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RP-HPLC METHOD DEVELOPMENT FOR QUANTITATION OF VALSARTAN IN NANO-STRUCTURED LIPID CARRIER FORMULATION AND IN VITRO RELEASE STUDIES

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Nanotechnology has surmounted various application in drug delivery despite divergent challenges in physical, surface properties and reproducibility of preparation method. Ultra-sensitive analytical methods are highly demanded in order to monitor the very minute drug concentrations obtained during drug release studies as a result of the extensive prolongation in the release rate achieved in all nano-particulate drug delivery systems. A simple, rapid, sensitive, reversed-phase isocratic RP-HPLC method for determination of valsartan (Val) was developed. The method was applied for quality control tests including entrapment efficiency and drug release for Val-loaded nano-structured lipid carriers (NLC). The method was carried out using Eclipsed XBD column (Agilent -PN 993967) C18 (150 mm x 3.0, 5µm particle size) with mobile phase composed of acetonitrile: phosphate buffer (60:40) at pH 3.6, 0.01 M. The flow rate was set at 1.0 ml/min and effluent was detected at 273nm. The retention time of valsartan was found to be 2.910 minute. The method was validated for specificity, accuracy, precision, linearity, and limit of detection, limit of quantification and robustness. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 6.0 and 25 ng/ml respectively. The calibration curve was linear in the concentration range of 39.06-2500 ng/ml with coefficient of correlation 0.9992. The percentage recovery for Val was found to be 99.86-100.06 and the % RSD was found to be less than 2 %. The proposed method was free from any interference and successfully applied for quantitative determination of valsartan in lipid-based nano-particulate systems.

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Keywords: Lipid, Nano particulate, Valsartan, HPLC, Validation

1. Introduction

Valsartan (Val) is angiotensin II receptor blocker used in the treatment of congestive heart failure, high blood pressure and post-myocardial infarction [1-4]. Val acts on receptor subtype AT1 causing vasodilation as well as reduction in vasopressin secretion and reduction in the production and secretion of aldosterone [2].

Nano-drug delivery is an emerging field trying to translate the beneficial attributes of nanotechnology into new advantageous products. Currently attention is given to nanomedicine because of improved bioavailability and less side effects. At the same time, it is important to evaluate therapeutic potential through a valid analytical procedure. Based on the nature of carrier system, many nano particulate systems have been introduced including polymeric, solid lipid, liposomes and micelles [5-8]. A common attribute among all types of nano-drug delivery, are their ability to induce extensive enhancement in the bio-distribution and prolonged drug residence time in the body [9-10]. The slow drug release rate results in very minute drug concentrations in both in vitro drug release studies and in vivo pharmacokinetics studies. The need for highly sensitive analytical methods is increasing for detection of such minute drug concentration.

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A couple of HPLC spectroscopic methods were reported earlier for the determination of Val. either in the form of raw drug or pharmaceutical dosage forms. [11-17]**.**

Others reports in the literature presented simultaneous determination of Val in combination with other drugs such as propranolol and amlodipine [18-19]. The chemical structure of Valsartan, (1-oxopentyl)-N-[2′-(1H-tetrazol-5-yl) [1,1′-biphenyl]-4-yl] methyl]-L-valine is shown as Fig-1.

The focus of this study was to develop and validate a sensitive, rapid, reproducible & accurate HPLC method for the estimation and quantitation of Val to be applied in the quality control tests of Val-loaded nano-structured lipid carriers (NLC) including entrapment efficiency and drug release.

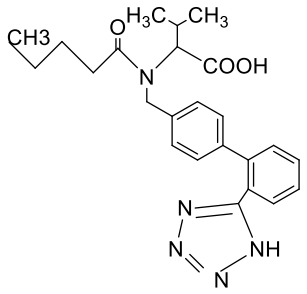

Fig. 1. Chemical Structure of Valsartan. 3 Methyl-2-(N{[2'(2H-1,2,3,4-tetrazole-5yl)biphenyl-4-yl]}pentamino) butanoic acid.

2. Materials and methods

2.1. Chemicals

Riyadh Pharma Medical and Cosmetic Products Co., Riyadh, Saudi Arabia supplied drug valsartan (VAL) Standard. HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany) and all other reagents were of analytical grade. Water obtained from the pure lab option-R, (ELGA Water system, UK) was used for the preparation of buffer and other aqueous solutions.

2.2. Instrumentation

The HPLC system consisted of Agilent 1200 series equipped with Diode Array Detector (DAD) of 1260 series (Agilent s, USA). An Eclipsed XBD column (Agilent -PN 993967) C18, 150 mm x 3.0 mm id. with particle size of 5µm was used for the separation and quantification. The preconditioned conditioned column was used at laboratory temperature (23±2 ^{0}C). An isocratic elution of an optimized mobile phase composition of (60:40 V/V) acetonitrile: 0.01 M phosphate buffer adjusted to pH 3.6, respectively was used for the separation of Val. The injection volume was set to 20 µL with a flow rate of 1 mL/min. throughout the analysis. The detection was attained at λmax 273 nm. The peak areas were integrated automatically by using HPLC Chem Station version B.03. .

