ABSTRACT

Objective: To develop a simple, rapid, economic, accurate and precise reverse phase-high performance liquid chromatographic (RP-HPLC) method for the determination of hydrochlorothiazide and candesartan in the pharmaceutical dosage form and to validate as per international conference on harmonization (ICH) guidelines.

Methods: The chromatographic separation was performed on Silanol BDS C18 column (250 x 4.6 mm, 5 μm), a mobile phase consisting of water (pH adjusted to 2.8 with orthophosphoric acid): acetonitrile (30:70 v/v), with a flow rate 1 ml/min and the detection wavelength of 210 nm using photodiode array (PDA) detector.

Results: The developed method resulted in elution of hydrochlorothiazide at 2.28 min and candesartan at 4.28 min. The calibration curves were linear (r²=0.999) in the concentration range of 6.25-18.75 μg/ml and 8-24 μg/ml for hydrochlorothiazide and candesartan respectively. The percentage recoveries were found to be 99.78-100.39 for hydrochlorothiazide and 99.87-100.64 for candesartan. The limit of detection (LOD) was found to be 1.367 μg/ml and 2.330 μg/ml for hydrochlorothiazide and candesartan respectively.

Conclusion: A simple, economic, accurate, precise, linear and rapid RP-HPLC method was developed for simultaneous quantitative estimation of hydrochlorothiazide and candesartan in bulk and pharmaceutical formulation and the method was validated as per ICH guidelines. Hence, the method holds good for the routine analysis of hydrochlorothiazide and candesartan in various pharmaceutical industries as well as in academics.

Keywords: Hydrochlorothiazide, Candesartan, RP-HPLC, Method development, Validation

INTRODUCTION

Hydrochlorothiazide [fig. 1] is chemically 6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide, belongs to the thiazide class of diuretics. The drug reduces extra fluid in the body caused by conditions such as heart failure and kidney disease.

Candesartan [fig. 2] is chemically 1-{[2-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl]ethyl}-2-ethoxy-1H-benz[d]imidazole-7-carboxylic acid, belongs to the class of angiotensin II receptor blockers. It is used for treating high blood pressure (hypertension).

MATERIALS AND METHODS

Chemicals and reagents

Pharmaceutical grade hydrochlorothiazide and candesartan were provided as a gift sample by Spectrum Pharma Labs, Hyderabad and the marketed formulation (Candesar-H, candesartan-16 mg, hydrochlorothiazide-12.5 mg) was purchased from local market, acetonitrile, orthophosphoric acid and HPLC grade water purchased from Merck.

Instrument

Chromatography was performed on Waters HPLC 2695 equipped with quaternary pumps with PDA detector. The chromatographic separation was performed using Silanol C18 column (4.5 x 250 mmx5 μm). The data acquisition and integration were performed using Empower 2 software.

A detailed literature survey revealed that there were analytical methods for the estimation of specified drugs with other combinations by ultraviolet spectroscopy (UV) [1-7], high-performance thin layer chromatography (HPTLC) [8], liquid chromatography/mass spectrometry/mass spectrometry (LCMS/MS) [9] and HPLC [10-12]. There were few RP-HPLC methods for the estimation of hydrochlorothiazide and candesartan [13-17] but the developed methods involve use of buffers as mobile phases which decrease the life of the column and has longer retention times, leading to more analysis time. Hence, an RP-HPLC method has been developed using simple mobile phase with less runtime.

In the present work an easy, rapid, accurate and specific method was developed by using low-cost solvent water and acetonitrile for the simultaneous estimation of hydrochlorothiazide and candesartan in bulk and pharmaceutical dosage form. The developed method was validated as per ICH guidelines. Hence, the method holds good for the routine analysis of hydrochlorothiazide and candesartan in various pharmaceutical industries as well as in academics.
Chromatographic conditions
The developed method used a reverse phase C<sub>18</sub> column, Silanol C<sub>18</sub> column (4.5 x 250 mm x 5 μm), a mobile phase consisting of water (pH adjusted to 2.8 with orthophosphoric acid): acetonitrile (30:70 %v/v), flow rate of 1.0 ml/min and a detection wavelength of 210 nm using PDA detector.

Preparation of standard solutions
Standard stock solutions of hydrochlorothiazide and candesartan were prepared by dissolving 12.5 mg of hydrochlorothiazide and 16 mg of candesartan in sufficient amount of mobile phase and sonicated for 5 min. After that, the solution was filtered and diluted to 10 ml with mobile phase. Further dilutions in five replicates were made to get the final concentration of 12.5 μg/ml and 16 μg/ml for hydrochlorothiazide and candesartan respectively. This has been treated as 100 % target concentration [18].

