Liquid Chromatography Method Development and Optimization for Valsartan: Pharmacokinetics of Oral Hydrogels in Rabbits

[1]Muhammad Sohail, [2]Mahmood Ahmad*, [2]Muhammad Usman Minhas, [4]Abubakar Munir
[2]Ikrima Khalid and [3]Haroon Rashid
[1]Department of Pharmacy, COMSATS Institute of Information Technology Abbottabad-22060, Pakistan.
[2]Faculty of Pharmacy and Alternative Medicine, the Islamia University of Bahawalpur-63100, Punjab, Pakistan.
[3]Institute of Chemistry, Martin Luther University, Von-Danckelmann-Platz 4, 06120, Halle (Saale) Germany.
[4]Department of Pharmacy Superior College Lahore, Pakistan.
ma786_786@yahoo.com*

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Summary: A simple, rapid, precise, accurate high performance liquid chromatography method has been developed and subsequently validated for determination of valsartan in rabbit plasma. The method was developed employing mixture of mobile phase consisting of 0.02 M potassium dihydrogen phosphate buffer and acetonitrile (45:55), pH was adjusted to 2.7 using 50% orthophosphoric acid and pumped thorough chromatographic system at a flow rate of 1 ml/min. An isocratic elution mode was carried out at HPLC system (Agilent Technologies, 1200 series, USA) fitted with variable wavelength detector and data processing software ChemStation. For sample analysis, 20µl sample was injected and eluate was monitored at 225nm wavelength. Pharmacokinetic evaluation was performed in rabbits after the oral administration of valsartan loaded PVA-co-poly(AA) hydrogels. The mean C_{max} of valsartan was 408.439ng/ml, T_{max} was 12h and half life ($t_{1/2}$) was 8.812h. The extraction procedure was simple with good response even at very low drug concentration, thereby making this method suitable for pharmacokinetic application. It is concluded that developed method is simple, fast, cost effective, and reproducible for the analysis of pharmacokinetic parameters in the rabbit plasma.

Key words: HPLC, Chromatography, Valsartan, Hydrogel, Rabbits, Pharmacokinetics.

Introduction

Cardiovascular (CV) diseases and hypertension are the major causes of morbidity and mortality globally, with the majority of burden occurring in developing countries, and epidemics of CV diseases is advancing across many regions of the world particularly those undergoing rapid social, economical and health transitions [1]. World Health Organization (WHO) has reported a 6-fold higher mortality rate among women and men due to cardiovascular diseases in Russian federation compared with France. The relationship between hypertension and the risk of CV diseases is directly proportional, consistent and continuous [2], thereby rendering reduction of hypertension as main goal to decrease the chances of CV diseases. Clinical studies have proved systolic blood pressure (SBP) as major risk factor CV diseases, thus main focus of treatment should be reduction of SBP [3]. Various studies have reported therapeutic potential of angiotensin-receptor blockers (ARBs) andangiotensin-converting– enzyme (ACE) inhibitors in reducing the incidence of diabetes and the risk of cardiovascular events in patients with hypertension and other cardiovascular disease [4]. Since the initial approval of valsartan, there has been a tremendous increase in its use worldwide, being mainly indicated for heart failure (HF), post-MI and to reduce mortality related to CV diseases. It has also been used for treating hypertension in children aged 6-16 years. Cardio-vascular (CV) risk associated with hypertension may be reduced with well-timed reduction of blood pressure [5, 6]. A number of studies have revealed that valsartan shows dose-dependent onset of BP decline [7, 8].

Valsartan is described chemically as N-valeryl-N [[2-(1H- tetrazol-5-yl) biphenyl-4-yl] methyl] valine (Fig. 1) [9]. It is a potent orally active drug, highly selective, being effective in lowering blood pressure and is mainly indicated for the management of mild to moderate essential hypertension. It is classified as specific angiotensin-II type-I receptor blockers [10]. Valsartan selectively inhibits angiotensin-II receptors, relaxing blood vessels as a result vessels are widened, lowering blood pressure and improving blood flow [11-13]. Valsartan is well tolerated after single oral dose up to 400 mg and multiple dosing with 200 mg per day [14, 15]

*To whom all correspondence should be addressed.

