Sci Pharm. 2008; 76: 439–450 doi:10.3797/scipharm.0808-01

Rapid Quantification of Valsartan in Human Plasma by Liquid Chromatography using a Monolithic Column and a Fluorescence Detection: Application for Pharmacokinetic Studies

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Abstract

A rapid high-performance liquid chromatographic (HPLC) method using a monolithic column has been developed for determination of valsartan in human plasma. The assay is based on protein precipitation using acetonitrile and fluorescence detection. The assay enables the measurement of valsartan for therapeutic drug monitoring with a minimum quantification limit of 20 $ngml^{-1}$. The method involves simple, one-step extraction procedure and analytical recovery was nearly complete. The separation was carried out in reversed-phase conditions using a Chromolith Performance (RP-18e, 100×4.6 mm) column with an isocratic mobile phase consisting of 0.01 M disodium hydrogen phosphate buffer-acetonitrile (60:40 v/v) adjusted to pH 3.5 with diluted phosphoric acid. The excitation and emission wavelengths were set at 230 and 295 nm, respectively. The calibration curve was linear over the concentration range 20-2000 $ngml^{-1}$. The coefficients of variation for inter-day and intra-day assay were found to be less than 6%. The assay was applied for the analysis of blood samples from a pharmacokinetic study.

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Keywords

Valsartan • Plasma • HPLC • Monolithic column • Pharmacokinetic study

Introduction

Valsartan is a potent and specific competitive angiotensin II antagonist which is used in the management of hypertension. Valsartan is rapidly absorbed following oral administration, with a rather poor bioavailability of about 23%. Peak plasma concentrations of valsartan occur 2 to 4 hours after ingestion. The drug is not significantly metabolized and is excreted mainly via the bile as unchanged drug [1, 2]. Several high-performance liquid chromatographic (HPLC) methods are available for determination of valsartan in plasma. All published assays employ native fluorescence of valsartan and use fluorimetric detection [3–10]. The sample preparation involves either liquid–liquid extraction with methyl-*tert*-butyl ether [3] and ethyl acetate [4] or solid-phase extraction using C8 [6–8] or cyclohexyl [3, 8] cartridges. The limit of quantification (LOQ) in these procedures is 5–130 $ngml^{-1}$, run times are typically 10–30 min. Macek et al [10] reported a rapid HPLC/fluorescence method for analysis of valsartan in human plasma. However, this method had low sensitivity (LOQ = 100 $ngml^{-1}$) and internal standard was not used. Accordingly, available pharmacokinetic profiles were only provided for the higher dose (160 or 320 mg) subjects. Recently, a LC-MS/MS method has been reported for the analyses of valsartan in human plasma by Koseki et al [11]. This method is highly selective and very sensitive, with low LOQ of 2 $ngml^{-1}$. However, this method is not practicable for most laboratories because of its special analytical requirements and financial reasons. Moreover, some purification steps are necessay before the samples are injected into chromatographic system such as liquid-liquid extraction, solid phase extraction, etc. Monolithic silica columns as stationary phases exhibit a tailor-made bimodal pore structure with both macropores or through pores and mesopores. The most unique feature of these columns is their high permeability, which is nearly twice as high as that of packed

columns. As a result of their high permeability monolithic silica columns are especially suitable for high throughput analysis. This is typically needed in pharmacokinetic studies where the decreasing concentration of a given drug is monitored [12]. As opposed to individual particles packed into chromatographic columns, monolithic supports are cast as continuous homogenous phases. They represent an approach that provides high rates of mass transfer at lower pressure drops as well as high efficiencies even at elevated flow rates. Therefore, much faster separations are possible and the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. This enhances the speed of the separation process and reduces backpressure and unspecific binding without sacrificing resolution [13]. This study described a fast HPLC method using a monolithic column with fluorescence detection, which enabled the determination of valsartan with good accuracy at low drug concentrations in plasma using single-step extraction procedure. The sample preparation only involved protein precipitation and no evaporation step was required. Also, the use of a smaller sample volume provided an advantage as compared with some previous methods [3, 6] that required large sample volume for analysis of valsartan. The overall time of analysis has been improved by the elimination of tedious extraction steps and optimization of chromatographic conditions using monolithic column (run time < 6 min).We also demonstrated the applicability of this method for pharmacokinetic studies in humans.

