Chromatographic method development and validation for the determination of valsartan in biological fluid

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Abstract: A swift, precise and simple HPLC bioanalytical technique with UV detection was established and validated for quantitative estimation of valsartan in human plasma. The analyte was separated from plasma by protein precipitation with acetonitrile and chromatographically separated on Zorbax SB-C18 (5µm, 4.6mm × 15cm) column. The solvent mixture system consisting of acetonitrile, water and glacial acetic acid (40:59:1 v/v), was pumped using isocratic mode at 1mL/min flow rate. Samples' detection of drug was made spectrophotometrically at a wavelength of 264nm. The analyte response was instituted to be linear from 0.06 to 8µg/mL with a regression value of 0.999. The accuracy of the proposed method was ranged between 97.2-100.3% with 5% RSD. The analytical recovery (>95%) was consistently observed and satisfactory sample stability was also found at different environmental conditions. In conclusion the reported bio-analytical method is easy and robust that was successfully utilized in estimation of valsartan in a pharmacokinetic study.

Keywords: HPLC, plasma, valsartan, method validation.

INTRODUCTION

Valsartan ($C_{24}H_{29}N_5O_3$) is an orally active, potent, non-peptide tetrazole derivative available in white, microcrystalline powder (Abraham *et al.,* 2011). The partition coefficient (P) of the drug is found to be 0.033 (log P = 1.499) with a melting point range of 105 to 110°C (Ahad *et al.,* 2011). The aqueous solubility of drug is low ($2.34e^{-02}$ g/L) with higher permeation rates (Drug Bank, 2013; Cheng and Chou, 2001). It is available in doses of 10, 20, 40, 80, 160 and 320mg to manage the complaints of post myocardial infarction (MI), congestive heart failure (CHF) and hypertension in pediatric, adolescents and the elderly patients (Siddiqui *et al.,* 2011; Flesch *et al.,* 1997).

The bioavailability of the compound is reported to about 23% and the peak plasma concentration is achieved upon oral dosing in 2 to 4 hours with a half-life of 6 hours (Ibrahim and El-Setouhy, 2010). Biologically it is non-competitive and highly selective angiotensin II-type I (AT1) receptor antagonist responsible to relax the blood vessels through widening of capillaries consequently lowering the blood pressure and improving blood flow (Siddiqui *et al.,* 2011; Ahad *et al.,* 2011). Owing to the mentioned properties, the model drug is utilized to be a first choice medication for treating uncomplicated hypertension, left ventricular hypertrophy and isolated systolic hypertension.

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It also inhibits the vasoconstriction and angiotensin II effects (aldosterone secretion) in the adrenal gland and smooth muscle. It is considered to be a first line agent for delaying advancement of diabetic nephropathy (Kumar *et al.,* 2013).

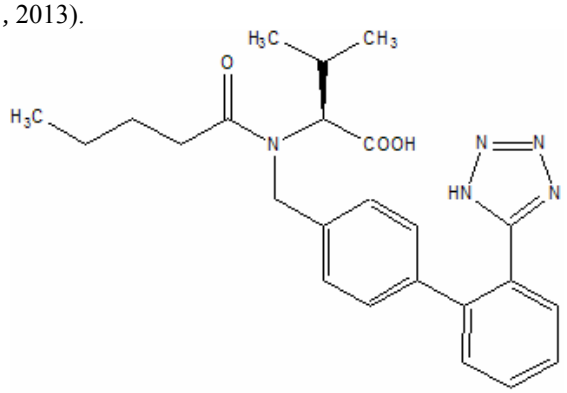

Fig. 1: Chemical structure of valsartan

Various techniques for analysis have been described for the quantitative assessment of the drug in pharmaceutical formulations and raw materials (Kesting *et al.,* 2010; Mehta *et al.,* 2010; Nissankararao *et al.,* 2013; Reddy *et al.,* 2010) using HPLC technique.

Few published HPLC methods are reported in literature for determination of valsartan in human plasma (Jones *et al.,* 2012; Duan *et al.,* 2012; Jiang *et al.,* 2011; Iqbal *et al.,* 2010). However, valsartan detection in biological fluids has not been addressed extensively. Serum/plasma estimation of the drug is deemed to be necessary for bioequivalence and pharmacokinetic studies.