2.3. Stock and Standard Solution

Standard stock solutions of Val were prepared in triplicate separately by dissolving 1.0 mg in 100 mL methanol and stored protected from daylight at 5^{o}C until use. A set of standards and quality control (QC) solutions were prepared by successive dilution of the stock solutions (10 µg/mL) of Val in methanol. All solutions were stored at 5^{0}C until used for the analysis. The final concentration of the standard Val solutions were 39.06, 78.125, 156.25, 312.50,1250 and 2500,5000 ng/mL obtained by further dilution with mobile phase. Finally, 20 µL of each solution was injected into the liquid chromatograph. The peak area for each concentration was recorded and then plotted against the corresponding concentration to obtain the calibration graph Fig -3. Calibration and linearity calibration curves were constructed in the ranges of 39.06 – 2500. (Fig-

3). To encompass the expected concentrations in the measured samples, triplicate 20 µL injections were made for each working standard solution.

2.4. Application of the method for the determination of Val entrapment efficiency into NLC:

NLC were successfully prepared by a hot emulsifying of Val in method mixture of triglycerides and castor oil using a combination of Tween 80 as surfactant and sodium deoxycholate as co surfactant [20]. The drug entrapment efficiency (EE) was indirectly calculated by determination of amount of Val in the supernatant obtained after centrifugation of the NLC dispersion using the following equation.

$$\% \text{ EE} = \frac{W \text{initial drug} - W \text{free drug}}{W \text{initial drug}} \text{ X } 100 \quad \text{(Eq. 1)}$$

2.5. Application of the method for determination of Val release profile from NLC formulation:

The drug release rate was determined using the dialysis tube method [20]. Certain weight of the freeze dried Val loaded NLC equivalent to 1 mg of Val was dispersed in 1 ml phosphate buffer and placed inside a dialysis tube cut off 12,000 DA, the closed tube was suspended in 20 ml of the buffer solution and placed in a shaking water bath adjusted at 37 ± 1^0C and 80 rpm shaking rate. Samples were obtained at different time intervals and Val concentration was determined.

2.6. Method Validation:

Chromatography is widely and perceptible separation technique used in pharmaceutical analysis. Column specification and Mobile phase buffers with pH are the major component to optimized robustness with fine separation [21].The stability of the HPLC method was exercised for the separation and determination of Nano Valsartan drug. To confirm its suitability for its intended purpose, the method was validated by linearity, detection and quantitation limits, robustness, and accuracy. Calibration curve representing the relation between the concentrations of drugs versus the peak area were constructed. Results show linear relationship in the expected range. All the validation studies were performed by replicate injections of standards and samples. The mobile phases containing water, methanol or acetonitrile: Water alone were found to elute the compounds unresolved. Increasing the acetonitrile concentration to more than 65% of buffer led to inadequate separation. Our objective of the chromatographic method development was to analyze in different lots of blank and control and sample matrix for the presence of potential interferences in the retention window of the peaks of interest.

2.7. Specificity

The specificity of the LC method was evaluated to ensure that there was no interference from the excipients contained in pharmaceutical product or from products resulting from Nano formation. In addition to that, of monitoring standard solutions of the drug in the presence of their impurities indicated a high degree of specificity of this method.

2.8. Detection and Quantitative limit:

The final concentration of drug may be detected in the sample is called (LOD). Formula used for the calculation of LOD = 3.3 SD/S. Where SD is standard deviation of y intercept and S is the mean of the slopes of three standard curves. The quantitation limit (QL) is the lowest amount of the analyte, which could be calculated by precision and accuracy of the sample.

2.9. Precision:

Injection repeatability: The RSD of drugs peak area in triplicate injections of standard drug solution determined each three consecutive days for intraday precision. On the same day, results was assessed using three concentration in ten replicates of each concentration. The

acceptance criteria of accuracy is within the range of 85 – 115% and percentage RSD. The relative standard deviation was calculated RSD= [SD/Mean] X 100.

2.10. Accuracy:

Accuracy of the measurements was determined after applying three quality control samples to the known amount of the sample. Each set of addition repeated thrice at each level. The result were expressed as Mean recovery ± (SD) and % RSD.