Preparation of sample solutions
20 tablets were weighed, crushed and quantity of powder equivalent to 12.5 mg of hydrochlorothiazide and 16 mg of candesartan were weighed and transferred into a 10 ml volumetric flask, sufficient amount of mobile phase was added and sonicated for 5 min. After that, the solution was filtered and the volume was made up to 10 ml with mobile phase. Further dilutions in five replicates were made to get the final concentration of 12.5 μg/ml and 16 μg/ml for hydrochlorothiazide and candesartan respectively.

Validation of the developed method
The proposed analytical method was validated for system suitability, linearity and range, precision, LOD, LOQ and accuracy in accordance with ICH guidelines for analytical procedures Q2 [R1] [19].

System suitability
System suitability parameters were studied to verify the system performance. Six replicate samples containing hydrochlorothiazide (12.5 μg/ml) and candesartan (16 μg/ml) were analyzed using the developed method. Factors such as theoretical plate count, tailing factor, percent relative standard deviation of peak area and retention time were taken into consideration for testing system suitability.

Linearity and range
The linearity was evaluated at six concentration levels in the range between 6.25-18.75 μg/ml and 8-24 μg/ml for hydrochlorothiazide and candesartan respectively. A calibration curve was obtained by plotting concentration against corresponding peak area and linearity was determined using least square regression analysis. The analytical range was established by the highest and lowest concentrations of analyte and the acceptable linearity was obtained with the specified analytical range.

Precision
The precision of the developed analytical method was carried out for same concentration level. Six determinations were performed and were expressed in terms of percent relative standard deviation [% RSD].

LOD and LOQ
Limit of detection and quantitation of the developed method were calculated from the standard deviation of the y-intercept and slope of the calibration curve of hydrochlorothiazide and candesartan using the following formula:

\[
\text{Limit of detection} = 3.3 \alpha/s \\
\text{Limit of quantitation} = 10 \alpha/s
\]

Where \(\alpha\) is the standard deviation of the y-intercept and 's' is the slope of the calibration curve.

Accuracy
Accuracy of the method has been studied by recovery experiment by applying the standard addition method. A known quantity of drug substance corresponding to 50%, 100%, and 150% of the label claim of drug were added, to determine if there are positive or negative interferences from excipients present in the formulation. Each set of addition were repeated three times. The accuracy was expressed as the percentage of analyte recovered by the assay.

Application of validated method for assay of hydrochlorothiazide and candesartan in pharmaceutical dosage form
Tablet powder equivalent to 12.5 mg of hydrochlorothiazide and 16 mg of candesartan were weighed and transferred into a 10 ml volumetric flask, 7 ml of diluent was added and sonicated for 5 min. After that, the solution was filtered and the volume was made up to the mark with diluent. From this solution, further dilution was made to get the final concentration of 12.5 μg/ml of hydrochlorothiazide and 16 μg/ml of candesartan. The prepared solutions were injected into the HPLC system, chromatograms were recorded and from the peak areas of hydrochlorothiazide and candesartan, the amount of drug present in the sample was computed.

RESULTS AND DISCUSSION
Method development
Different chromatographic conditions were tried for better separation and resolution. Silanol BDS C<sub>18</sub> column (250×4.6 mm, 5 μm) was found satisfactory. Detection of analytes was carried out using PDA detector and 210 nm was considered satisfactory for detecting both the drugs with adequate sensitivity. A number of trials were performed with different solvents in the different ratios over a wide range of pH, with different flow rates and column temperatures.