The objective of the current research is the development and the validation of an economical, precise and reproducible HPLC-UV technique that can be used for the determination of drug in blood samples. All experimental work was planned and conducted as per ICH monograph (ICH, 2005). The underlying method employs a single step protein precipitation technique to extract the drug in plasma with short retention time (RT) values (4.1min) enabling the procedure to be time saving.

MATERIALS AND METHODS

Chemicals

Valsartan (pure) was a gift from Tabros Pharma, (Pvt. Ltd., Karachi, Pakistan); acetonitrile, methanol, glacial acetic acid, all chemicals were of HPLC grade and products of Merck, (Darmstadt, Germany).

Instruments

A High Performance Liquid Chromatography (LC-20A, Shimadzu, Kyoto, Japan) consisting of auto-sampler SIL-20A, column oven CTO-20A and PDA detector SPDM-20A equipped with Zorbax SB-C18 column (5µm, 4.6mm × 15cm) was used to obtain chromatographic separation. LC solution software was used for chromatogram generation and data analysis.

Method of preparation of chromatographic solutions

In our study, assay of valsartan in plasma was performed by modification of the high performance liquid chromatographic method previously reported by Reddy *et al.,* and Srinath Nissankararao *et al.,* (Reddy *et al.,* 2010, Srinath Nissankararao *et al.,* 2013).

Mobile phase

Solvent system is a mixture of acetonitrile, water and glacial acetic acid in the ratio of 40:59:1 with a pH of 2.9. It was filtered through 0.45µm membrane filter and then degassed by sonication prior to use.

Preparation of standard & stock solutions

Valsartan stock solution (1000µg/mL) was made in methanol and sonicated for approximately 20min. For calibration purpose working solution of 100µg/mL was prepared in plasma and subsequently diluted to concentrations of 0.06, 0.12, 0.25, 0.5, 1, 2, 4 and 8µg/mL. The samples for quality control were made in the strengths of 0.18, 3 and 6µg/mL. Calibration graph was constructed by plotting drug peak areas against the corresponding nominal concentrations.

Stock solution stability

Valsartan stock solution in methanol (1mg/mL) was diluted to a concentration of 1µg/mL for stock solution stability testing. Analysis of five samples of the same concentration was made at the end of second week, third week and finally for the fourth week of storage. Mean, standard deviation, % CV (variance coefficient) and % accuracy were measured with reverence to its preliminary strength.

Preparation of sample for determining valsartan in plasma

The local blood bank was accessed to obtain drug free plasma. Spiking of valsartan was done in plasma through addition of specific amount of working solution to obtain 500µL and then volume was made up in eppendorf micro centrifuge tubes with plasma. For the precipitation of proteins, addition of 500µL of acetonitrile was done and the mixture was vortexed (vortex) for 1min followed by centrifugation for 15 minutes at 8000rpm. A volume of 0.4mL of the supernatant was transferred for injection into auto sampler vials.

Chromatographic settings

The solvent system was pumped to the column at a flow rate of 1mL/min from the solvent reservoir; the column temperature was maintained at 40°C. The PDA detector was used to monitor the eluate at 264nm and the injection volume was 20µL. The mobile phase was employed as diluent for the making of system suitability samples.

Method validation

The developed bio-analytical system was validated for accuracy, selectivity, linearity, precision, stability and sensitivity (ICH, 2005).

Selectivity

For distinction between medication and other endogenous constituents in the sample, process selectivity was examined by studying 6 different blank plasma samples with LLOQ of valsartan and assessed for any interfering peak at the analyte's retention time.

Linearity & calibration curve

Linearity of the proposed technique was assessed from 0.06µg/mL to 8µg/mL. Spiking was done 3 times, concentration curves were plotted and correlation co-efficient (r^2) was calculated. Similar strengths of drug was made in mobile phase and also utilized for calibration. Back calculation was used to estimate the drug's concentration in mobile phase and plasma both. Standard deviation, % CV (coefficient of variance) and % accuracy were measured for each concentration of samples.

Precision & accuracy

Intraday precision

Five samples of three dissimilar strengths (0.18, 3 and 6µg/mL) of valsartan were analyzed in one day at different timings. The drug concentration in plasma was estimated by standard calibration curves and for determination of intraday precision for the three concentrations; various parameters like SD, % CV (within 15%), % accuracy (within 15%) and LLOQ (within 20%) were calculated.

