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# ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF PHARMACEUTICAL DRUGS BY RP-HPLC METHOD

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**ABSTRACT**
Developing a single analytical method for estimation of individual drug from a multidrug composition is a very challenging task. A simple, rapid, precise, and reliable reverse phase HPLC method was developed for the separation and estimation of two drugs hydrochlorothiazide and eprosartanin bulk drug mix and pharmaceutical dosage forms. The estimation was carried out using column; mobile phase consisting of methanol, orthophosphoric acid and buffer at pH 5; the flow rate of 1.7 mL/min and ultraviolet detection at 232 nm. All the drugs were properly resolved having run time of 2.5 and 4.3 minutes for hydrochlorothiazide and eprosartan, respectively. The method was validated as a final verification of method development with respect to precision, linearity, accuracy, ruggedness, and robustness. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible result.

**KEYWORDS:** RP-HPLC, hydrochlorothiazide and eprosartan, method development.

## 1.0 INTRODUCTION

Human life starting from birth to death mostly depends on medicines like vaccines,pharmaceutical dosages, drugs, vitamin tablets, mineral supplements, energy drinks,boosters etc. Because of advent development of the medical and pharmaceutical scienceswere now leading a long span of healthy life.[1,2] Medicines enhanced the life expectancy of humans and are considered to be the most important necessity of mankind which plays a vital role in curing the diseases and health issues. People who are suffering with hypertension, diabetes etc. are completely depending on the medicines throughout their tenure of life. Thus, the medicine is basically concerned with the health and welfare of the human.[3,4]

During our ancestor's period pollution was low, food habits are hygiene, lot of physical work was involved, no significant mental stress or work pressures etc., and hence their health was not affected much with various kinds of diseases. But today, rapid increase of population, change of food habits, lack of physical work, extensive work pressure, mental stress, various kinds of pollutions etc., are causing the diseases and health problems. As the increase of diseases, the usage of medicines is also enormously increased.[5,6] For the effective treatment of various diseases and health issues, proper medicines orpharmaceutical dosages in the right combinations are required. Medicines are there sincefrom the ancient times and most of them are prepared by using herbals, natural ingredientsand by the other home remedy methods. Preparation of the medicines is mainly concernedwith the efficiency of the drug and the existing technology. The medicines should maintaincertain requirements - like purity, effective reaction time, lack of side effects etc.[7,8] Pharmaceutical companies look at qualitative and quantitative analysis to verify the qualityof final product and to check that their raw materials meeting their indeed specifications. Qualitative analysis concerns with the identification of elements, functional groups orcompounds in a sample, whereas the quantitative analysis helps in the determination ofamount of a particular element, species or compound in the given sample, which enable usto maintain permissible limits of impurities in the sample.[9,10]

### 1.1 Analytical Techniques

For the qualitative and quantitative analysis of various drug products the following analytical techniques are readily available.

#### 1.1.1 Titrimetric Techniques

Titrimetric analysis implies in the determination of concentration of a standard solution to react quantitatively with a measured volume of a solution of the substance to be determined. It is also used for the determination of degradation products of the pharmaceuticals.[11,12]

#### 1.1.2 Spectroscopic Techniques

Spectroscopy is the study of absorption, emission, or scattering of electromagnetic radiation with matter.

#### 1.1.3 Electrochemical Techniques

These techniques are concerned with the interaction between electricity and chemistry for the measurements of electrical quantities such as electric current, voltage or potential, charge and their relationship with the chemical parameters.

#### 1.1.4 Kinetic method of Analysis

Chemical kinetics provides a major tool for the study of chemical mechanisms, and the detailed account of how reactions take place. The basic concept of the reaction kinetics is "law of mass action" and it states that the rate of a chemical reaction is proportional to the active masses of the reacting substances each raised to the power of its coefficient in the stoichiometric equation.

#### 1.1.5 Electrophoresis Techniques

The principle of electrophoresis is very simple, namely that a charged ion or group migrates towards one of the electrodes when placed in an electric field and this technique operates on a principle which is different from the principle of chromatography and is alternative technique for separating the closely related charged substances, and after electrophoresis the substances are located as a number of separate discrete zones.[13,14]

#### 1.1.6 Flow injection analysis and Sequential injection analysis

In flow injection analysis (FIA) definite volume of the liquid sample is injected into a moving, non-segmented continuous carrier stream of a suitable liquid15. The injected sample forms a zone, which is then moves towards a detector that continuously records the changes in absorbance, electrode potential, or other physical parameter resulting from the passage of the sample through the flow cell. The flow injection technique is used as an automated solution handling system for went chemical analysis16, sensors and electrodes, chromatography18, atomic absorption spectrometry etc. Sequential injection analysis (SIA) is the development of flow injection analysis, and it provides a robust methodology than the traditional flow injection.

