Research Article

Rapid HPLC-UV Method for Quantification of Valsartan in Plasma and Intestinal Perfusate for Pharmacokinetic Studies

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ABSTRACT

A simultaneous HPLC method using UV detector was developed and validated for quantification of valsartan in plasma and intestinal perfusate samples of rats. Plasma samples were purified with a simple protein precipitation procedures. An isocratic separation of valsartan was performed on reversed-phase C18 (4.6-mm × 250-mm) column. Acetonitrile-water (adjusted to pH 3.3 with formic acid)-methanol (50:40:10, v/v) was the mobile phase used at column temperature of 37°C, flow rate of 0.9 ml/min and under pump pressure of 78 bar. Wave length of detection was 230 nm. Linear responses of R^2>0.999 in the concentration range of 100-10,000 ng/ml were observed with a short time for separation of less than 10 minutes. The method was highly accurate and precise, and the recoveries were more than 95%. Same results were seen upon analysis of samples spiked before extraction, which indicates the suitability of the developed method for application in pharmacokinetic studies.

Keywords: Valsartan, HPLC-UV, Validation, Plasma, Intestinal perfusate.

INTRODUCTION

Valsartan is a nonpeptide specific angiotensin II receptor blocker acting on the AT1 receptor subtype, used in the management of hypertension. Valsartan is official in USP and chemically described as l-Valine, N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-N-[p-(o-1H-Tetrazol-5-ylphenyl)benzyl]-N-valeryl-l-valine.[1] Its molecular formula is C24H29N5O3, with a molecular weight of 435.5 g/mol. Structural formula is shown in Figure 1.

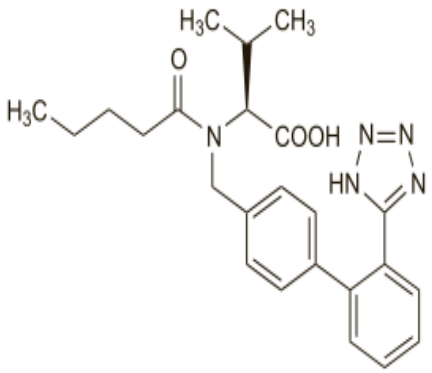


Figure 1: Structural Formula of Valsartan

Valsartan is rapidly absorbed after oral doses, with a bioavailability of about 23%. The initial dose is 80 mg once daily. This may be increased, if necessary, to 160 mg once daily; and the maximum dose is 320 mg once daily.[2]

Many analytical methods were validated to estimate valsartan in pharmaceutical dosage forms and plasma. These included fluorimetric detection,[3,4] spectrophotometric detection,[5,6] and LC-MS/MS methods.[7,8] LC-MS/MS method had the higher selectivity and sensitivity with low limit of quantitation, but the special analytical requirements and the high cost may limit its application. This work was conducted to provide an accurate, simple and less expensive method for quantification of valsartan in large number of rat's intestinal perfusion solution and plasma samples, in a short time, for pharmacokinetic studies. FDA, 2014; Analytical Procedures was the guidance for this validation.[9-11]

MATERIALS AND METHODS

Instrumentation

UFLC XR Schimadzu HPLC system consisted of two HPLC pumps, with a UV detector, and a vacuum degasser. A Knalier Vertex Plus C18, 250×4.6 mm, Eurospher 100-S, 5 µm Column (Berlin, Germany) was used. Chromatograms were recorded by a computer and were treated with the Realtime Analysis software (Kyoto, Japan). Water for HPLC was obtained from Siemens Labostar water filteration system (Alpharetta, USA).

Chemicals

Valsartan (purity 99.7%) was supplied from Zhuhai Rundu Pharmaceutical Co Ltd (Zhuhai, China). Acetonitrile and methanol of HPLC grade, along with other salts and reagents of ACS grade were purchased from Carl Roth (Karlsruhe, Germany).

Sample Preparation

Intestinal Perfusion Solution Samples

Krebs- Henseleite Bicarbonate buffer was the intestinal Perfusion solution. It was consisted of NaCl, 118 mM; $NaHCO_3$, 24.9 mM; $CaCl_2.6H_2O$, 2.5 mM; KCl, 4.7 mM;

$MgSO_4.7H_2O$, 1.2 mM; KH_2PO_4, 1.2 mM; and contained 16.7 mM glucose and 0.01 mM sodium taurocholate, adjusted to pH7.4 using NaOH). Rats were obtained from experimental animals facility, Faculty of Pharmacy, Damascus University. An intramuscular injection of Ketamine 60 mg/Kg and Diazepam 2.5 mg/Kg combination was used for anaesthesia.[12,13] Abdomen midline incision was performed and the small intestine was canalized starting from duodenum to the end of jejunum. Buffer solution was perfused through the intestine at a flow rate of 0.5 ml/min after flushing with warmed normal saline. The intestinal perfusion solution was collected and centrifuged at 3500 rpm for 10 minutes. Polypropylene tubes were used to collect the supernatant and frozen at -80°C until analysis.[14]