But, either peak shape was broad or resolution was not good. Repeated trials to obtain good, sharp peaks with better retention times and efficient resolution between hydrochlorothiazide and candesartan were performed on Silanol BDS C<sub>18</sub> column. The runtime was good in isocratic trial with a flow rate of 1 ml/min using mobile phase containing water (pH adjusted to 2.8 with orthophosphoric acid):acetonitrile (30:70 %v/v) and the detection wavelength of 210 nm using PDA detector gave satisfactory results in terms of retention time, resolution, symmetry and sensitivity. A typical RP-HPLC chromatogram for simultaneous determination of hydrochlorothiazide and candesartan from standard preparation and sample preparation was shown in fig. 3 and 4.
On comparison with literature, it is found that the mobile phase used by Rahul et al. [13] was phosphate buffer and acetonitrile. Quatab et al. [14] and Anand Rao et al. [15] used phosphate buffer and methanol. Whereas Mathrusri et al. [16] and Balamuralikrishna et al. [17] used 0.01 M tetrabutylammonium hydrogen sulphate: methanol and 0.02% triethylamine: acetonitrile respectively. All the methods used buffers in their mobile phases takes more preparation time and also the usage of buffers reduces the life of column. The retention time of drugs in the method developed by Rahul et al. [13] was 4.3 min and 16.1 min, Anand Rao et al. [15] was 2.1 min and 7.2 min and Mathrusri et al. [16] was 2.7 min 8.1 min thereby increasing the analysis time. In the present study, a simple mobile phase consisting of water (pH adjusted with OPA) and acetonitrile was used which elute the hydrochlorothiazide and candesartan with lower retention time.

**Method validation**

**System suitability**

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and tailing factor were evaluated. The system suitability parameters were summarized in table 1. All the parameters were found to be within the limits.

| Parameters                  | Acceptance limits | Hydrochlorothiazide | Candesartan |
|-----------------------------|-------------------|---------------------|-------------|
| Retention time* (min)       | -                 | 2.30±0.16           | 4.40±0.18   |
| Resolution*                 | NLT 2             | -                   | 14.2±0.03   |
| Theoretical plates*         | NLT 2000          | 6493±2              | 1017±4      |
| Tailing factor *            | NMT 2             | 1.23±0.02           | 1.05±0.03   |

* = results of six determinations

**Linearity and range**

The linearity of the test solutions for the assay method was prepared from hydrochlorothiazide and candesartan standard stock solution at five concentration levels from 50% to 150% of assay concentration. The peak area versus concentration data was treated by least-square linear regression analysis. The results showed an excellent correlation between peak areas and concentration within the concentration range of 6.25-18.75 μg/ml and 8-24 μg/ml for hydrochlorothiazide and candesartan respectively. The results of linearity of hydrochlorothiazide and candesartan were summarized in table 2. The correlation coefficients were found to be 0.999 for both hydrochlorothiazide and candesartan, which meet the method validation acceptance criteria and hence the method was said to be linear for both the drugs. The results showed that a linear relationship between peak area and concentration of the drug in the calibration curve.

| % level | Hydrochlorothiazide concentration (μg/ml) | Hydrochlorothiazide peak area | Candesartan concentration (μg/ml) | Candesartan peak area |
|---------|------------------------------------------|-------------------------------|-----------------------------------|-----------------------|
| 50      | 6.25                                     | 292718                        | 8                                 | 626266                |
| 75      | 9.37                                     | 408707                        | 12                                | 909947                |
| 100     | 12.50                                    | 544937                        | 16                                | 1213266               |
| 125     | 15.62                                    | 681175                        | 20                                | 1516578               |
| 150     | 18.75                                    | 812002                        | 24                                | 1881404               |
| Correlation coefficient     | 0.999                                   | 0.999                         | 0.999                             | 0.999                 |
| Slope  |                                         | 43031                         | 77306                             |                       |

**Accuracy**

The accuracy of the method was determined by recovery studies by the determination of % mean recovery of both the drugs at three different levels (50 %, 100 % and 150%). At each level, three determinations were performed. The percentage recovery and mean percentage recovery were calculated for the drugs and presented in table 3. The observed data were within the specified range. Hence good recovery values indicate that the developed method is accurate for the determination of specified drugs.

**Precision**

**Method precision**

The precision of the method was verified by precision method studies. The sample solution was prepared at working concentration and analysis was carried out at replicates. The sample solutions of hydrochlorothiazide and candesartan were prepared as per the test method and injected six times into the column. The results of precision were tabulated in table 4. The mean of peak area was calculated, % RSD was calculated and reported. Method precision %
RSD values lower than 2% clearly assured that the developed method was found to be fairly precise and reproducible.