#### 1.1.7 Hyphenated technique

Hyphenation involves the union of two or more instrumental methods in a single run with the help of proper interface20. A significant preglen have been noticed in past two decades which has broadened their applications in the analysis of biomaterials, especially natural products21. Hyphenated analytical methods provide more compatible information in less time leading to faster and accurate results. Here the combinations include separation, separation-identification and identification-identification techniques.15,16

## 2.0 MATERIAL AND METHODS

### 2.1 Chemicals and Solvents

Methanol (HPLC grade, Merck Ltd),Ortho-phosphoric acid (GR Grade, SD Fine Chem. Ltd), Eprosartan& Hydrochlorothiazide (Abbott, USA) and all other chemicals are of the highest grade commercially available unlessotherwise specified.

### 2.2Equipment

The Chromatographic equipment consists of a Shimadzu Class VP binary pump LC-10ATvp, SIL-10ADvp auto sampler, CTO-10Avp column temperature oven, SPD-10Avp UV-visible detector. All the constituents of the system were controlled by using SCL- 10Avp system controller. Data acquisition was done by using the software LC solutions.

### 2.3 Preparation of diluent solution

50% of methanol and 50% of milli-Q water were mixed well to obtain 50:50 % (v/v) of methanol and water. This mixture was mixed well and it is sonicated in an ultrasonic bath for 20 minutes and then used for the analysis. The solution is labelled and it is used within 7days from the preparation date.

### 2.4 Preparation of standard solutions

Stock solutions of Eprosartan and Hydrochlorothiazide (5mg/mL) were preparedseparately in a volumetric flask and labelled accordingly. The dilute solutions of therespective drugs were prepared by using 50:50 % (v/v) methanol & milli-Q water as diluentSolution. The calibration curve containing 6 non-zero standards for each drug wereprepared by using diluent solution in the concentration range of 5.01-50.16 µg/mL and5.03 - 50.31 for Eprosartan and Hydrochlorothiazide respectively. Samples for specificity(sample with drug - blank sample) were also prepared accordingly.

### 2.5 Preparation of sample solutions

In order to prepare the quality control samples, a separate stock containing relevant to the same concentration of the drug substance was prepared and labelled as quality control stock. From this stock, quality control samples were prepared in three concentration levels namely LQC (12.54 and 12.57 µg/mL for Hydrochlorothiazide and Eprosartan) and MQC (25.08 and 25.15 µg/mL), HQC (37.62 and 37.73 µg/mL) so as to obtain low, median and high concentration quality control samples. The linear

calibration curve was then evaluated using quality control samples.

**Table 1: Preparation of Standard Solutions.**

| | Hydrochlorothiazide | Eprosartan |
|---|---|---|
| **Weighed Amount (mg)** | 22.8 | 21.5 |
| **Volume of Solvent (MeOH used) (mL)** | 5 | 5 |
| **Stock Concentration ( µg / mL)** | 4560 | 4300.00 |
| **Stock** | HCTZD-ST | EPR-ST |
| **Volume of Stock Taken (mL)** | 1.1 | 1.17 |
| **Final Volume (mL)** | 5 | 5 |
| **Concentration Of Intermediate Dilution (µg / mL)** | 1003.20 | 1006.20 |
| **Intermediate Dilution** | HCTZD-INT | EPR-INT |

### 2.6 Preparation of Mobile Phase

50 parts of methanol is mixed with 50 parts of 0.1% orthophosphoric acid to obtain 50:50 % (v/v) of Methanol and 0.1% orthophosphoric acid. The mixture is mixed well, sonicated in an ultrasonic bath for 20 min and then used for experiment. The solution is labelled and used within 7 days from the date of preparation.

