INTRODUCTION

Valsartan (VAL) is chemically N-{1-Oxopentyl}-N-[2′-(1H-tetrazol-5-yl) [1,1′-biphenyl]-4-yl] methyl]-L-valine.[1] It is a new potent, highly selective and orally active antihypertensive drug belonging to the family of Angiotensin II type I receptor antagonist. Angiotensin II receptor type I antagonists have widely used in the treatment of hypertension, heart failure, myocardial infarction and diabetic nephropathy.[2] Literature survey revealed that HPLC, LC-MS, protein precipitation, capillary electrophoresis and simultaneous UV-spectrophotometric methods[3-15] are reported for estimation of VAL alone or on combination with other agents. As the published literature and knowledge of the molecule suggest, reversed phase liquid chromatography (RP-HPLC) is suitable for analysis of VAL, various methods are available for estimation of VAL in isocratic mode, but as the main aim was to resolve the compound from degraded products and impurities if any, the gradient method was chosen.

MATERIALS AND METHODS

Chemicals and reagents

A reference standard sample of VAL was obtained as a gift from Macleod's Pharmaceuticals Ltd and commercial dosage form containing the studied drug were purchased from local market. HPLC grade methanol, orthophosphoric acid, triethylamine, and water were HPLC grade purchased from E. Merck, Mumbai, India. All the other chemicals and reagents used were of analytical grade and purchased from SD Fine Chemicals, Mumbai, India.

Instrumentation and chromatographic system

The chromatographic system consisted of a JASCO (Japan) chromatograph equipped with an LC-Net II/ADC, an MU–2010 plus PDA detector, a PU–2089 plus quaternary pump, an online degasser and a rheodyne model 7725 injector valve with 20 µl sample loop. The chromatograph is coupled with "Chrompass" software (version 1.7.403.1).

The chromatographic separations were performed at ambient temperature using Symmetry C18 column (4.6 mm×150 mm, particle size 5.0 µm). Separation was achieved using a gradient method. Mobile phase A comprised of orthophosphoric acid buffer (the pH of the solution was adjusted to 4.2±0.05 with triethylamine). Mobile phase B was methanol, orthophosphoric acid buffer (pH 4.2) was prepared by adding 5.5 ml of orthophosphoric acid in 1000 ml of HPLC grade water, adjusting the pH to 4.2±0.05 with triethylamine, diluting to 1000 ml with HPLC-grade water, and filtering through 0.45 µm membrane filter. For the preparation of diluent, a mixture of buffer pH 4.2 and methanol (50:50/v/v) filtered and degassed for 30 min prior to use. The eluent was monitored with PDA detector at 225 nm with a flow rate of 1.0 ml/min, run time of 65 min; sample size of 20 µl was carried out at room temperature all over the study.

ABSTRACT

Objective: The objective of this research work was to develop a sensitive, precise, specific, linear and stability-indicating gradient HPLC method for the estimation of valsartan in bulk drug and in pharmaceutical preparations.

Methods: Chromatographic separation was achieved on C-18 stationary phase with a gradient mobile phase consisting of orthophosphoric acid buffer (the pH of the solution was adjusted to 4.2±0.05 with triethylamine) and methanol. The eluent was monitored with PDA detector at 225 nm with a flow rate of 1.0 ml/min, run time of 65 min.

Results: The method was linear over the range of 20-120µg/ml. The correlation coefficient was found to be 0.9994±0.02. In order to check the selectivity of the method for pharmaceutical preparations, forced degradation studies were carried out. Valsartan was found to be stable at light and oxidation experiments. The performance of the method was validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision and robustness. The LOQ was found to be 0.26µg/ml and the LOD was found to be 0.79µg/ml. Valsartan showed good correlation coefficient in the concentration range of 20-120µg/ml. The developed method was compared statistically by applying two-way anova and student's t-test to correlate with an isocratic method and was applied to bulk drug and tablet dosage form. There was no significant difference between the two methods.

Conclusion: The proposed method was found to be accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of valsartan in pharmaceutical formulation and for analysis of stability samples obtained during accelerated stability study.

Keywords: RP-HPLC, Valsartan, Degradation products, Pharmaceutical dosage forms, Two-way ANOVA and student's t-test

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DOI: http://dx.doi.org/10.22159/ijpps.2016v8i9.12581
Preparation of stock solution
Stock solutions were prepared by accurately weighing 10 mg of VAL and transferring to 10 ml volumetric flasks containing 6 ml of methanol. The flasks were sonicated for 10 min to dissolve the solids. Volumes were made up to the mark with diluents, which gave 1000μg/ml the drug. Aliquots from the stock solutions were appropriately diluted with diluents to obtain working standards of 100μg/ml of VAL.