After oral administration, valsartan is rapidly absorbed having relatively poor bioavailability of 23% and 95% of the drug is bound to serum proteins mainly albumin [13, 16]. Peak plasma concentrations (C_{max}) is achieved within 2-4 hours of ingestion. The drug is not significantly metabolized and is excreted mainly as unchanged drug via bile [17, 18].

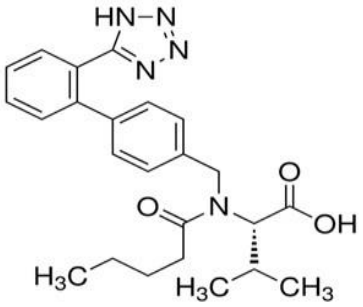

Fig. 1: Structural formula of Valsartan.

Previously very few high-performance liquid chromatographic (HPLC) methods have been developed for determination of valsartan in plasma, but these methods employexpensive solvents with longer run times, thereby limiting their use. Most of the reported assays employ native fluorescence of valsartan and fluorimetric detectors for its detection [13, 15, 19-20]. Sample preparation was performed usually by liquid-liquid extraction using methyl-*tert*-butyl ether [19], ethyl acetate [15] or solid-phase extraction with C8 [20-21] and cyclohexyl [22] cartridges. In these methods, limit of quantitation has reported to be 5-130 ng/ml with run time between 10-30 min.

The purpose of current study is todevelop and validate a simple, rapid and sensitive HPLC-UV method for unified quantification and determination of valsartan in rabbit plasma and to evaluate its pharmacokinetics parameters after the administration of valsartan loaded PVA-co-poly(AA) hydrogels to healthy rabbits. The overall speed of analysis is improved by eliminating complex and tedious extraction procedures and by optimizing chromatographic conditions. HPLC method was validated for specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision and accuracy.

Experimental

Chemicals and Reagents

Valsartan was obtained as a kind gift from Werrick Pharmaceuticals (Pvt.) Ltd. Islamabad, Pakistan. Acetonitrile and potassium dihydrogen phosphate was obtained from Merck (Germany). Orthophosphoric acid was purchased from Sigma-Aldrich. HPLC grade water was prepared in our laboratory at the Islamia University of Bahawalpur. Rabbit plasma was obtained from animal house of our department.

Instrumentation and Chromatographic Conditions

An isocratic high performance liquid chromatography system of Agilent technologies (series 1200) consisting of a pump and variable wavelength detector (VWD) with data processing software ChemStation was used for the analysis of prepared plasma samples. Chromatographic separation was performed at room temperature on BDS hypersil C18 (5µm, 4.6mm × 250 mm) (Thermo-Electron, USA) stainless steel column. The mobile phase used for the elution was composed of 0.02 M potassium dihydrogen phosphate buffer and acetonitrile (45:55). pH of the mobile phase was adjusted to 2.7 using 50% orthophosphoric acid. The mobile phase was degassed and filtered through 0.45µm millipore filters. Mobile phase was eluted at a flow rate of 1.0 ml/min.

Sample of 20µl was injected in to the system and effluent was monitored at a wavelength of 225 nm. The analyte was eluted using various C_{18} stationary phases e.g. hypersil BDS, hypersil ODS and Nucleosil C_{18} and various mobile phases. Different combinations of mobile phases were investigated by changing its components, component ratios (aqueous and organic). Buffers of various types like acetate, phosphate and sulphate in pH range of 2-8 were also studied as mobile phase. Effect of various organic modifiers like acetonitrile, methanol and ethanol on separation of valsartan in plasma was also investigated.