Interday precision

Five samples of three dissimilar strengths were assessed at different and the same times for 3 successive days. For calculating the concentration of valsartan an average of 2 calibration curves was used and SD, % CV (less than 15%), % accuracy (within 15%), LLOQ (within 20%) and % RSD were also determined.

Limit of detection (LOD) & lower limit of quantification (LLOQ)

Limit of detection (LOD) and quantification (LLOQ) were estimated using four lower drug concentrations (0.03, 0.06, 0.12, & 0.25µg/mL) with SD, % CV, % accuracy and precision. LOD is the lowest amount of drug that could be identifiable and it is taken when the signal to noise ratio is noticed thrice the baseline noise. LLOQ is considered to be the lowermost detectable and quantifiable strength against standard. The signal to noise ratios were determined for the lowermost strength chromatogram and found to be satisfactory when response would be seven times of the noise for limit of quantification.

Analytical recovery of method

The extraction efficiency of valsartan was determined through comparative investigation of drug spikes in plasma and mobile phase solvent system to establish absolute recovery. Five samples of different concentrations (0.5, 1, 2 & 4µg/mL) were prepared in plasma and compared with those of mobile phase samples and the results were expressed in % recoveries.

Drug stability in plasma

The stability of the samples were estimated by "freeze and thaw cycle" in plasma using high (6µg/mL) and low (1µg/mL) strengths. A set of frozen samples (at -40^{o}C for 24 hours) of all strengths was thawed and analyzed however keeping the rest of the samples frozen. Other 2 sets with 5 samples of each strength were assessed in the similar fashion whereas the last set was re-frozen for next day to complete 3 cycles of freeze-thaw. Each time the samples were assessed with reference to freshly made samples. 15 samples in plasma of high and low drug strengths were prepared to perform long term stability and the samples were stored at -40^{o}C. At the end of second week storage, 5 samples of each strength were examined and at the termination of third week of storage, the next 5 samples of each concentration were examined in comparison to the initial concentration.

Bench top and auto-sampler stability of spiked plasma samples

The spiked plasma samples stability (bench top stability) was assessed for 24h which were stored at room temperature. The spiked plasma samples stability was estimated for 65h, stored in autosampler at 2-8°C (autosampler stability). The extracted plasma samples injected instantaneously (time 0h) were compared with the samples subjected to be re-injected after storage at specified conditions in order to assess the stability.

Application of validated method for the pharmacokinetic study of valsartan 160mg tablet (Diovan®)

The applicability of this validated method was evaluated by performing a bioavailability study of immediate release valsartan (160mg) tablet in one healthy male subject. Single healthy male volunteer was selected for the determination of applicability of this validated method on the basis of previously reported literature (Tanam *et al.,* 2015; Ben-Eltriki *et al.,* 2013; Nemutlu *et al.,* 2009). This design was single center, open label, single dose study. The protocol of the study was approved by the Institutional Bioethics Committee (IBC), University of Karachi. This study was conducted in the University of Karachi under the supervision of principal investigator and physician. A written signed informed consent was obtained by the volunteer before study. Diovan®, a marketed product of valsartan, was administered to the healthy male volunteer after taking his informed consent. Plasma samples were withdrawn at regular time intervals for 48h. These samples were analyzed to determine valsartan concentration in order to measure various pharmacokinetic parameters by using Kinetica® version 5.1 Thermoelectron Corp., USA.

RESULTS

Selectivity was investigated by pretreatment and chromatographic separation of blank plasma from six different sources. There was no interfering peak noticed at valsartan's retention time (fig. 2). Linearity of the method was established in the intended range i.e. 0.06-8µg/mL with r^2 values 0.999 and accuracy within ±15 % for all the selected concentrations (table 1 and 2). For the investigation of lower limit of detection (LLOD) and limit of quantification (LLOQ) four dissimilar strengths 0.03, 0.06, 0.12 & 0.25µg/mL were analyzed and with respect to accuracy and precision the LLOQ was set at 0.06µg/mL (fig. 2) while 0.03µg/mL was considered to be the limit of detection (LLOD) (table 3).