Calibration standards and quality control sample
Different calibration standards ranging from 20, 40, 60, 80, 100 and 120μg/ml were prepared by appropriate dilution of standard solution (1000μg/ml) with methanol. Three quality control samples at concentrations 60, 80 and 100μg/ml representing 50, 100 and 150% respectively of assay concentration (40μg/ml) were prepared from the standard solution. An aliquot of 20μL of the solution was injected into HPLC system.

Preparation of assay solution
To determine the VAL content of tablet formulations, twenty tablets were weighed, to determine the average weight of the tablets, and then crushed and mixed using a mortar and pestle. A portion of powder equivalent to 1000μg/ml was accurately weighed into each of three 10 ml volumetric flasks and 5 ml methanol was added. Each solution was sonicated for 20 min to achieve complete dissolution of the VAL and the solutions were then diluted to volume with diluents, to yield concentrations of 100μg/ml and filtered through a 0.22μm nylon membrane filter. The solution obtained was analyzed by HPLC.

Method validation
The method was validated according to International Conference on Harmonization [17] guidelines for validation of analytical procedures.

RESULTS AND DISCUSSION
HPLC method development and optimization
Some important parameters, like pH of the mobile phase, concentration of buffer solution, percentage and type of organic modifiers (acetonitrile and methanol), different columns (CN column, C8 and C18), flow rates (0.5 to 2.0 ml/min) were attempted to resolve the good chromatographic separation of VAL VAL and degraded products could not be separated without pH adjustment of the mobile phase. Various pH ranges (3 to 6) for mobile phase were tried, and the best resolution was obtained with pH 4.2.

The method was optimized to provide a good separation of the components (acceptable theoretical plates and resolution between peaks) with a sufficient sensitivity and suitable peak symmetry (peak tailing factor<2). Symmetry C18 column (4.6 mm×150 mm, 5.0 μm particles were preferred over the other columns. The mobile phase consisted of 55% phosphate buffer and 45% methanol to 80% buffer and 20% methanol within 40 min with a 1 ml/min flow rate. Retention times for VAL under these conditions were 27.04 (fig. 2). The quantitative analysis of the drug and its degradation products were performed at 225 nm.

Gonzalez et al. [5] developed a HPLC method coupled to fluorescence detection using gradient elution mode and was applied to plasma samples obtained from hypertensive patients under clinical studies after oral administration of a therapeutic dose of some of these angiotensin II receptor antagonists. The objective of this research work reported was to develop a simple, precise, accurate gradient stability-indicating LC method for analysis of VAL in bulk and its formulations in the presence of degradation products and impurities.

Method validation
System suitability
System suitability test is an integral part of method development and is used to ensure adequate performance of the chromatographic system. Retention time, number of theoretical plates, asymmetrical factor, and peak area were evaluated for five replicate injections of
the drug at a concentration of 80μg/ml. The results given in table 2 were within acceptable limits.

Table 2: System suitability of HPLC method for VAL

| Parameters                      | (Mean±%RSD) |
|---------------------------------|-------------|
| Retention time                  | 27.04±0.59  |
| No. of theoretical plates       | 9154.2±0.72 |
| Asymmetrical factor             | 1.05±0.47   |
| Peak area                       | 705360±0.68 |

*Mean of five determinations

Linearity

VAL showed good correlation coefficient in the concentration range of 20-120μg/ml (fig. 3). Linearity was calculated by determining six standard working solutions containing VAL in triplicate (table 3). For the method, the linearity of calibration graph was validated by the high value of correlation coefficient and the RSD for slope and intercept value was less than 2%.

Table 3: Linear regression data for calibration curve of VAL

| Parameters                      | VAL         |
|---------------------------------|-------------|
| Linearity range(μg/ml)          | 20-120      |
| r±RSD%                          | 0.9994±0.02 |
| Slope±RSD%                      | 94265±0.26  |
| Intercept±RSD%                  | 453560±1.60 |

*Mean of three determinations

Accuracy

The accuracy of the method was checked by recovery study using standard addition method known the amount of standard VAL was added into the pre-analyzed sample and subjected it to the proposed high performance liquid chromatographic method.

These studies were carried out at three levels i.e. (50, 100 and 150%). The recovery studies were carried out and the % recovery and standard deviation of the % recovery were calculated and presented in table 4.