Formulation Development and Pharmacokinetic Studies

A controlled release formulation, valsartan loaded PVA-co-poly(AA) hydrogels were developed and administered to rabbits for pharmacokinetic studies [23]. Plasma drug concentration in rabbit plasma was determined through the constructed calibration curves. After hydrogel administration, estimation of drug concentrations in rabbit plasma was performed by using Microsoft® Office Excel 2007 program. Pharmacokinetic parameters (AUC, Cmax, Tmax, $t_{1/2}$, Cl, MRT and Kel) were calculated using scientific application package Kinetica® version 4.1.1 (Thermo Electron Corporation).

Standard Solutions

The stock solution of valsartan (1000 µg/ml) was prepared by dissolving 100mg pure valsartan in 100 ml of deionized distilled water. Using stock solution, further serial dilutions of 0.1, 1, 2, 4, 6, 8, 10, 12, 16, 20µg/ml were prepared for the construction of calibration curve.

Plasma Samples

Rabbit plasma (Blank) was obtained from animal house of the Faculty of Pharmacy & Alternative Medicine, the Islamia University of Bahawalpur. The blank plasma was stored at -20^{0}C and used for method development and validation. The study was performed with the prior approval of Pharmacy and Research Ethics Committee (PREC) (Ref. No. 112-2013/PREC) the Islamia University of Bahawalpur.

Plasma Spiking and Sample Preparation

A series of valsartan solutions were prepared in distilled water. To prepare the specific concentration range, 500µl plasma was spiked with 50µl drug solution. Extraction of drug from plasma was performed by the addition of 500µl acetonitrile followed by vortexing for 10 min. The precipitated samples were centrifuged (centrifuge machine by EBA-20, Hettich-Germany) at 3500 rpm for 15 min. The transparent supernatant layer was separated in clean and dried amber glass vials. The extracted drug solution (20 µl) was injected directly into the HPLC system.

Calibration and Calculation

A calibration curve was prepared for developing and validating the analytical method. Ten points calibration curve was prepared in rabbit plasma from 0.1-20 µg/ml. First, working drug solutions were injected in to HPLC followed by rabbit plasma samples for analysis. In order to identify interfering peaks of plasma, blank plasma was injected before each analysis. Each calibration curve was constructed in triplicate to confirm the precision and accuracy.

Method Validation

Limit of Detection(LOD) and Quantification (LOQ)

For evaluating the sensitivity of analytical methods, LOD and LOQ are regarded as essential parameters. The LOD is the concentration or amount of analyte corresponding to a measurement level three units above the value for zero analyte while the LOQ is concentration of analyte quantifiable with a variation coefficient not greater than 10% and its value is always higher than the LOD [24]. The LOD and LOQ were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD was calculated using the equation as described by [25];

LOD = (3N/S) x amount found

Analysis Time

Overall cost of the analytical method is affected by the consumption of chemicals or solvents, which in turn are affected by the total analysis time. For any analytical method, total analysis time is determined from the total rum time. Commonly, the run time in the range of 5-10 min is considered optimum for a standard analytical method but variations may occur due to the nature of analyte. The total run time of the method has been optimized to 10 min.

Linearity

The linearity of an analytical method is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. The above mentioned concentration ranges for drug were repeatedly injected (n=3) in to the system, in order to establish the linearity of the proposed method. Obtained peak areas were plotted against the anticipated drug concentrations. Correlation coefficient, slope and intercept parameters were calculated by the least-squares linear regression analysis.

Accuracy and Precision

In analytical method development, the closeness of agreement between the accepted reference or true value and the value obtained is termed as accuracy. Accuracy of the proposed method was evaluated by comparing obtained drug concentration with nominal concentrations. Four different QC samples were analyzed to estimate inter- and intraday precision and accuracy. The intraday precision and accuracy was calculated by analyzing six replicates of QC samples on same day, while each sample was analyzed on six different days to evaluate inter-day precision and accuracy. To evaluate the precision and accuracy within acceptable limits, relative standard deviation (R.S.D) was calculated. The R.S.D for precision must be equal or less than 15% [26, 24].