3. Results and discussion

3.1. HPLC method: Development and optimization

After several preliminary investigatory of column type, mobile phase, different temperatures, the chromatographic runs with 20 µL volume, blank, standard and samples were injected. The composition of the mobile phase was optimized and a mobile phase consisted of phosphate buffer (pH 3.6, 0.01M), acetonitrile: phosphate buffer (60:40V/V) was observed the best to provide a rapid and sharp separation. Val was eluted at a retention time of 2.910 min. The HPLC chromatogram is presented in Figure 2. Mobile phase solvent was filtered through 0.45 µm film. Analyses were run at a flowrate of 1 mL min-1 at laboratory temperature (23 ± 2^0C). Photodiode Array detector was set at wavelength 273nm. Under the described experimental conditions, all peaks were well defined and free from tailing. The effects of small deliberate changes in the mobile phase composition, pH, and flow rate were evaluated as a part of testing for method robustness [22]. Moreover, percentage RSD did not exceed more than 2%, thus indicating the ruggedness of the method. The retention time of 2.910 min is comparatively shorter than those reported elsewhere. All the parameters were validated according to the guideline described elsewhere. [11,23,24].

3.2. Validation of the method

The analytical method was validated with respect to parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, robustness selectivity and recovery [23,25,26].

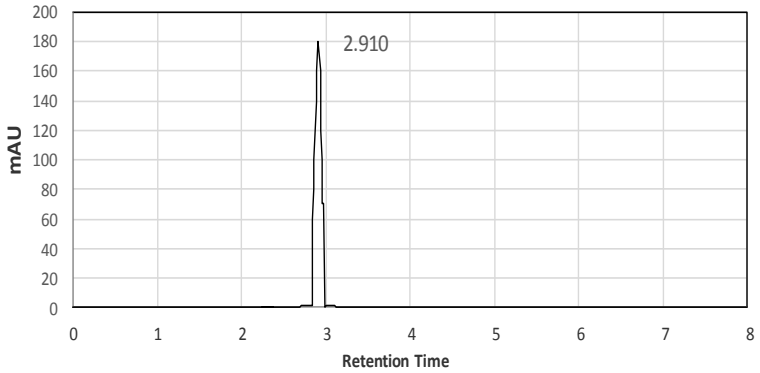

Fig. 2. A typical HPLC chromatogram for Val

3.3. Linearity

The range of an analytical method is the interval between the upper and lower analytical concentration of a sample where the method has shown to demonstrate acceptable accuracy, precision, and linearity [23,27]. Linearity was studied by preparing serial diluted standard samples at different concentration levels. Peak areas of val. was plotted against their respective concentrations and linear regression analysis performed on the resultant curve. The least squares linear regression analysis of the calibration curve was employed for assessing the linearity. Table 1 presents the mean and RSD for peak areas obtained with each concentration. It was observed that

the constructed calibration curves were linear over the concentration range from 39.06 ng mL^{-1} to 2500 ng mL^{-1}. Correlation coefficient (r^2, *n*=3) was found to be more than 0.99998 with mean % RSD=0.6300 % as shown in Table 1,which indicating high consistency across the concentration ranges studied (Fig. 3).

Table 1. Calibration data of Valsartan.

Calibration data of Val of Concentration (ng mL−1)	Mean area (± SD) (n=3)	% RSD (n=3)
39.06	85.10±1.73	1.57
78.125	170.92 ± 1.52	0.89
156.25	330.643±1.15	0.35
312.5	648.995±0.41	0.06
625.0	1285.33±3.53	1.38
1250	2528.34±0.26	0.01
2500	4891.87±7.50	0.15

Method characteristic Valsartan: Linearity Range (ng / ml) 39.06-10000: Regression equation, $y = 9.99998*10^{-2} X – 3.9362$: Retention time 2.91; Correlation coefficient (r^2) 0.99998; SD of intercept 39.35, mean RSD=0.6300

Table 2. Results of intermediate precision study.

Level	**Inter-day repeatability (%RSD) (*n* = 5)**			**Intra-day repeatability (%RSD) n=10**
	Day-1	Day-2	Day-3	Fresh injection
LQC (39.06)	38.72±0.55	38.21±1.08	38.60±1.45	38.85±1.87
LQM (625.0)	624.06±0.18	623.51±0.30	623.24±0.16	623.45±0.24
HQC (5000)	4980.54±0.27	4959.25±0.20	4980.91±0.13	4969.83±0.35

Percentage relative standard deviation (RSD) ≤ 2%.

3.4. Limit of Detection and Limit of Quantitation

The limit of quantitation (LOQ) of the present method that can be determined with acceptable precision and accuracy under the stated experimental conditions was found to be 25 ng/ml with a resultant % RSD of 0.4% (*n* = 5). Signal to noise ratio as limit of detection (LOD) was calculated to be 6 ng/ml (n=10).