Material and methods

1. Chemicals

Valsartan and ranitidine (internal standard), were supplied by Osveh Pharmaceuticals (Tehran, Iran). Valsartan is available as oral tablet containing 80 mg of valsartan and other inactive ingredients. HPLC-grade acetonitrile and all

other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation and purified additionally with a Milli-Q system.

2. Instruments and chromatographic conditions

The chromatographic apparatus consisted of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2600 fluorescence detector connected to a model Eurochrom 2000 integrator, all from Knauer (Berlin, Germany). The separation was performed on a Chromolith Performance (RP-18e, 100×4.6 mm) column from Merck (Darmstadt, Germany). The excitation and emission wavelengths were set at 230 and 295 nm, respectively.

The mobile phase was a mixture of 0.01 M disodium hydrogen phosphate buffer-acetonitrile (60:40 v/v) adjusted to pH 3.5 at a flow rate of 2 ml/min. The mobile phase was prepared daily and degassed by ultrasonication before use. The mobile phase was not allowed to recirculate during the analysis.

3. Standard solutions

Stock solutions (2 $mgml^{-1}$) of valsartan were prepared in methanol and 20 μgml^{-1} and 2000 $ngml^{-1}$ solutions were made by dilution with methanol. Then 20, 50, 500, 1000, 1500 and 2000 $ngml^{-1}$ standard samples were prepared in plasma from the 2000 $ngml^{-1}$ solution and stored at +4 ^{o}C.

4. Sample preparation

To 450 µl of plasma in a glass-stoppered 5 ml centrifuge tube were added 50 µl of atorvastatin as internal standard (12 μgml^{-1}) and 500 µl of acetonitrile. After mixing (30 s), the mixture was centrifuged for 10 min at 8000 rpm. Finally 20 µl of supernatant was injected into the liquid chromatograph.

5. Biological samples

24 male healthy volunteers were included in this study. The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and written informed consent was obtained from the volunteers. Valsartan

was administered in a single dose of 80 mg to the volunteers after over night fasting. Plasma samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 7, 10 and 24 h after dosing and then frozen immediately at −20 °C until assayed.

6. Stability

The stability of valsartan was assessed for two sets of spiked plasma samples (with a low and high concentration) stored at −20 °C for up to two months and at ambient temperature for at least 24 h. The stability of stock solutions stored at −20 °C and ambient temperature were determined by injecting appropriate dilutions of stocks in methanol and comparing their peak areas with fresh stocks prepared on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

7. Plasma standard curve

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at –20 °C. After thawing, stock solution of valsartan was added to give final concentrations of 20, 50, 100, 250, 500, 1000, 1500 and 2000 ng ml^{-1}. Internal standard solution was added to each of these samples to yield a concentration of 600 ng ml^{-1}. The samples were then prepared for analysis as described above.

8. Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of valsartan to pooled plasma (quality control samples). For intra-day precision and accuracy five replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days.

9. Limit of quantification (LOQ) and recovery

For the concentration to be accepted as LOQ, the percent deviation from the nominal concentration (accuracy) and the relative standard deviation must be ±10%

and less than 10%, respectively, considering at least five-times the response compared to the blank response. The relative analytical recovery for plasma at three different concentrations of valsartan (100, 800 and 2000 ng ml^{-1}) was determined. Known amounts of valsartan were added to drug-free plasma and the internal standard was then added. The relative recovery of valsartan was calculated by comparing the peak areas for extracted valsartan from spiked plasma and a standard solution of valsartan in methanol containing internal standard with the same initial concentration (six samples for each concentration level).

10. Selectivity and specificity

Control human plasma, obtained from 24 healthy volunteers, was assessed by the procedure as described above and compared with respective plasma samples to evaluate selectivity of the method. Losartan, Sotalol, atenolol, carvedilol, lovastatin and hydrochlorthiazide were also tested for potential interferences.