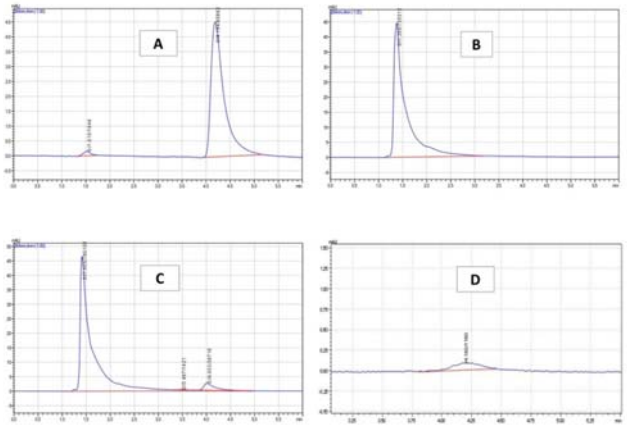

Fig. 2: Chromatogram of A) valsartan in mobile phase B) blank human plasma C) valsartan in spiked plasma (1 µg/mL) D) valsartan at LLOQ in spiked plasma (0.06 µg/mL).

Table 1: Standard calibration curve for linearity in mobile phase

Concentration (µg/mL)	Calculated Concentration (µg/mL)			Mean	SD	% CV	% Accuracy
	Sample 1	Sample 2	Sample 3				
8	8.11	8.27	8.19	8.190	0.080	0.976	102.375
4	4.18	4.02	4.21	4.136	0.102	2.469	103.416
2	2.25	2.01	2.01	2.090	0.138	6.629	104.500
1	1.01	1.07	1.03	1.036	0.030	2.946	103.666
0.5	0.56	0.53	0.51	0.533	0.025	4.718	106.666
0.25	0.256	0.259	0.251	0.255	0.004	1.582	102.133
0.12	0.124	0.126	0.121	0.123	0.002	2.034	103.055
0.06	0.064	0.062	0.06	0.062	0.002	3.225	103.333

Regression (r^2) 0.9993

Table 2: Standard calibration curve for linearity in plasma

Concentration (µg/mL)	Calculated Concentration (µg/mL)			Mean	SD	% CV	% Accuracy
	Sample 1	Sample 2	Sample 3				
8	8.010	8	8	8.003	0.006	0.072	100.042
4	3.910	4	4	3.970	0.052	1.309	99.250
2	2	2.010	2.010	2.007	0.006	0.288	100.333
1	1.010	1	0.990	1.00	0.010	1.00	100.00
0.5	0.500	0.500	0.501	0.500	0.001	0.115	100.067
0.25	0.250	0.240	0.251	0.247	0.006	2.463	98.800
0.12	0.120	0.120	0.110	0.117	0.006	4.949	97.222
0.06	0.061	0.059	0.056	0.058	0.002	4.289	97.777

Regression (r^2) 0.9989

Table 3: LLOQ & LOD of drug in plasma samples

Limit of quantification (LLOQ) of valsartan in plasma							
Concentration (µg/mL)	Calculated Concentration (µg/mL)			Mean	SD	% CV	% Accuracy
	Sample 1	Sample 2	Sample 3				
8	8.17	8.14	8.19	8.166	0.025	0.308	102.083
4	4.19	4.14	4.16	4.163	0.025	0.604	104.083
2	2.12	2.11	2.09	2.106	0.015	0.725	105.333
1	1.01	1.00	1.05	1.020	0.026	2.593	102.000
0.5	0.53	0.53	0.51	0.523	0.011	2.206	104.666
0.25	0.230	0.250	0.231	0.237	0.011	4.755	94.800
0.12	0.120	0.120	0.130	0.123	0.006	4.681	102.778
0.06	0.061	0.059	0.056	0.058	0.002	4.289	97.777
Limit of detection (LOD) of valsartan in plasma							
0.12	0.11	0.12	0.12	0.116	0.005	4.948	97.222
0.06	0.061	0.059	0.056	0.058	0.002	4.289	97.777
0.03	0.027	0.02	0.024	0.023	0.003	14.838	78.888

Table 4: Accuracy and precision of valsartan in plasma

Selected concentrations in method validation (µg/mL)			
	0.18 (µg/mL)	3 (µg/mL)	6 (µg/mL)
Intraday Accuracy & Precision			
Mean (n=5)	0.180	3.004	5.750
% Accuracy	100.00	100.13	95.88
SD	0.005	0.036	0.384
% CV	3.042	1.214	6.673
Interday Accuracy & Precision			
Mean (n=5)	0.175	2.916	5.620
% Accuracy	97.666	97.2	93.666
SD	0.005	0.063	0.187
% CV	2.993	2.177	3.333

