in equation, Y = 0.0002x+0.0156 and Y = 0.0002x+0.0142, respectively. Each of the obtained linear regression equations was evaluated for % RE, random scatter and homoscedasticity for selection of appropriate calibration model [table 2]. From this, it was concluded that although all calibration equations gave random scatter residuals, the total % RE was minimal when weighted regression with weighting factor $1/x^2$ was applied. Further, when the $F_{calculated}$ values were compared with $F_{tabulated}$ (α = 0.05), it became evident that a weighting factor of $1/x^2$ was suitable to homogenize the variance of the residuals. Thus, it was decided to adopt calibration model of weighted linear regression with weighing factor $1/x^2$ in the calibration range of 500 to 16000 ng/ml of drug.

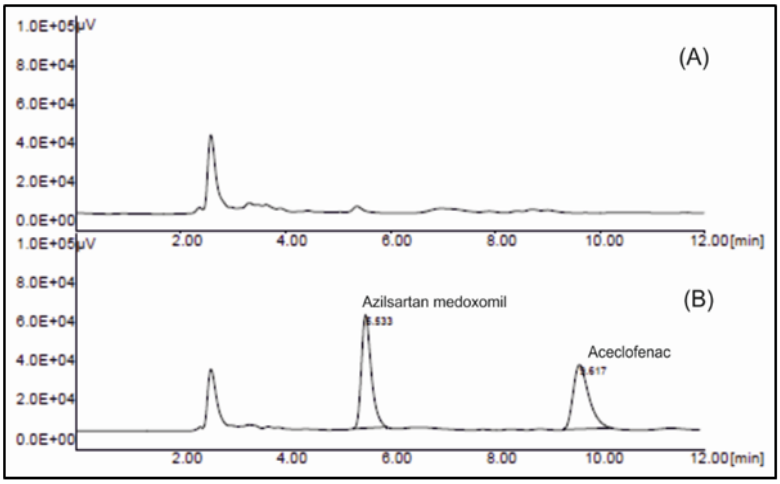

Fig. 2(a): Chromatogram of blank plasma extracted in tert-Butyl methyl ether, 2(b): Chromatogram of Azilsartan medoxomil and Aceclofenac extracted in tert-Butyl methyl ether

Table 1: Area ratios from calibration experiments

CC	Amount of drug in ng/ml	Area ratio (mean±SD) (n=6)
1	500	0.1190±0.013
2	1000	0.2108±0.020
3	2000	0.4498±0.037
4	4000	0.8663±0.047
5	8000	1.5918±0.154
6	16000	3.3145±0.254

Table 2: Results of evaluation of various calibration models

Unweighted regression			Weighted regression (1/x)			Weighted regression ($1/x^2$)		
∑% RE	Nature of residuals plot	$F_{5,5}$* value	∑% RE	Nature of residuals plot	$F_{5,5}$** value	∑% RE	Nature of residuals plot	$F_{5,5}$** value
36.18	Random scatter	349.207	-3.796-E	Random scatter	0.341	-8E-13	Random scatter	0.003

* F value calculated as ratio of variances at the extremes of the calibration range, ** F value calculated as ratio of variances of the weighted residuals

During validation studies, it was found that the peak areas for the lower limit of quantification (LLOQ) samples were more than five times the blank responses obtained using six different plasma sources which concluded that the method was deemed to be selective for an LLOQ of600 ng/ml. From [table 3], it was concluded that % nominal values of the back-calculated concentrations of CC standards were between 97-107 %, which were in acceptable limits.

Table 3: Standard curve parameters for Azilsartan medoxomil

CC	Nominal conc. (ng/ml)	Back calculated concentrations (ng/ml)						Mean	±SD	% R. S. D.	% Accuracy
		1	2	3	4	5	6				
1	500	499	431.5	599	449	537.5	575.5	515.2	67.53	13.10	103.05
2	1000	1037	927.5	876	848	1094	1062.5	974.2	103.6	10.64	97.42
3	2000	2136	1926	1981	2249	2236	2395.5	2154	176.7	8.20	107.7
4	4000	4326	3926	3991	4427	4499	4342.5	4251.9	236.5	5.56	106.2
5	8000	8035.5	6926	9207	7659	8020.5	7426.2	7879.0	770.6	9.78	98.48
6	16000	15926.5	15543.5	15927	16649	17170.6	18982	16699.8	1262.0	7.55	104.3

The evaluation of accuracy and precision showed that the intra-day % RE was between±15 %, while the % RSD was less than 15 %. The US-FDA Guidelines require that % RE be between±15 %, while % RSD should be less than 15 %. The results of precision and accuracy,

as well as extraction recovery for Azilsartan medoxomil at LQC, MQC and HQC and for Aceclofenac, are presented in [table 4]. Further, the intermediate precision of the method was determined by using a one-way ANOVA. For each QC level, *within mean square* and *between mean square* values were determined. The total variance was taken as a sum of *within* and *between mean squares* and the standard deviation was determined as a square root of total variance and F value was determined. The $F_{calculated}$ was found less than $F_{tabulated}$ (α = 0.05), [table 5] indicates that there is no significant difference between intra-day and inter-day precision.

Table 4: Results of accuracy and precision studies

Intraday n= 5					**Interday n = 5**			
QC Level	**Conc in ng/ml**	**Mean conc found ng/ml**	**%RE**	**%RSD**	**Mean conc found ng/ml**	**%RE**	**%RSD**	**% Recovery**
LQC	1500	1513.2	0.82	4.64	1563.32	5.27	5.15	41.08
MQC	8000	8003.6	0.03	3.12	8378.5	4.70	4.99	58.4846
HQC	16000	17366.5	8.54	2.9	16970.1	6.06	4.54	46.88
IS	-	-	-	-	-	-	-	60.25

Table 5: Results of one-way ANOVA at each QC level

QC Level	**Source**	**Sum of squares**	**Df**	**Mean squares**	**Total Variance**	**±SD**	**F value**
LQC	Within run	168784	20	8439.2	12435.4	111.5	3.8
	Between run	129666	4	32417			
MQC	Within run	4586655	20	229333	3911007	625.3	5.2
	Between run	4799763	4	1199941			
HQC	Within run	1.490Eto7	20	745214	1230856	1109.4	4.9
	Between run	1.463Eto7	4	3659064			

Table 6: Results of stability studies for Azilsartan medoxomil

QC Level	**Stability at RT**		**Stability at-20°C**		**Freeze-thaw stability**	
	%Nominal	**% RSD**	**%Nominal**	**% RSD**	**%Nominal**	**% RSD**
LQC	102.21	3.37	102.3	3.85	101.26	4.9
HQC	101.73	3.22	100.6	5.66	100.23	6.92

The results of stability evaluation of Azilsartan medoxomil are presented in [table 6]. Analysis cycles *viz.* three freeze-thaw cycles, stability at-20 °C for 30 d and stability at room temperature for 6 h indicated that Azilsartan medoxomil was stable in human plasma under these conditions. The developed method is sensitive and convenient for use. The developed method does not require expensive chemicals and solvents and does not involve complex instrumentation or sample preparation methods and hence it is simple and economical as compared to previously reported methods.

CONCLUSION

In this report, a simple, rapid, selective and accurate HPLC-UV method was described for the quantification of Azilsartan medoxomil in spiked human plasma using liquid-liquid extraction. The developed bioanalytical method is capable of quantifying Azilsartan medoxomil from spiked human plasma in the concentration range of 500–16000 ng/ml. The method meets the requirements of the US-FDA guidelines.

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CONFLICT OF INTERESTS

Declared none

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