11. Pharmacokinetic Analysis

Valsartan pharmacokinetic parameters were determined by non compartmental methods. Elimination rate constant (K) were estimated by the least-square regression of plasma concentration-time data points lying in the terminal log-linear region of the curves. Half-life ($T_{1/2}$) was calculated as 0.693 divided by K. The area under the plasma concentration-time curve from time zero to the last measurable concentration at time t (AUC_{0-t}) was calculated using the trapezoidal rule. The area was extrapolated to infinity ($AUC_{0-\infty}$) by addition of C_t/K to AUC_{0-t} where C_t is the last detectable drug concentration. Peak plasma concentration (C_{max}) and time to peak concentration (T_{max}) were derived from the individual subject concentration- time curves. Apparent oral clearance (Cl) and apparent volume of distribution (Vd) were calculated by the following equations:

$$Cl = Dose/AUC_{0-\infty}$$

$$Vd = Cl/K$$

Results and discussion

Under the chromatographic conditions described, valsartan and the internal standard peak were well separated. Endogenous plasma components did not give any interfering peaks. Figure 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of valsartan and ranitidine were 3.3 and 5.2 min, respectively. None of the drugs mentioned above interfered with analytes peaks as well. The calibration curve for the determination of valsartan in plasma was linear over the range 20-2000 ng ml^{-1}. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (R) for calibration curves were equal to or better than 0.997.The relative standard deviation (RSD) values of the slope were equal to or better than 6%. For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves (Table 1).

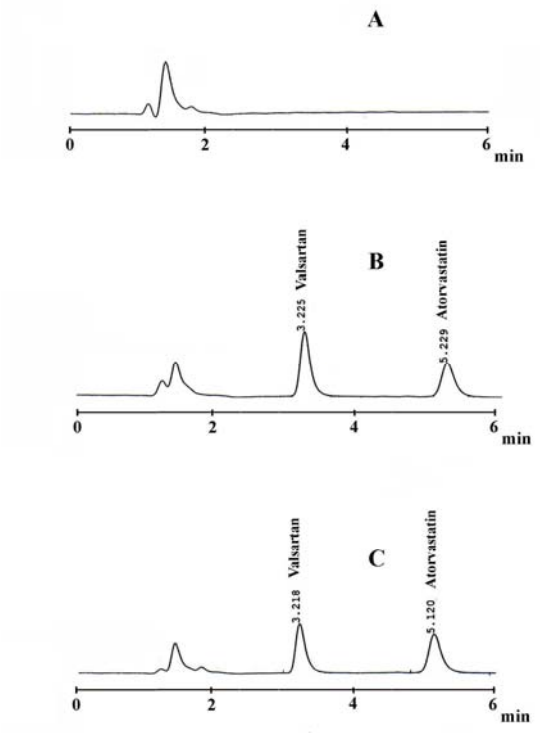

Fig.1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 500 ng ml^{-1} valsartan and 600 $ngml^{-1}$ atorvastatin (internal standard); (C) plasma sample from a healthy volunteer 0.5 after oral administration 80 mg of valsartan.

Tab. 1. Assay linearity

Coefficient of the linear Regression analysis (R±SD)		Slope±SD	Intercept±SD
Intra-assay	$0.9971 \pm 8.65 \times 10^{-4}$	$(8.0 \pm 0.3) \times 10^{-4}$	0.0117±0.0006
n= 6	RSD= 0.0867%	RSD= 3.75%	
Inter-assay	$0.9982 \pm 9.55 \times 10^{-4}$	$(8.0 \pm 0.4) \times 10^{-4}$	0.0120±0.0007
n= 9	RSD= 0.0957%	RSD= 5.0%	

The limit of quantification (LOQ), as previously defined, was 20 $ngml^{-1}$ for valsartan. This is sensitive enough for drug monitoring and pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of valsartan. As shown in Table 2, coefficients of variation were less than 6%, which is acceptable for the routine measurement of valsartan. The relative analytical recovery for plasma at three different concentrations of valsartan was determined.

Tab. 2. Reproducibility of the analysis of valsartan in human plasma (n= 5)

Concentration added ($ngml^{-1}$)	Concentration measured (mean ±S.D.)	
	Intra-day	Inter-day
100	108.2 ± 4.5 (4.2	107.4 ± 5.6 (5.2)
800	778.4 ± 33.5 (4.3)	798.3 ± 27.1 (3.4)
2000	1938.1 ± 50.3 (2.6)	1990.8 ± 59.7 (3.0)

Values in parentheses are coefficients of variation (%).

The average recovery was 96.3± 1.3%. Stability was determined for spiked plasma samples under the conditions as previously described. The results showed that the samples were stable during the mentioned conditions (Table 3).