Selectivity

The selectivity was evaluated by the resolution of valsartan from nearest interfering peaks of plasma proteins and other components of mobile phase. Maximum resolution of analyte was obtained by treating plasma samples with protein precipitants and pH stabilizers. The proposed method showed resolution greater than 2.

Results and Discussion

Optimization of Method

An optimum and desired chromatographic method is the one capable of separating analyte of interest with good resolution, sensitivity, precision, accuracy and reproducibility. Valsartan can be assayed by conventional HPLC-UV method [27-29]. The maximum wavelength for the valsartan was confirmed by UV-spectrometric (UV-spectrometer 1601 Shimadzu, Japan) scan analysis of 10 µg/ml solution of valsartan in double distilled water. The UV-visible scan was performed from 500-200 nm and peak response was recorded. The maximum absorbance was recorded at 225 nm and λ_{max} was then confirmed on HPLC by analyzing reported λ_{max} values of 235 nm [30] and 230 nm [10].

In order to achieve an optimum separation of valsartan in plasma samples, several mobile phases were evaluated. The main problem with valsartan chromatographic analysis was early elution of valsartan (short retention time of 2-4 min). Short retention time caused merger of drug peak with the peaks of plasma protein when spiked in plasma [31]. The short retention time has been shown in the representative chromatogram in Fig. 2. Various Buffers (different molarities) and organic solvents were investigated initially in different ratios and pH ranges. Potassium dihydrogen phosphate buffer (0.02M) and methanol showed good peak resolution but elution was carried out too early. Later on, potassium dihydrogen phosphate buffer (0.02M) combined with acetonitrile at pH 2.7 showed good resolution and separation after 5 min, as shown in representative chromatogram in Fig. 3. Previously many researchers have developed HPLC method using mobile phases of various compositions. Flesch *et al.*, [13] developed HPLC method for valsartan using acetonitrile and trifluoroacetic acid (45:55) as mobile phase and Neucleosil C_{18}as column in binary isocratic elution mode, results showed that peak plasma levels are achieved after 2 hours upon oral administration with terminal $t_{1/2}$ of 7.0 hours and AUC 8.54mg/L, while our study showed higher values for theses parameters, thereby concluding controlled delivery of drug from the fabricated hydrogels. Shah *et al.* [32] developed LC-MS method for simultaneous determination of valsartan and hydrochlorothiazide employing acetonitrile: methanol: aqueous ammonia (75:15:10) as mobile phase using C_8analytical column, this study had limitation in terms of using multiple solvents in the preparation of mobile phase making process tedious, in addition ammonia is a pungent solvent thereby limiting the use of this method.

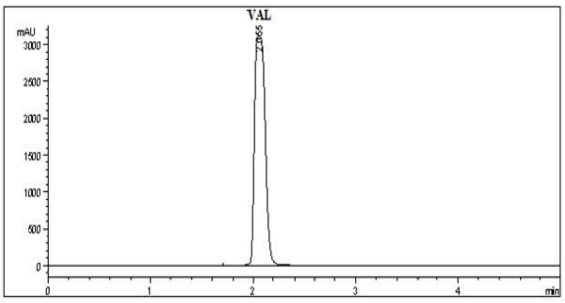

Fig. 2: Chromatogram of pure valsartan solution in mobile phase of 0.02M Potassium dihydrogen Phosphate: Methanol (80:20) at pH 2.7 adjusted with perchloric acid), shows the short retention time.