### 2.7 Detection of wavelength

The spectra of diluted solutions of the Eprosartan and Hydrochlorothiazide in Methanol were recorded on UV spectrophotometer. The peaks of maximum absorbance wavelengths were observed. The spectrum of the Eprosartan and Hydrochlorothiazide showed that a balanced wavelength was found to be 232 nm.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Retention time of Eprosartan and Hydrochlorothiazide

With the above optimized conditions, observed retention times for eprosartan and hydrochlorothiazide were 4.3 min and 2.5 min respectively. A typical chromatogram showing the separation of eprosartan and hydrochlorothiazide is presented in figure 1. After a thorough study of the various parameters following the optimized conditions mentioned in table 2.8 were followed for the determination of eprosartan and hydrochlorothiazide in bulk samples and pharmaceutical formulation.

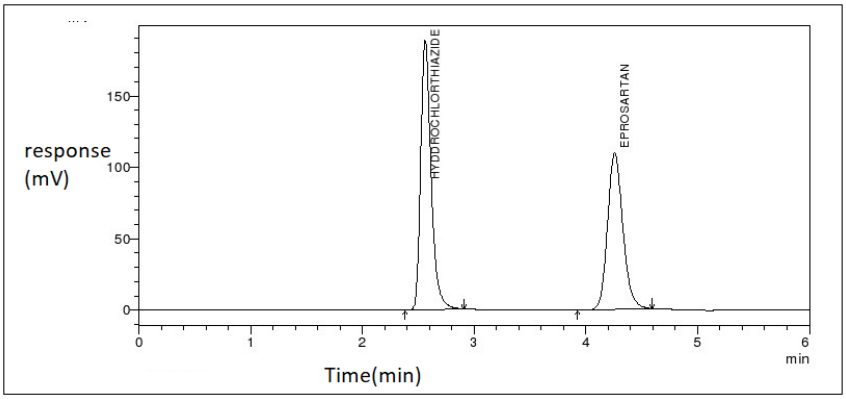


**Figure 1: Chromatogram showing the separation of Hydrochlorothiazide &Eprosartan.**

### 3.2 Method Validation

#### 3.2.1 Specificity

The method specificity can be estimated by comparing the chromatograms obtained from the drug with those obtained from the blank solution. The blank solutions were prepared by mixing the excipients in the mobile phase without drug. These mixtures allow to filter by passing through 0.45 µ membrane filter before injection. Now performed the experiment and there are no extra peaks were formed near the drugs peak. This indicates that the proposed method was specific. The chromatograms which show the overall separation for specificity was given in figure 2 for hydrochlorothiazide, eprosartan and sample containing both the drugs respectively.

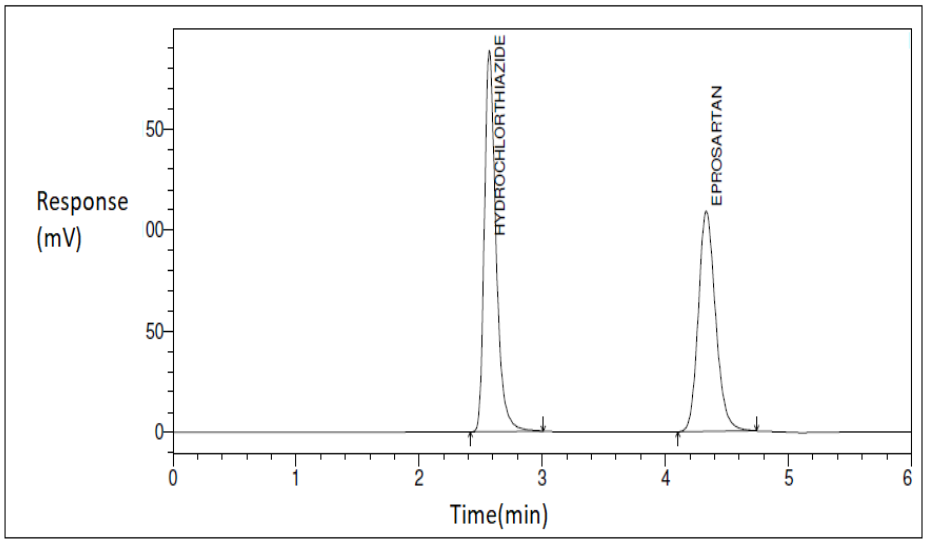


**Figure 2: Chromatogram of the sample containing Hydrochlorothiazide &Eprosartan.**

### 3.2.2 Construction of calibration curve and linearity

Mention in Figure 3 & 4.