Plasma Samples

To prepare plasma matrix, Blood samples were obtained from 6 rats,[15] collected by cardiac puncture after anaesthesia, individually sonicated for 5 minutes to lyse RBCs, and centrifuged at 3500 rpm for 10 minutes. Plasma supernatant was carefully separated and collected in polypropylene centrifuge tubes. Acetonitrile was added in the ratio 3:1 (acetonitrile:plasma) to precipitate plasma proteins. The matrix was thoroughly mixed and centrifuged for 10 minutes at 6000 rpm. Upper clear solution layer was collected. Plasma aliquot tube was kept on ice until freezing at -80°C.[16]

Calibration Standards

100 mg of valsartan was dissolved in 100 ml of methanol to prepare 1.0 mg/ml stock solution. The maximum concentration of valsartan in intestinal perfusion solution for PK studies in rats was calculated depending on the maximum single oral dose in human.[17] Therefore, the calculated dose in 300-g rat was found to be 1.37 mg. Although, the intestinal perfusion solution of 30-ml volume had the concentration of 45.67 µg/ml (104.86 µM), which was the maximum concentration, the maximum concentration in the calibration curve which proved a linearity was 10 µg/ml, so a 5 times dilution should be performed. The lower limit of quantitation (LLOQ) was determined through the bioanalytical method. The final concentrations of calibration standards were 100, 200, 250, 500, 1000, 2000, 2500, 5000, 7500, 10000 ng/ml for Valsartan. Standard solutions with concentrations of 100,000, 10,000, 1,000, 100 and 10 ng/ml were prepared by serial dilution in methanol.

Quality Control Samples

Precision and accuracy standards were prepared in the same manner. Standard solutions were used to prepare calibration and quality control (QC) samples in intestinal perfusion solution and plasma by spiking after extraction. LLOQ, LQC (3LLOQ), medium (MQC), high (HQC), and diluted sample (5 times dilution factor) were prepared to yield the following concentrations: 100, 300, 450, 1000, 6500, 8000, 10000, and a concentration of 50000 ng/ml diluted with solvent 5 times to 10000 ng/ml.

Extraction Recovery

Recovery was calculated using the results of QCs used for precision and accuracy on the first day run. Recovery was determined by comparing the response of spiked matrix with the response of standard solution.

Method Validation

The bioanalytical method was validated for selectivity, precision, accuracy, linearity, stability and recovery. According to ICH Q2B guideline on validation,[18] standard deviation of the y-intercept and the slope of the regression line were used to calculate lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). Three separate batches of calibration standard samples were prepared for evaluation of linearity, and five replicates of QC samples of valsartan in matrix were evaluated for Intra- and inter-batch precision and accuracy.

Accuracy was reported as percentage bias (%RE), whereas precision was expressed as the percentage coefficient of variance (%CV) or relative standard deviation (RSD) of the replicate measurements. Deviation of the LLOQ from concentration should not exceed 20%, and 15% of standards other than LLOQ. Selectivity was investigated by analysis of blank matrix and spiked matrix samples. Stock solution stability, short-term stability, long-term stability, and freeze-thaw stability were studied.

RESULTS AND DISCUSSION

Chromatographic Conditions

Chromatographic conditions were tested to achieve the desired selectivity, sensitivity and symmetric peak shape. Isocratic separation was performed on a reversed-phase 250×4.6 mm, 5 µm Vertex Plus C18 Column maintained at 37°C. Sonication of the mobile phase was performed for degassing before application. Injection volume was 10 µL. Mobile phase solutions consisted of acetonitrile: water (adjusted to pH3.3 using formic acid): methanol (50:40:10, v/v) were connected to the two inlets of the binary HPLC pump with flow rate adjusted to 0.9 ml/min. Pump pressure was 78 bar. Valsartan was detected at 230 nm.

Linearity

10 concentrations of valsartan ranging between 100 and 10,000 ng/ml were used for evaluation of linearity, each concentration was measured in triplicate. LLOQ was selected observationally at the beginning to be 100 ng/ml. Microsoft Excel was used to generate linear regression equations for the calibration curves (A=aC+b, where A is the absorbance, a is the slope, b is the intercept and C is the concentration). All curves showed good linear response with R^2>0.999 and a slope value of 0.0005 in solvent (methanol), intestinal perfusion solution, and plasma (Figure 2). The calibration curve equation of valsartan in solvent was A=0.0005C + 0.0115 with a standard deviation (SD) of intercept equal to

0.0031. In intestinal perfusion solution, the equation was A=0.0005C + 0.013 with a SD of intercept equal to 0.005. And in plasma, the equation was A=0.0005C + 0.011 with a SD of intercept equal to 0.005. Retention time values were 6.1, 5.9, and 6.2 minutes in solvent, intestinal perfusate and plasma, respectively.