Analysis Time

In the development of chromatographic method for valsartan, initially short retention time (2-4 min) was observed at various pH values, which was then optimized and retention time was delayed. A mobile phase of 0.2M phosphate buffer: acetonitrile (45:55) having pH 2.7, adjusted with ortho-phosphoric acid delayed the retention time of valsartan upto 5-6 min, which resolved the problem of interference of plasma protein peaks with the drug peak. Total analysis time was 10 min only for a single run which was an optimum time for the analysis of several plasma samples of valsartan. In previously reported studies, Zarghi *et al.* [10] have reported 3.3 min retention time of valsartan. Iriarte *et al*., [9] observed 8.3 min retention time for the valsartan in their analytical method development, while Chitlange *et al*. [30] reported 6.2 min retention time for valsartan in developed method.

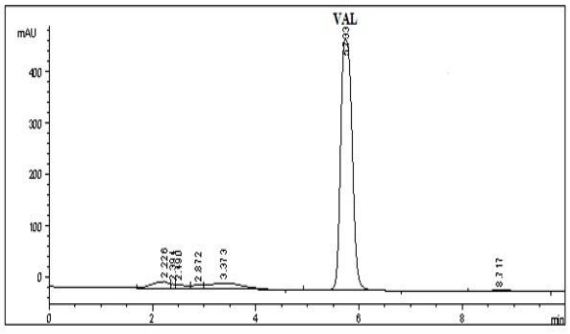

Fig. 3: Chromatogram of pure valsartan solution in mobile phase of 0.02M Potassium dihydrogen Phosphate: acetonitrile (45:55) at pH 2.7 adjusted with Ortho-phosphoric acid), shows retention time after 5 min.

Chromatographic Separation and Selectivity

In current chromatographic method, the run time was not more than 10 min and valsartan was successfully separated from plasma. The retention time of valsartan was 5-6 min with no interference with plasma proteins. In the chromatographic conditions described above, the analysis of valsartan in rabbit plasma showed excellent separation. The representative chromatograms of blank and spiked rabbit plasma have been shown in Fig. 4 and 5.

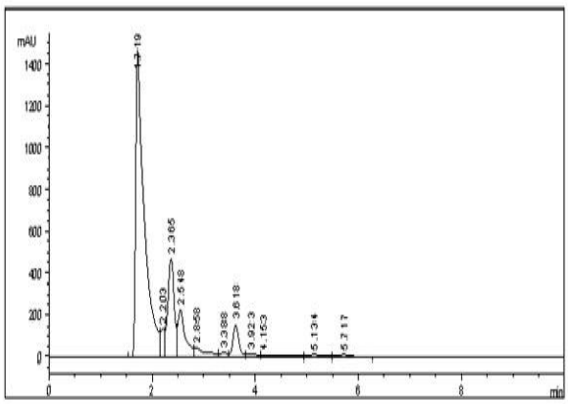

Fig. 4: Chromatogram of rabbit blank plasma (without drug).

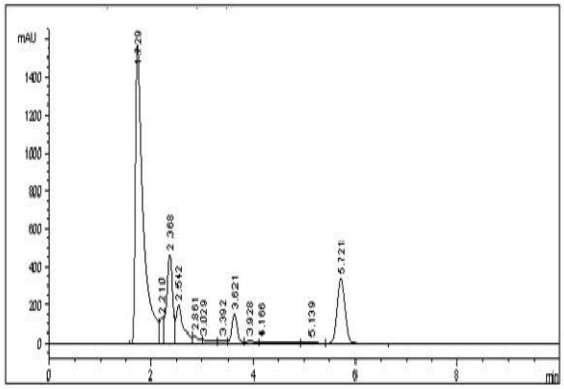

Fig. 5: A representative chromatogram of spiked rabbit plasma (5µg/ml).

Linearity of Calibration Curves, LOD and LOQ

Calibration curves of rabbit plasma showed good linearity ($r^2 \geq 0.995$) over the specified concentration range. The regression correlation equations for calibration curves and their regression coefficients are summarized in Table-1.The obtained LOD and LOQ values for rabbit plasma were 105ng/ml and 119ng/ml, respectively.