Table 4: Recovery study by standard addition method for VAL

| Sample   | Amount taken (μg/ml) | Amount % | Amount added (μg/ml) | Mean amount present (μg/ml) | Recovery mean* (%)±SD |
|----------|----------------------|----------|----------------------|----------------------------|-----------------------|
| Valzaar-80 | 40                    | 50       | 20                   | 60                          | 99.98±0.25            |
|           | 40                    | 100      | 40                   | 80                          | 99.90±0.35            |
|           | 40                    | 150      | 60                   | 100                         | 99.82±0.55            |
|           | 40                    | 50       | 20                   | 60                          | 100.21±0.47           |
| Diavon-80 | 40                    | 100      | 40                   | 80                          | 99.94±0.48            |
|           | 40                    | 150      | 60                   | 100                         | 100.26±0.45           |

*Mean of five determinations

Precision

The precision of the method was determined by repeatability (intraday precision) and intermediate precision (inter-day precision) of VAL standard solutions. Repeatability was calculated by assaying three samples of each three different concentration levels on the same day. The inter-day precision was calculated by assaying three samples of each at three different concentration levels on three different days. The results are expressed as a relative standard deviation. The relative standard deviations were below 2% which signifies the precision of both the methods (table 5).

Table 5: Precision of the method

| Name of the formulation | Intra-day precision (n=6) | Inter-day precision (n=6) |
|-------------------------|---------------------------|----------------------------|
|                         | Mean*±RSD                 | Mean*±RSD                  |
| Valzaar-80              | 99.97±0.35                | 99.91±0.72                 |
| Diavon-80               | 100.11±0.33               | 100.13±0.61                |

*Mean of three determinations injected six times at each concentration level

Limits of detection (LOD) and quantification (LOQ)

The LOQ and LOD were determined based on the 10 and 3.3 times the standard deviation of the response, respectively, divided by the slope of the calibration curve. The LOQ was found to be 0.26μg/ml, and the LOD was found to be 0.79μg/ml.

Robustness

In order to measure the extent of the method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged, and in parallel, the chromatographic profile was observed and recorded. The chromatographic parameters were interchanged within the range of 1-10% of the optimum recommended conditions. The studied parameters were: mobile phase pH, flow rate, and detector wavelengths. The results indicated that the small change in the conditions did not significantly affect the determination of VAL. Under all deliberately varied conditions, the %RSD for the assay values (n=3) for VAL was found to be well within the acceptance limit of 2%. The results are reported in table 6.
Table 6: Results from testing the robustness of the method

| Condition                      | Modification | Mean area* ±SD   | RSD (%) | Mean Rₜ* (min) ±SD |
|--------------------------------|--------------|------------------|---------|--------------------|
| Mobile phase flow rate (mL/min) | 0.8          | 7078494±16792    | 0.23    | 29.95±0.05        |
|                                | 1            | 7062968±54957    | 0.77    | 27.08±0.04        |
|                                | 1.2          | 7097530±57017    | 0.8     | 26.95±0.15        |
| Mobile Phase pH                | 4            | 7033714±34615    | 0.49    | 27.04±0.10        |
|                                | 4.2          | 7070556±17718    | 0.25    | 27.08±0.04        |
|                                | 4.4          | 7074327±26629    | 0.37    | 27.05±0.08        |
| Detector wavelength (nm)       | 223          | 702746±32695     | 0.46    | 26.94±0.06        |
|                                | 225          | 7067339±20404    | 0.28    | 26.94±0.11        |
|                                | 227          | 7077785±13108    | 0.18    | 27.13±0.10        |

*Mean of three determinations

Specificity and degradation studies

When establishing the stability-indicating properties of analytical methods, the intermediate degradation products should not interfere with any stage of drug analysis. VAL was found to be stable at light and oxidation experiments. In acidic condition VAL degraded up to 9.2%, in basic condition up to 6.5% and in thermal condition 12.2% degradation was observed for VAL. The results from forced degradation studies are given in Table 7.

Chromatograms obtained from after degradation under different stress conditions are shown in Fig. 4, respectively. No peaks co-eluted with the drug peak, suggesting the method enabled specific analysis of VAL in the presence of its degradation products.