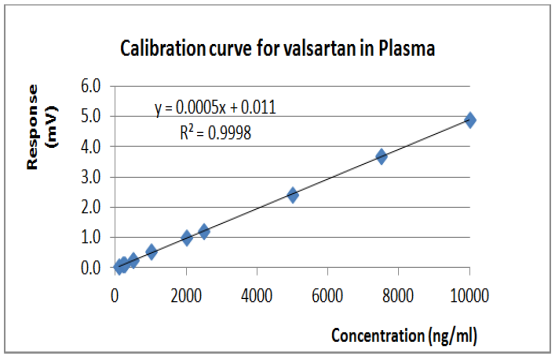


Figure 2: Calibration curve for valsartan in plasma

Determination of Limit of Detection and Limit of Quantitation

Lower limits of detection and quantitation were calculated in solvent, perfusate and plasma. The range of linearity was between 100 and 10,000 ng/ml. The Lower limits of detection (LLOD) were 20.138, 32.309, and 30.434 ng/ml in solvent, perfusate and plasma, respectively. While, the lower limits of quantitation (LLOQ) were 61.025, 97.905, and 92.224 ng/ml in solvent, perfusate and plasma, respectively.

Selectivity and Sensitivity

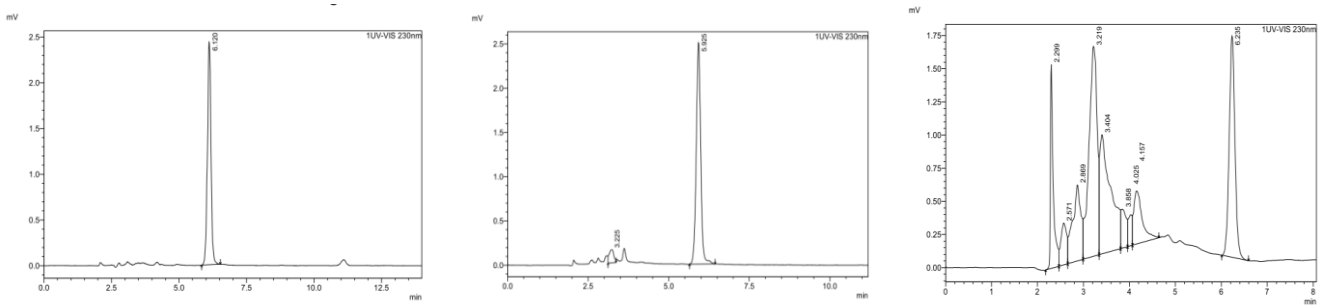
Chromatograms showed no peaks from endogenous compounds were observed on the same retention time of valsartan (6±0.24 minutes) (Figure 3).

Carry-over in blank sample following high concentration standard was convenient with the FDA requirements for bioanalytical methods since it was not greater than 20% of the LLOQ.

Precision and Accuracy

Statistical evaluation of the results established high precision and accuracy. It was applied on seven concentration levels covering the calibration curve range; 3 low concentration levels including the LLOQ and three times the LLOQ; lower QC (LQC), two concentration levels within the medium calibration curve range, upper calibration curve range (HQC), and a five-times diluted sample using the mobile phase.

Each concentration level was repeated 5 times within the same day, and in three consecutive days. Results for perfusate and plasma samples were within the acceptance criteria and were shown in Table 1.



(a) (b) (c)

Figure 3: Chromatograms of valsartan in solvent (methanol) (a), Intestinal perfusion solution (b) and plasma (c) under the selected chromatographic conditions.

Table 1: Intra- and Inter-day Precision and Accuracy in Perfusate and Plasma

Added Concentration (ng/ml)	Precision and Accuracy											
	Perfusate						Plasma					
	Intra-day			Inter-day			Intra-day			Inter-day		
	Found Conc. (ng/ml)	RSD (%)	%RE	Found Conc. (ng/ml)	RSD (%)	%RE	Found Conc. (ng/ml)	RSD (%)	%RE	Found Conc. (ng/ml)	RSD (%)	%RE
100	91.6	4.37	-8.40	87.6	2.232	-10.40	99.2	4.73	-0.80	96.00	3.15	-4.000
300	301.6	0.66	0.53	268	6.106	-5.82	268	5.73	-10.67	272.67	1.53	-9.111
450	436	4.77	-3.11	424	1.481	-4.71	422.4	12.66	-6.13	434.40	2.76	-3.467
1000	954	5.28	-4.60	960	0.625	-4.00	938.8	8.57	-6.12	958.93	1.89	-4.107
6500	6578	2.32	1.20	6366	2.354	-1.39	6330.8	4.08	-2.60	6408.93	1.06	-1.401
8000	7850	4.15	-1.88	7882	0.568	-1.98	7910	1.97	-1.13	7914.00	0.48	-1.075
10000	9854	1.02	-1.46	9862	0.589	-1.75	9476	4.14	-5.24	9662.67	2.00	-3.373
10000 (diluted)	9674	3.77	-3.26	9822	1.076	-2.09	9898	2.02	-1.02	9844.67	0.51	-1.553