Table-1: Calibration curves parameters of valsartan in rabbit plasma.

Run Number	Equation Form: Y=BX+A		Correlation Coefficient (r^2)
	B	A	
1	0.049	0.040	0.995
2	0.048	0.042	0.996
3	0.049	0.041	0.995
N	3	3	3
Mean ± SD	0.049±0.00058	0.041±0.001	0.995±0.00058

Precision and Accuracy

The intra-day and inter-day precisions were determined as coefficient of variation (CV%) and ranged between 1.50% to 2.30% and 1.92% to 2.65%, respectively for valsartan in rabbit plasma. Similarly, the intra-day and inter-day accuracy was in the range of 99.25% to 99.88% and 98.71% to 99.63% for valsartan in rabbit plasma. The values confirmed the good precision and accuracy of developed method in rabbit plasma. All values are presented in Table-2.

Table-2: Intra-day and Inter-day precession and accuracy of valsartan in rabbit plasma.

Intra-day (n=6)			
Parameters	LQC(ng/ml)	MQC(ng/ml)	HQC(ng/ml)
Nominal Conc.	110.0	150.0	200.0
Mean	109.18	149.29	199.76
±S. D.	2.112	3.441	3.011
Precision CV%	1.934	2.305	1.507
Accuracy (%)	99.25	99.53	99.88
Inter-day (n=6)			
Parameters	LQC (ng/ml)	MQC (ng/ml)	HQC (ng/ml)
Nominal Conc.	110.0	150.0	200.0
Mean	108.58	148.91	199.27
±S. D.	2.887	2.868	4.119
Precision CV%	2.659	1.926	2.067
Accuracy (%)	98.71	99.27	99.63

Plasma Concentration Quantification and Pharmacokinetic Profiling

Plasma concentration versus time curves after the administration of valsartan loaded hydrogel (3mg/kg) is shown in Fig. 6. The mean C_{max} of valsartan was 408.439ng/ml, T_{max} was 12 h and half life ($t_{1/2}$) was 8.812h. Pharmacokinetic parameters of valsartan loaded hydrogels in rabbit plasma are summarized in Table-3.

Table-3: Pharmacokinetic parameters of (Mean ± SEM, n=6) of valsartan loaded hydrogels (3 mg/kg) in rabbit plasma.

S. No.	Pharmacokinetic Parameters	Valsartan Loaded Hydrogel (Mean ± SEM)
1	T_{max} (hr)	12.00±0.000
2	C_{max} (ng/ml)	408.439±2.96
3	$t_{1/2}$ (hr)	8.8125±0.068
4	AUC_{tot} (ng.h/ml)	7374.38±34.558
5	K_{el} (hr^{-1})	0.07866±0.001
6	Cl (L/hr)	0.000814±0.006
7	MRT (hr)	20.870±0.085

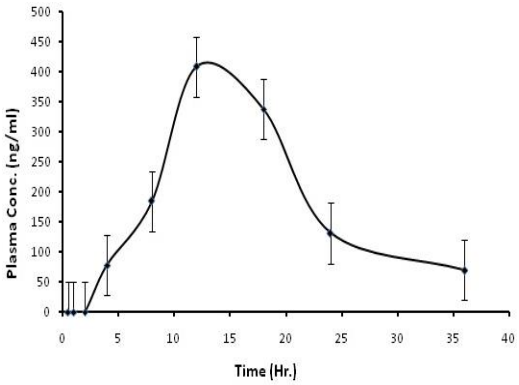

Fig. 6: Mean plasma concentration-time after oral administration of valsartan (03 mg/kg) to healthy rabbits

Conclusion

A reproducible, specific, raid and sensitive chromatographic method has been developed and validated for the analysis of valsartan in rabbit plasma. In present study, simple processing (mobile phase and sample preparation) and validation in rabbit plasma will lead to an enhancement of its applicability in routine analysis of valsartan in preclinical/clinical analysis and drug delivery research.

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