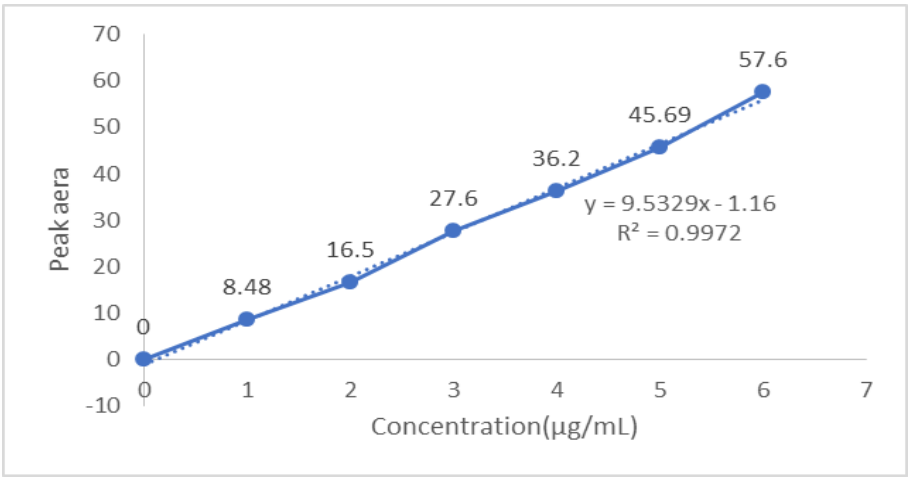


**Figure 3: Linearity curve of Hydrochlorothiazide.**

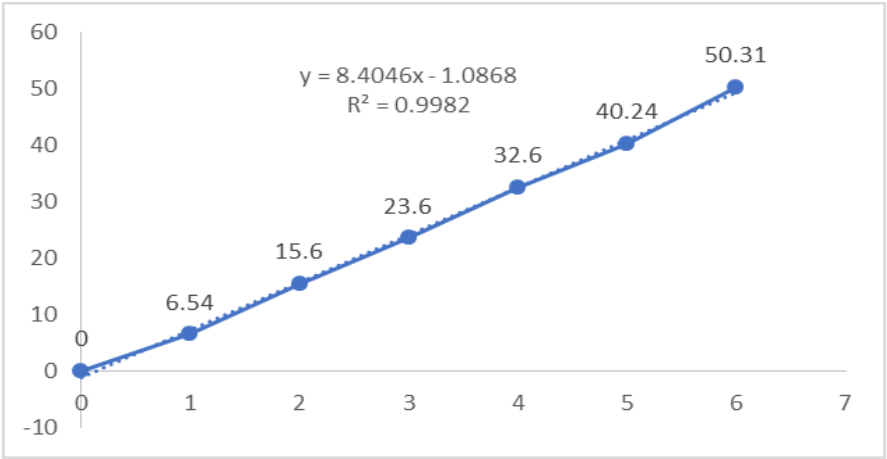


**Figure 4: Linearity curve of Eprosartan.**

### 3.2.3 Accuracy

Accuracy of the method can be determined by using standard addition method. These sample solutions were injected in triplicate to HPLC system for the analysis at the proposed conditions of the method. The individual % of recovery and % RSD for recovery at each level were calculated. A recovery ranged from 98.00 - 102.00% has been obtained by the method indicates its accuracy.

### 3.2.4 Precision

Precision is the measure of degree of repeatability or reproducibility of an analyticalmethod under normal working conditions Table 2.

**Table 2: Results of inter & intra-day accuracy & precision (% CV) for Hydrochlorothiazide.**

| Sample ID | LQC | MQC | HQC |
|---|---|---|---|
| **Nominal Concentration (µg/mL)** | 12.72 | 25.44 | 37.50 |
| **DAY 1** | | | |
| **Mean Concentration (µg/mL)** | 12.62 | 25.12 | 37.37 |
| **SD** | 0.22 | 0.23 | 0.19 |
| **% CV** | 1.72 | 0.92 | 0.51 |
| **DAY 2** | | | |
| **Mean Concentration (µg/mL)** | 12.58 | 25.32 | 37.25 |
| **SD** | 0.21 | 0.25 | 0.49 |
| **% CV** | 1.67 | 0.99 | 1.32 |
| **DAY 3** | | | |
| **Mean Concentration (µg/mL)** | 13.12 | 24.89 | 37.56 |
| **SD** | 0.25 | 0.35 | 0.56 |
| **% CV** | 1.91 | 1.41 | 1.49 |

### 3.2.5 Limit of Detection and Limit of Quantification (LOD and LOQ)

Limit of detection (LOD) is the lowest amount of analyte in a given sample which gives a measurable response under operational conditions. LOD is determined typically by three times of signal to noise ratio (S/N) for HPLC methods. The limit of quantification (LOQ) is defined as the lowest concentration which can be quantitatively measured with a specified level of accuracy and precision. LOD & LOQ of Hydrochlorthiazide & Eposartan is mention in table 3,4,5 & 6, respectively.