Tab. 3. Stability of plasma samples (n=6)

Spiked concentr. (ng ml^{-1})	Storage conditions	Concentration found (ng ml^{-1})	RSD (%)
100	24h/22^{o}C	96.6	2.7
100	2 months/−20 ^{o}C	97.1	3.0
2000	24h/22^{o}C	2011.1	1.6
2000	2 months/−20 ^{o}C	1978.6	2.4

The aim of our study was to develop a rapid and sensitive method for the routine analysis of biological samples in pharmacokinetic valsartan research. This method is well suited for routine application in the clinical laboratory because of the speed of analysis and simple extraction procedure. Owing to use of the monolithic column, which has lower separation impedance comparing to the particulate packings, much faster separations are possible the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. Accordingly, the chromatographic elution step was undertaken in a short time (less than 6 min) with high resolution. The sample preparation only involved protein precipitation and no evaporation step was required. Also, the use of a smaller sample volume provided an advantage as compared with some previous methods that required 1-2 ml of plasma for analysis of valsartan. Over 700 plasma samples were analyzed by this method without any significant loss of resolution. No change in the column efficiency and back pressure was also observed over the entire study time, thus proving its suitability. In this study plasma concentrations were determined in 24 healthy volunteers, who received 80 mg of valsartan each. Fig 2. Shows the mean plasma concentration-time curve of valsartan: plasma concentration reached a maximum 1.54±0.25 h after administration with a mean peak concentration of 1096.2±242.3 ng ml^{-1}. The derived pharmacokinetic parameters of 24 healthy volunteers were summarized in Table 4. These pharmacokinetic parameters were in accordance with those found previously [3, 5].

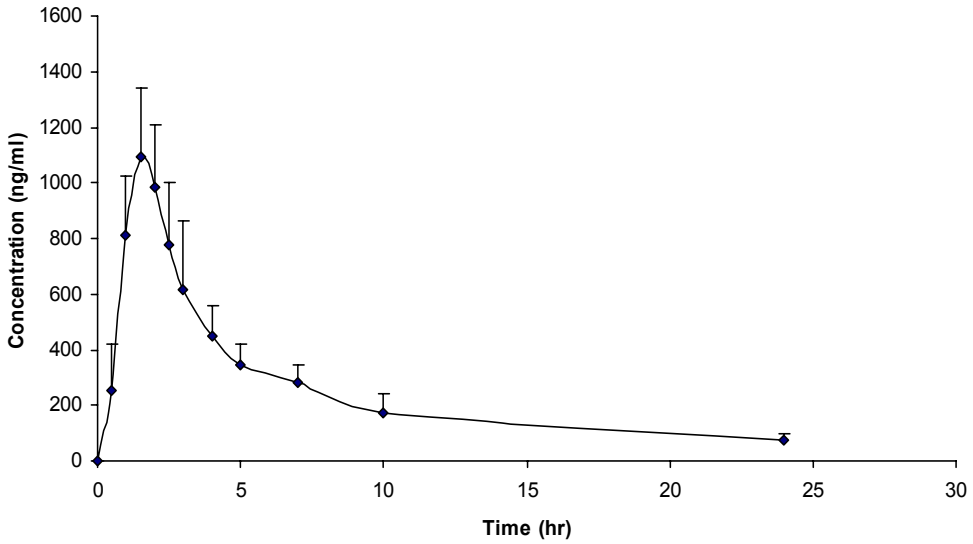

Fig. 2. Mean plasma concentration-time profile of valsartan in healthy volunteers (n=24) after a single 80 mg valsartan.

Tab. 4. Pharmacokinetic parameters of valsartan in healthy volunteers following a single oral dose of 80 mg of valsartan

Parameter	**Result (mean±SD)**
T_{max} (h)	1.54±0.25
C_{max} (ng mL^{-1})	1096.2±242.3
AUC_{0-t} (ng.h mL^{-1})	5361.7±546.1
K_{el} (h^{-1})	0.070±0.005
$T_{1/2}$ (h)	10.71±1.1
Cl_{tot} (L Kg^{-1} h^{-1})	0.175±0.022
Vd (L Kg^{-1})	2.50±0.37

Acknowledgement

This work was supported by Noor Research and Educational Institute.

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Received August 2nd, 2008
Accepted (after revision) August 20th, 2008
Available online at www.scipharm.at August 22nd, 2008