Table 2: Stability of Valsartan in Intestinal Perfusate and Plasma (n=3) at Various Conditions

Matrix	Stability	QC Spiked Concentration (ng/ml)	Mean Found Concentration ± S.D. (ng/ml)	R.S.D. (%)	Relative Error (%)	% of Initial Concentration
Intestinal Perfusate	Short-Term Stability (6 h)	300	291.3 ± 8.33	2.86	-6.62	93.36
		1000	1064 ± 96.44	9.06	12.32	112.32
		8000	7794 ± 255.34	3.28	-0.09	99.92
	Three Freeze-thaw Cycle	300	275.3 ± 4.62	1.68	-11.75	88.25
		1000	964.7 ± 30.02	3.11	1.83	101.83
		8000	7740.7 ± 152.75	1.97	-0.77	99.23
	Long-Term Stability (6 months)	300	266.7 ± 3.05	1.15	-14.53	85.47
		1000	1007.3 ± 70.24	6.97	6.33	106.33
		8000	7807.3 ± 57.74	0.74	0.09	100.08
Plasma	Short-Term Stability (6 h)	300	276 ± 3.46	1.26	0.98	100.98
		1000	1018 ± 72.11	7.08	2.55	102.55
		8000	7771.3 ± 144.68	1.86	-0.77	99.23
	Three Freeze-thaw Cycle	300	267.3 ± 4.62	1.73	-2.2	97.81
		1000	926 ± 24.33	2.63	-6.72	93.28
		8000	7431.3 ± 230.94	3.11	-5.11	94.89
	Long-Term Stability (6 months)	300	258.67 ± 19.22	7.43	-5.37	94.63
		1000	974.7 ± 56.86	5.83	-1.81	98.19
		8000	7218 ± 140	1.94	-7.83	92.17

Stability

Short-term, long-term, and freeze-thaw stability were studied at three concentration levels; LQC, MQC, and HQC levels. Concentrations after experiment were compared with concentrations of freshly prepared QCs (Table 2). Stock solution showed stability at room temperature for 6 hours (RSD < 1.8%).

All percent deviation values indicated stability of valsartan in all study matrices under the experimental conditions. In perfusate, valsartan showed RSD<10% and RE<13% at all QC levels for short-term stability (6 h), RSD<4% and RE<12% for three freeze-thaw cycle stability, and RSD<7% and RE<15% for long-term stability (6 months). In plasma, valsartan showed RSD<8% and RE<3% at all QC levels for short-term stability (6 h), RSD<4% and RE<7% for three freeze-thaw cycle stability, and RSD<8% and RE<8% for long-term stability (6 months).

Recovery

The mean recovery rates of valsartan in biological matrices spiked after extraction were calculated using the measured responses to valsartan in QC samples prepared in solvent. Six replicates of each concentration level were applied. In perfusate, recovery values were 101.57, 96.85, and 97.96% for the concentrations 300, 1000, and 8000 ng/ml, respectively. The mean recovery in intestinal perfusate was 98.8±4.2%. In plasma, recovery values were 91, 95.44, and 99.23% for the concentrations 300, 1000, and 8000 ng/ml, respectively. the mean recovery was 95.28 ± 5.17%.

Robustness

No significant changes in retention time, peak height and shape were observed upon minor alteration in the pH, mobile phase ratio, injection volume, and column temperature.

Sample Analysis

Samples of intestinal perfusion solution and samples of plasma were spiked with valsartan before extraction and analyzed using the validated HPLC method. The calculated recovery for the studied perfusate samples were 96.9, 101.47, and 99.32% for the concentrations 300, 1000, and 8000 ng/ml, respectively. The mean recovery in perfusate was 99.23%. While, the recovery values were 99.1, 98.22, and 97.45% in plasma. The mean recovery in plasma was 98.25%. The results indicated the suitability of the validated method for the analysis of valsartan in intestinal perfusion solution and plasma for PK studies.

CONCLUSION

A highly precise and accurate HPLC method with UV detection for analysis of valsartan in plasma and intestinal perfusion solution of rats was developed and validated. Simple and rapid sample preparation with liquid-liquid extraction and high recovery rate was achieved. In addition to high selectivity, sensitivity and short run time, this method was cost and time preservative which makes it suitable for pharmacokinetic studies.

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