**Table 3: LOD of Hydrochlorothiazide.**

| Hydrochlorothiazide | | |
|---|---|---|
| **Sr. No.** | **Retention Time** | **Peak Area** |
| 1 | 2.58 | 6543 |
| 2 | 2.58 | 6425 |
| 3 | 2.58 | 6632 |
| Mean | 2.58 | 6533.3 |
| Std. Dev. | 0.00 | 103.83 |
| % CV | 0.00 | 1.58 |

**Table 4: LOQ of Hydrochlorothiazide.**

| Hydrochlorothiazide | | |
|---|---|---|
| **Sr. No.** | **Retention Time** | **Peak Area** |
| 1 | 2.58 | 10386 |
| 2 | 2.57 | 10081 |
| 3 | 2.58 | 10224 |
| Mean | 2.57 | 10230.3 |
| Std. Dev. | 0.0057 | 152.59 |
| % CV | 0.22 | 1.49 |

**Table 5: LOD of Eprosartan.**

| Eprosartan | | |
|---|---|---|
| **Sr. No.** | **Retention Time** | **Peak Area** |
| 1 | 4.41 | 6652 |
| 2 | 4.41 | 6628 |
| 3 | 4.39 | 6515 |

| | | |
|---|---|---|
| Mean | 4.4 | 6598.3 |
| Std. Dev. | 0.011 | 73.33 |
| % CV | 0.26 | 1.11 |

**Table 6: LOQ of Eprosartan.**

| Eprosartan | | |
|---|---|---|
| **Sr. No.** | **Retention Time** | **Peak Area** |
| 1 | 4.39 | 10471 |
| 2 | 4.39 | 10236 |
| 3 | 4.37 | 10119 |
| Mean | 4.38 | 10275.33 |
| Std. Dev. | 1.011 | 179.26 |
| % CV | 0.25 | 1.74 |

### 3.2.6 Ruggedness

The prepared MQC mix was injected and performed by different analysis shown in Table 7.

**Table 7: Ruggedness results of Hydrochlorothiazide and Eprosartan.**

| Sr. No. | Hydrochlorothiazide | | | Eprosartan | | |
|---|---|---|---|---|---|---|
| | **Retention Time** | **Peak Area** | **Tailing Factor** | **Retention Time** | **Peak Area** | **Tailing Factor** |
| 1 | 2.59 | 1336820 | 1.31 | 4.44 | 1141821 | 1.16 |
| 2 | 2.59 | 1348701 | 1.32 | 4.43 | 1118063 | 1.17 |
| 3 | 2.59 | 1319984 | 1.32 | 4.43 | 1102644 | 1.17 |
| Mean | 2.59 | 1335168.3 | 1.31 | 4.43 | 1120843 | 1.16 |
| Std. Dev. | 0.00 | 14429.57 | 0.01 | 0.0057 | 19735.86 | 0.005 |
| % CV | 0.00 | 1.08 | 0.44 | 0.12 | 1.76 | 0.43 |

### 3.2.7 Robustness

A study was performed to determine the effects obtained in the results by varying the proposed chromatographic conditions like composition of the mobile phase, flow rate level and pH range of the mobile phase etc., and observed the effect of these changes on the parameters like tailing factor, number of theoretical plates and assay. A single condition was varied at a time keeping all other parameters constant. The results were found to be within the allowed limits which indicate that the method is robust.