Table 5: Post extraction stability of valsartan from plasma

Spiked plasma concentration (µg/mL)	Bench-top stability		Auto sampler stability	
	24 h		65 h	
	Concentration measured (n=6) (µg/mL) (mean ±S.D)	CV (%) (n =6)	Concentration measured (n=6) (µg/mL) (mean ± S.D)	CV (%) (n=6)
0.5	0.49 ± 0.21	1.32	0.47 ± 0.34	1.68
4	3.8 ±1.0	2.78	3.7 ±1.8	2.89

Table 6: Freeze and thaw stability of valsartan

Sample No	Low Concentration (1µg/mL)				High Concentration (6µg/mL)			
	Fresh Sample	*FT Cycle 1	FT Cycle 2	FT Cycle 3	Fresh Sample	FT Cycle 1	FT Cycle 2	FT Cycle 3
1	1.010	0.980	0.980	0.970	5.930	5.830	5.640	6.000
2	1.030	0.990	0.960	0.960	6.000	5.980	6.000	5.550
3	0.990	1.000	0.980	0.960	5.840	6.000	5.930	5.630
4	1.000	1.010	0.990	0.980	5.980	5.820	5.830	5.540
5	1.020	0.990	1.000	0.970	6.010	5.780	5.420	5.760
Mean	1.010	0.994	0.982	0.968	5.952	5.882	5.764	5.696
SD	0.015	0.010	0.014	0.008	0.069	0.101	0.235	0.191
% CV	1.565	1.147	1.510	0.864	1.724	1.710	4.081	3.360
% Accuracy	101.00	99.400	98.200	96.800	99.200	98.030	96.066	94.933

*FT Cycle = Freeze-thaw cycle

Table 7: Long term stability of valsartan in plasma

Sample No	Low Concentration (1µg/mL)			High Concentration (6µg/mL)		
	Fresh Sample	After 2 Weeks	After 3 Weeks	Fresh Sample	After 2 Weeks	After 3 Weeks
1	1.000	0.960	0.910	6.050	5.950	5.970
2	1.030	0.980	0.960	5.950	6.040	5.760
3	1.060	1.050	1.000	6.032	6.010	5.790
4	1.050	0.950	1.030	5.888	5.880	6.000
5	0.980	0.960	0.950	6.090	6.000	5.890
Mean	1.024	0.980	0.970	6.002	5.976	5.882
SD	0.033	0.040	0.046	0.081	0.062	0.106
% CV	3.282	4.144	4.780	1.359	1.049	1.804
% Accuracy	102.400	98.000	97.000	100.033	99.600	98.030

Table 8: Stock solution stability of valsartan in methanol

Sample No	Fresh Sample (1µg/mL)	After 2 Weeks	After 3 Weeks	After 4 Weeks
1	0.993	0.996	0.991	0.996
2	1.003	0.98	0.978	0.989
3	1.006	0.986	0.995	0.975
4	1.014	0.998	0.99	1.01
5	0.992	1.01	0.986	0.989
Mean	1.002	0.994	0.988	0.992
SD	0.008	0.010	0.006	0.011
% CV	0.825	1.042	0.583	1.147
% Accuracy	100.16	99.4	98.8	99.18

Table 9: Recovery of plasma samples

Concentration (µg/mL)	Mean Peak Area in Plasma	Mean Peak Area in Mobile Phase	% Recovery
4	34634	35030	98.871
2	17947	18092	99.201
1	9516	9751	97.586
0.5	5058	5230	96.711
		Mean Recovery	98.092

Table 10: Pharmacokinetic parameters of Diovan® tablet

T_{max}	C_{max}	AUC_{0-t}	AUC_{0-inf}	AUMC	MRT	$T_{1/2Kel}$
h	µg/mL	µg/mL.h	µg/mL.h	µg/mL.$(h)^2$	h	h
2.601	3.071	23.650	29.618	716.661	9.971	5.962

Precision and accuracy of the suggested technique is evident from the % accuracy and % CV results well within 15% of the nominal concentrations (table 4). Stability studies were spanned from the time of first sample collection in a clinical study till the last sample analysis. Selected concentrations of analyte were assessed for stability at varying environmental conditions like bench top, autosampler (table 5), freeze-thaw (table 6) and long term plasma stability (table 7) while stock solution stability was determined for 4 weeks in methanol. Valsartan was found stable under these conditions as presented in table 8.