Table 3: Results of accuracy

| Level (%) | Hydrochlorothiazide | Candesartan |
|-----------|---------------------|-------------|
|           | % recovery          | % mean recovery | % recovery | % mean recovery* |
| 50        | 99.59               | 99.78        | 99.89      | 99.87            |
| 50        | 99.75               | 99.78        | 99.78      |                  |
| 50        | 100.01              | 99.57        | 99.87      | 101.08           |
| 100       | 99.99               | 101.63       |            |                  |
| 100       | 99.23               | 100.76       |            |                  |
| 150       | 100.10              | 99.96        | 101.45     | 100.64           |
| 150       | 101.00              | 100.66       |            |                  |
| 150       | 100.08              | 99.83        |            |                  |

*Average of triplicate determinations; Acceptance criteria: % recovery must be 98%-102%

Table 4: Method precision data for hydrochlorothiazide and candesartan

| No. of injections | Hydrochlorothiazide | Candesartan |
|-------------------|---------------------|-------------|
|                   | Rt                  | peak area   | Rt                  | peak area   |
| Injection 1       | 2.286               | 544936      | 4.462               | 1213265     |
| Injection 2       | 2.287               | 544933      | 4.485               | 1213266     |
| Injection 3       | 2.291               | 544940      | 4.491               | 1213267     |
| Injection 4       | 2.301               | 544936      | 4.488               | 1213259     |
| Injection 5       | 2.302               | 544939      | 4.487               | 1213258     |
| Injection 6       | 2.299               | 544938      | 4.480               | 1213262     |
| Mean*±SD          | 544937±2.52         | 0.4         | 1213263±3.76        | 0.3         |

% RSD *

*The value is represented as a mean±SD of 6 observations (n=6). SD: Standard Deviation, #RSD: Relative Standard Deviation, Acceptance criteria:<2

Limit of detection and limit of quantitation

LOD and LOQ were estimated from the standard deviation of the y-intercept and slope of the calibration curve of hydrochlorothiazide and candesartan.

The LOD and LOQ were found to be 0.410 μg/ml and 1.367 μg/ml for hydrochlorothiazide and 0.699 μg/ml and 2.330 μg/ml for candesartan. The results of LOD and LOQ specified the sensitivity of the developed method.

Robustness

Robustness of the developed method was determined by deliberately altering the experimental conditions and the system suitability parameters were evaluated. The solutions prepared as per the test method and injected at different variable conditions like flow rate (0.8, 1.2 ml/min) and wavelength (208 nm, 212 nm), system suitability parameters were compared with that of method precision.

The results were tabulated in table 5. Even though, small changes were made in the conditions there was no significant effect on tailing, plate count and retention time of specified drugs. Hence, the developed method was found to be robust.

Application of validated method for assay of hydrochlorothiazide and candesartan in pharmaceutical dosage form

The developed method was successfully implemented in the assay of hydrochlorothiazide and candesartan in pharmaceutical dosage form. Assay of hydrochlorothiazide and candesartan was found to be 99.16% and 99.60% respectively. The results of the assay were summarized in table 6.

Table 5: Results of robustness

| Parameter                        | Hydrochlorothiazide | Candesartan |
|----------------------------------|---------------------|-------------|
|                                  | Plate count*        | Tailing*    | Plate count* | Tailing* |
| Less flow rate (0.8 ml/min)      | 5416                | 1.21        | 9606         | 1.11     |
| More flow rate (1.2 ml/min)      | 6692                | 1.19        | 8807         | 1.04     |
| Less wavelength (208 nm)         | 3284                | 1.09        | 9075         | 1.12     |
| More wavelength (212 nm)         | 6217                | 1.17        | 9294         | 1.09     |

Acceptance criteria (Limits): #Peak Asymmetry: >2000, *Tailing: <2

Table 6: Assay of hydrochlorothiazide and candesartan in pharmaceutical dosage form

| Name                | Standard average area* | Sample average area* | % Assay |
|---------------------|------------------------|----------------------|--------|
| Hydrochlorothiazide | 544837                 | 534811               | 99.16  |
| Candesartan         | 1213262                | 1208408              | 99.60  |

*Mean of six determinations
CONCLUSION

In the present study, a simple and efficient RP-HPLC method was developed for the simultaneous analysis of hydrochlorothiazide and candesartan in bulk and in pharmaceutical dosage form. The method was validated as per ICH guidelines and found to be applicable for routine quality control analysis. The results of analysis of pharmaceutical formulation by the proposed method were highly reproducible and reliable. Hence, it can be concluded that the proposed RP-HPLC method was simple, cost-effective, specific, accurate, precise, robust and rapid.

ACKNOWLEDGMENT

The authors wish to express their gratitude to the Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam (Women’s University), Tirupati and the Spectrum Pharma Lab, Hyderabad for providing necessary facilities to carry out the research work.

CONFLICT OF INTERESTS

Declared none

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