**Table 8: Robustness results of flow rate at 0.7 mL/min.**

| Sr. No. | Hydrochlorothiazide | | | Eprosartan | | |
|---|---|---|---|---|---|---|
| | **Retention Time** | **Peak Area** | **Tailing Factor** | **Retention Time** | **Peak Area** | **Tailing Factor** |
| 1 | 2.94 | 1421649 | 1.32 | 4.89 | 1374060 | 1.18 |
| 2 | 2.96 | 1431432 | 1.33 | 5.03 | 1335902 | 1.17 |
| 3 | 2.96 | 1437424 | 1.32 | 5.03 | 1329434 | 1.16 |
| Mean | 2.953 | 1430168.3 | 1.32 | 4.98 | 1346465.3 | 1.05 |
| Std. Dev. | 0.0115 | 7963.06 | 0.0057 | 0.0808 | 24115.51 | 0.01 |
| % CV | 0.39 | 0.55 | 0.43 | 1.62 | 1.79 | 0.95 |

**Table 9: Robustness results of flow rate at 0.9 mL/min.**

| Sr. No. | Hydrochlorothiazide | | | Eprosartan | | |
|---|---|---|---|---|---|---|
| | **Retention Time** | **Peak Area** | **Tailing Factor** | **Retention Time** | **Peak Area** | **Tailing Factor** |
| 1 | 2.29 | 1110125 | 1.34 | 3.85 | 1075014 | 1.18 |
| 2 | 2.29 | 1108516 | 1.35 | 3.81 | 1077938 | 1.18 |
| 3 | 2.29 | 1100195 | 1.34 | 3.80 | 1068857 | 1.18 |
| Mean | 2.29 | 1106278.7 | 1.34 | 3.82 | 1073936.3 | 1.18 |
| Std. | 0.00 | 5329.68 | 0.0057 | 0.0265 | 4635.42 | 0.00 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Dev. | | | | | | |
| % CV | 0.00 | 0.48 | 0.42 | 0.69 | 0.43 | 0.00 |

### 3.2.8 Stability

To determine the stability of the drug MQC samples of the respective drugs were kept aside for 12 hrs at room temperature and perform the HPLC analysis by injecting the stability sample to the instrument. The chromatograms obtained were compared with the chromatograms obtained from the analysis of freshly prepared drug samples. Further, the stability can be studied up to 24 hrs and established (Table 10 &11).

**Table 10: Room temperature stability results of Hydrochlorothiazide.**

| Sr.No. | Fresh Sample | | Stability Sample | |
|---|---|---|---|---|
| | Retention Time | Peak Area | Retention Time | Peak Area |
| 1 | 2.59 | 1315677 | 2.58 | 1175097 |
| 2 | 2.59 | 1363821 | 2.58 | 1181751 |
| 3 | 2.59 | 1361213 | 2.58 | 1162699 |
| 4 | 2.59 | 1336820 | 2.58 | 1186109 |
| 5 | 2.59 | 1348701 | 2.58 | 1172300 |
| 6 | 2.59 | 1319984 | 2.58 | 1172967 |
| Mean | 2.59 | 1341036.0 | 2.58 | 1175153.8 |
| Std. Dev. | 0.00 | 20440.45 | 0.00 | 8143.60 |
| % CV | 0.00 | 1.52 | 0.00 | 0.69 |

**Table 11: Room temperature stability results of Eprosartan.**

| Sr. No | Fresh Sample | | Stability Sample | |
|---|---|---|---|---|
| | Retention Time | Peak Area | Retention Time | Peak Area |
| 1 | 4.45 | 1129576 | 4.41 | 1028700 |
| 2 | 4.44 | 1149207 | 4.42 | 1034396 |
| 3 | 4.44 | 1155932 | 4.41 | 1019896 |
| 4 | 4.44 | 1141821 | 4.41 | 1037992 |
| 5 | 4.43 | 1123019 | 4.41 | 1030746 |
| 6 | 4.43 | 1114571 | 4.42 | 1011914 |
| Mean | 4.43 | 1135687.6 | 4.41 | 1027274.0 |
| Std. Dev. | 0.0075 | 15962.35 | 0.0051 | 9695.89 |
| % CV | 1.6 | 1.40 | 0.11 | 0.94 |

### 3.2.9 Stress degradation for fresh sample

Shown in figure 5 & 6.