Analytical recovery was assessed by comparison of peak area of selected valsartan strengths prepared in mobile phase to those prepared in plasma (Zhang *et al.,* 2014; Thürmann, 2000). Recovery was found consistent and reproducible in the range 96.7- 99.2% (table 9).

Healthy volunteer was recruited as per FDA guidelines for pharmacokinetic evaluation of valsartan (marketed product Diovan®) (FDA, 2003). Chromatograms of Diovan® 160 mg in plasma at 0.5h and 3h are shown in fig. 3. Plasma valsartan concentration versus time profile for Diovan® 160mg tablet is illustrated in fig. 4 and pharmacokinetic parameters are presented in table 10.

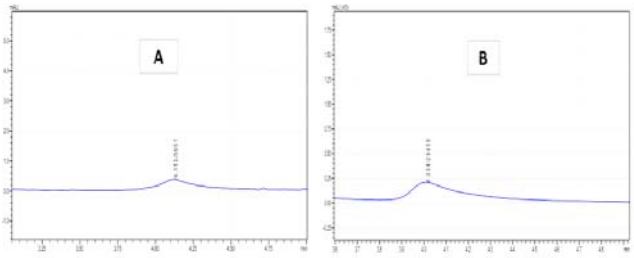

Fig. 3: Chromatogram of Diovan® 160mg in plasma at A) 0.5h and B) 3h.

DISCUSSION

In this study valsartan was accurately quantified in human plasma by applying a simple protein precipitation technique using acetonitrile in the ratio of 1:1 to avoid unnecessary dilution of drug and to achieve satisfactory lower limit of quantification (LLOQ). The chromatographic conditions were optimized so that no interfering peak was found at the analyte's retention time. Since valsartan is a lipophilic drug (Saseen and Carter, 2005) hence deemed necessary to select a mobile phase composition that facilitates suitable retention of analyte and early elution of plasma proteins and phospholipids. The most appropriate ratio of aqueous phase to organic phase was thus 1:1.

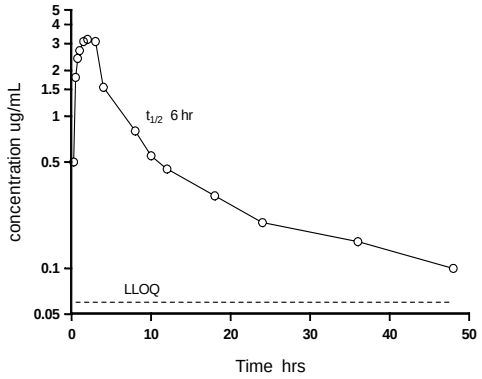

Fig. 4: Plasma concentration vs. time profile of Diovan® 160mg tablet in healthy volunteer

From practical point of view analytical method validation should be aimed at establishing that an analytical method is 'Fit for purpose' (Saydam and Takka, 2007). Therefore the presented method is validated to be applied in a full scale bioavailability or bioequivalence studies.

Applying the method to a pharmacokinetic study with healthy volunteer

The sensitivity and specificity of the developed method were found to be sufficient for accurately characterizing the plasma pharmacokinetics of valsartan in humans. This validated method was successfully applied to the pharmacokinetic study of immediate release valsartan 160mg tablet in human plasma. The evaluated pharmacokinetic parameters includes area under the plasma concentration-time curve (AUC_{0-t}), the area under the plasma concentration–time curve extrapolated to infinity ($AUC_{0-\infty}$), maximum plasma concentration (C_{max}) and time to achieve the maximum concentration (t_{max}), elimination half-life $t_{1/2}$, area under first moment plasma concentration (AUMC) and mean residence time (MRT). The plasma concentration–time profile for a healthy volunteer after an oral administration of valsartan tablet 160mg was shown in fig. 4. Like our study, Abdallah and Zeid, also measured the pharmacokinetic parameters by using non-compartmental methods via Kinetica® 2000 software. The values of the main pharmacokinetic parameters are shown in table 10 which agreed with the published pharmacokinetic studies (Biswas and Kuotsu, 2017; Sohail *et al.,* 2015; Zaid *et al.,* 2014; Kim *et al.,* 2014).

CONCLUSION

The defined technique is precise and sensitive with short retention time values and sufficient limit of quantitation thus making it economical and suitable for use in bioavailability and bioequivalence studies for valsartan.

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