Table 7: Summary of forced degradation results of VAL

| Condition                                      | Time  | % Assay of active substance | % Degradation |
|-----------------------------------------------|-------|----------------------------|---------------|
| Treated with 5 ml of 0.1 N HCl solution and kept at 80 °C on water bath | 2 h   | 90.3                       | 9.2           |
| Treated with 5 ml of 0.1 N NaOH solution and kept at 80 °C on water bath | 2 h   | 93.5                       | 6.5           |
| Treated with 5 ml of 10.0 % H₂O₂ solution and kept at 80 °C on a water bath | 2 h   | 98.2                       | 1.8           |
| Heated at 80 °C in oven                       | 24 h  | 87.8                       | 12.2          |
| Exposed in the UV chamber                     | 24 h  | 98.8                       | 1.2           |

Fig. 4: LC chromatograms of Valsartan (a) after acidic degradation (b) after basic degradation (c) after oxidative degradation (d) after thermal degradation (e) after photodegradation (f) formulation I (g) Formulation II
Use of the method for analysis of marketed formulations

Two marketed samples have been analyzed to see the performance of the method. The first formulation taken was Valzaar-80 which contains 80 mg of VAL; the second formulation taken was Diavon-80 contains 80 mg of VAL. Results obtained have been summarized in the table 8.

Table 8: Results from assay of VAL in marketed formulation

| Formulation | Mean±SD | RSD (%) | Recovery (%) |
|-------------|---------|---------|--------------|
| Valzaar-80  | 80.04±0.33 | 0.41    | 100.05       |
| Diavon-80   | 79.93±0.28  | 0.35    | 99.91        |

*Mean of six determinations

Statistical comparison of gradient HPLC method and Isocratic HPLC

To test the difference between the developed gradient liquid chromatographic method and our previous work isocratic HPLC method for estimation of VAL [16] statistical tests were performed for the level of confidence 95% (P = 0.05). Two-way ANOVA and One-way ANOVA was applied to test both methods–sample interaction and differences in method precision. Two-way ANOVA was used for comparison of two formulations by two chromatographic methods (table 9). One way ANOVA was used for comparison of each formulation by chromatographic methods (table 9). To test means between different chromatographic methods paired student's t-test was applied. The test removes any variation between samples. For VAL, the two formulations were compared for the chromatographic methods (table 10).

Table 9: Comparison of chromatographic methods between valzaar-80 and diavon-80

| Source of Variation | SS    | df | MS    | F-stat | P-value | F-crit |
|---------------------|-------|----|-------|--------|---------|--------|
| Sample              | 0.0082| 1  | 0.0082| 0.0868 | 0.7713  | 4.3512 |
| Columns             | 0.0031| 1  | 0.0031| 0.0331 | 0.8573  | 4.3512 |
| Interaction         | 0.0455| 1  | 0.0455| 0.4786 | 0.4969  | 4.3512 |
| Within              | 1.9010| 20 | 0.0950|        |         |        |

ANOVA: Single Factor [VAZZAR-80]

| Source of Variation | SS    | df | MS    | F-stat | P-value | F-crit |
|---------------------|-------|----|-------|--------|---------|--------|
| Between Groups      | 0.0363| 1  | 0.0363| 0.3221 | 0.5828  | 4.9646 |
| Within Groups       | 1.1269| 10 | 0.1126|        |         |        |

ANOVA: Single Factor [DIAVON-80]

| Source of Variation | SS    | df | MS    | F-stat | P-value | F-crit |
|---------------------|-------|----|-------|--------|---------|--------|
| Between Groups      | 0.0123| 1  | 0.0123| 0.1595 | 0.6979  | 4.9646 |
| Within Groups       | 0.7741| 10 | 0.0774|        |         |        |

Table 10: Student's t-test for different chromatographic methods

| Parameters          | Valzaar-80 | Diavon-80 |
|---------------------|------------|-----------|
| t-stat              | -0.5097    | -0.4082   |
| Pearson correlation | 0.5053     | 0.3460    |
| t Critical two-tail | 2.5705     | 2.5705    |

CONCLUSION

A gradient stability-indicating reversed phase high performance liquid chromatographic method for valsartan in bulk and pharmaceutical dosage form was undertaken in the present research work. The method was found to be specific, as there was no interference of any co-eluting impurities after stress degradation study. The method was validated according to ICH guidelines and correlated by statistical analysis. The proposed method is found to be accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of valsartan in pharmaceutical formulation and for analysis of stability samples obtained during accelerated stability study.

ACKNOWLEDGMENT

The authors express their sincere thanks to Macleod's Pharmaceuticals Ltd., India for providing gift sample of valsartan, Thanks are also due to the Professor and Chairman Sïksha 'O' Anusandhan University, Bhubaneswar for providing laboratory facility for carrying out the present research work.

CONFLICTS OF INTERESTS

Declared none

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How to cite this article

- Tripti Sharma, Sudan Chandra SI. A validated gradient stability-indicating LC method for the analysis of valsartan in pharmaceutical dosage form. Int J Pharm Pharm Sci 2016;8(9):128-133.