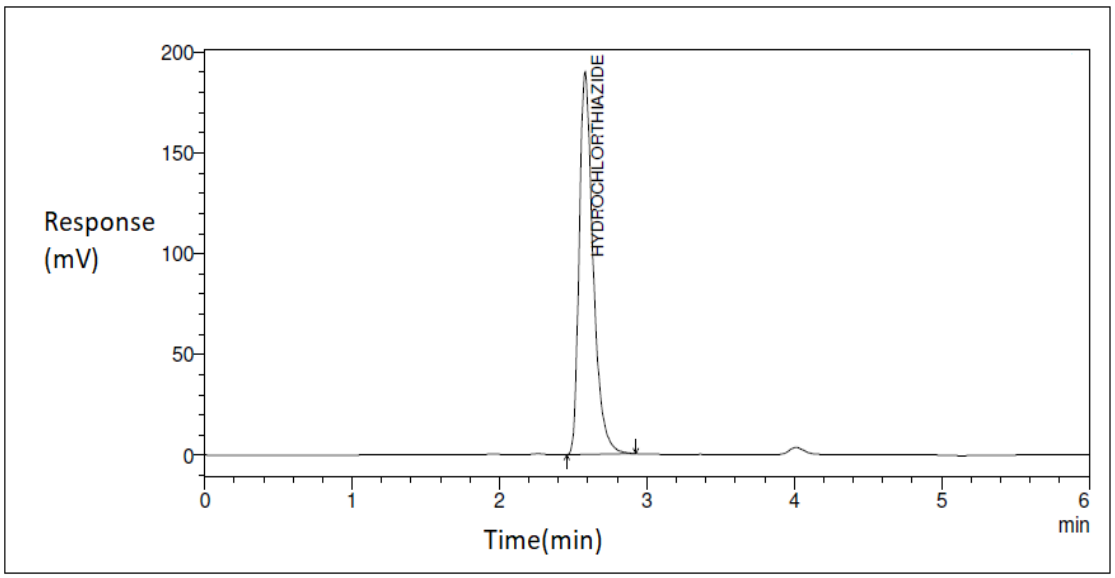


**Figure 5: Chromatogram of stress degradation studies of Hydrochlorothiazide.**

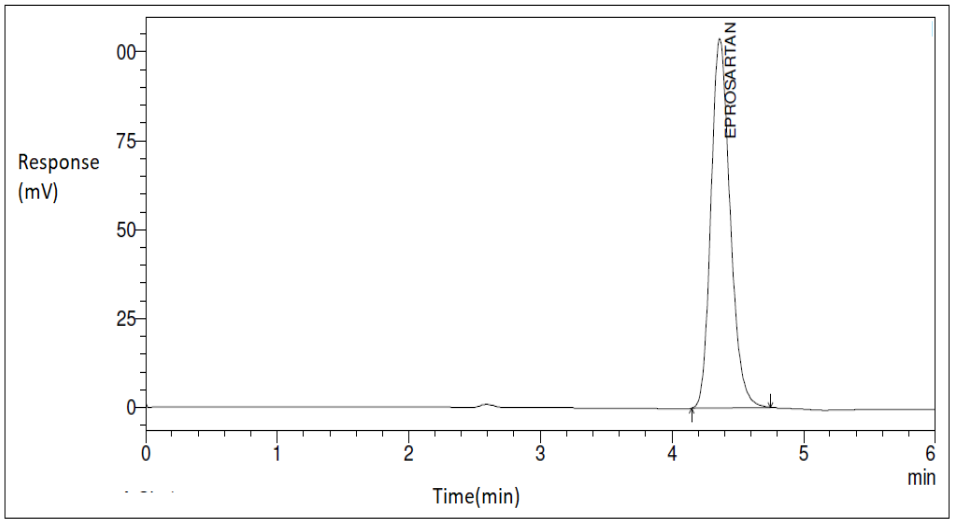


**Figure 6: Chromatogram of stress degradation studies of Eprosartan.**

### 3.2.10 Oxidative stress conditions

Shown in Figure 7 & 8.