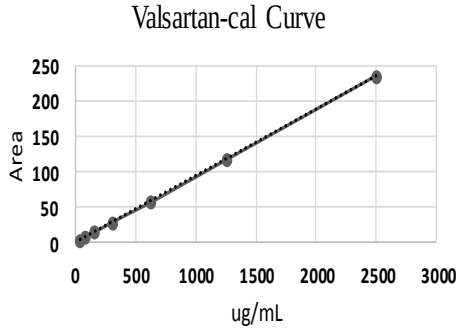

Fig. 3. Standard calibration curve of Val. at λ = 273nm.; RT: 2.910; DAD A, Ref= 360,100; Res std. Dev.: 0.56222; Equation of the line, $y = 9.40859 *10^{-2} X – 3.93620$, $R^2 = 0.99998$.

3.5. Precision: Intraday (Repeatability) and Inter day (Intermediate Precision)

Precision of the assay was investigated with respect to both repeatability and reproducibility. Repeatability was investigated by injecting ten replicate samples of each of the

39.06, 625 and 5000 ng/ml standards. Where the mean Concentration (percentage ± RSD) was observed to be 38.85 (±1.87), 623.45 (±0.24) and 4969.83(±0.35) ng/mL respectively. Inter-day precision assessed by injecting the same three concentrations over three consecutive days. The % RSD values for intra- and inter-day assays of Val. loaded into in various NLC formulations was found to be less than 2% as shown in Table -2. The result found to be an indication of good repeatability and reproducibility, which support the method optimization as being reliable for various Val. nano-particulate systems.

3.6. Specificity and Selectivity

Retention times of the peaks in the chromatograms of Val.-loaded NLC formulations was observed to be the same as that of standard drugs and no interference from excipients of lipid components.

Ten replicates of three different concentrations were injected to find out the specificity of the HPLC method. The mean RSD was found less than 2 % which is the clear indication of successful application to determine the drugs in various NLC samples. The results are summarized in Table 3. All the materials (excipients or solvents) studied were well separated from the analyte peak, demonstrating that the developed method put forward was observed as specific and selective for evaluation of Val. in lipid based nano-particulate systems.

Table 3. Specificity for the proposed method.

Levels	Predicted concentration (ng mL−1) n=10			*Mean accuracy ± SD
	Range	Mean (± SD)	%RSD	
LQC (39.06 ng mL−1)	38.24 – 40.06	38.82 ± 0.78	2.00	100.23±1.23
MQC (625 ng mL−1)	621.14 – 625.65	623.45 ± 1.51	0.24	99.96±0.22
HQC (5000 ng mL−1)	4936.22 - 4995.85	4969.84 ± 17.34	0.35	99.98±0.34

*Means of ten replicates

For the evaluation of the method robustness, some parameters were interchanged such as pH (± 0.2), organic phase ratio of mobile phase (± 0.5mL) and column oven temperature (±2 ^{0}C). The flow rate 0.9 mL/min was also tested to observe the difference in retention time and other factors. It was observed that the capacity remained unaffected by small deliberate variations indicating that the method was robust as no detrimental effect on method performance have been identified [21].

3.7. Accuracy (Recovery):

The recovery test was employed to evaluate the accuracy of the method. To calculate recovery, fixed amount of standard drug was added to per analyzed sample, resulting different level concentration of spikes samples. The analyze results of accuracy in percentage are shown in Table 4. All solutions were prepared in triplicate and analyzed. The % mean recovery for the assay following the determination of the compound of interest were 99.89 % indicating high level of accuracy. The coefficient of variation (%RSD) of three different concentrations (1:2; 1:4; 1:8) support that the method is accurate within the desired range.

Table 4. Results of Accuracy of Valsartan

Drug	spiked level	Amount of drug added($ngmL^{-1}$)	Amount of drug found($ngmL^{-1}$)	%Recovery* (% RSD)**	Mean % Recovery* %RSD**
Valsartan	Spiked Neat	625	625.35	100.06±0.245	99.89±0.620
	1:2	625	936.38	99.87±0.155	
	1:4	625	780.25	99.86±0.37	
	1:8	625	701.51	99.77±1.71	

Recovery percentage*, %RSD**

Generally, RP HPLC methods have been the first choice for the analysis of many pharmaceutical formulations [11,15,16]. In our study, the desired sensitivity with a LOQ of 25 ng/mL was achieved, which was proved to be superior in sensitivity in comparison to the methods reported previously [12-14]. The present conditions employed mild, simple, inexpensive and readily available chemicals and instruments.

4. Conclusions

The developed RP-HPLC method for quantitation of Val. has proven to be specific, rapid, and sensitive method. The levels of accuracy and precision of the method were among the standard accepted range as per the ICH guidelines. The limited volume demand, the very low LOQ, the absence of interference with many common lipid excipients make it an attractive option for quantitation of Val. in various lipid based nano-particulate systems including; NLC, solid lipid nanoparticle, and nano-emulsions. The method can be readily acclimatized to routine pharmaceutical quality control purposes.

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