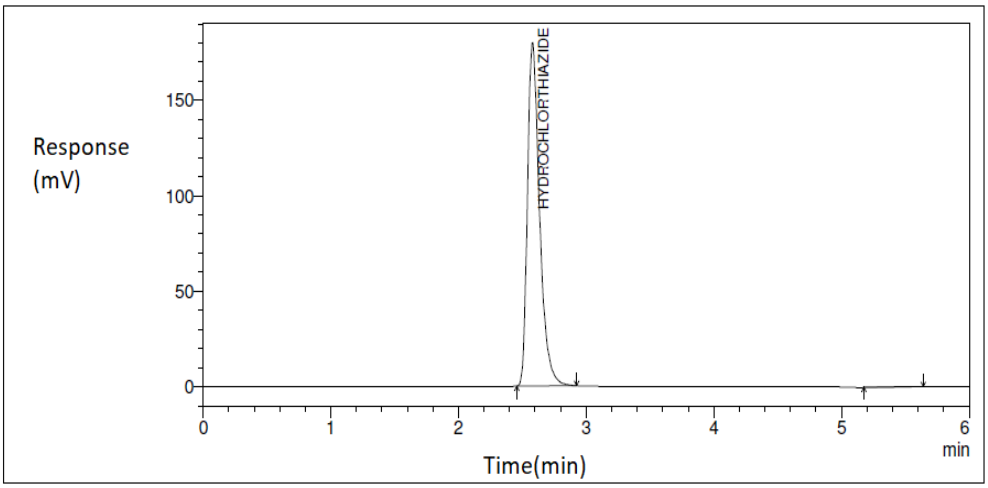


**Figure 7: Chromatogram of degradation studies of Hydrochlorothiazide-Oxidative Stress.**

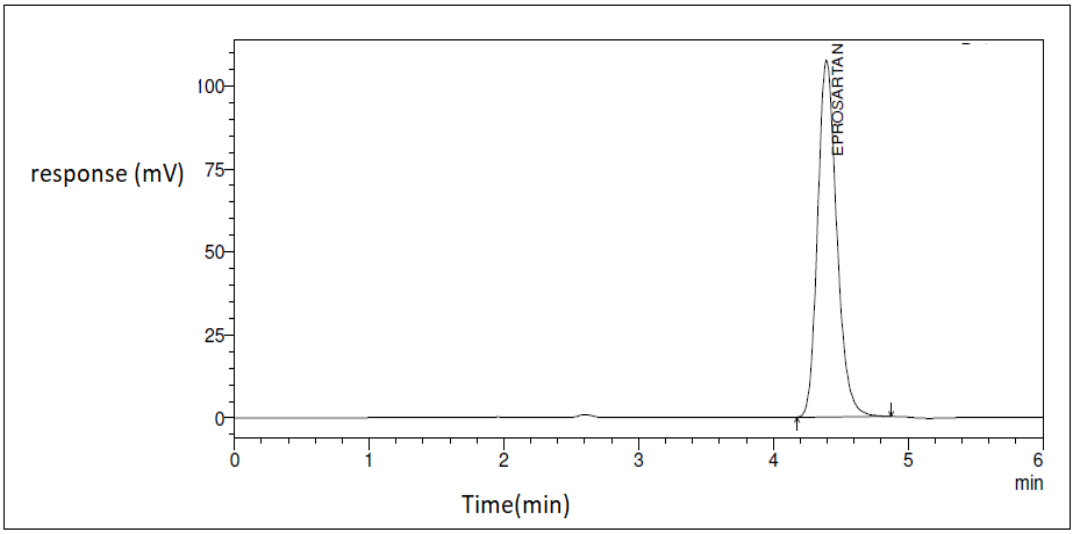


**Figure 8: Chromatogram of degradation studies of Eprosartan - Oxidative Stress.**

### 3.2.11 Stress degradation under acidic conditions

Shown in Figure 9 & 10.

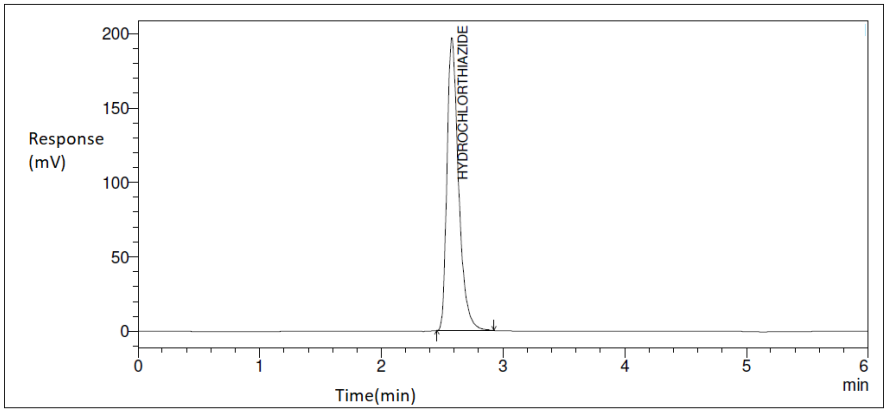


**Figure 9: Chromatogram of degradation studies of Hydrochlorothiazide - Acidic Stress.**

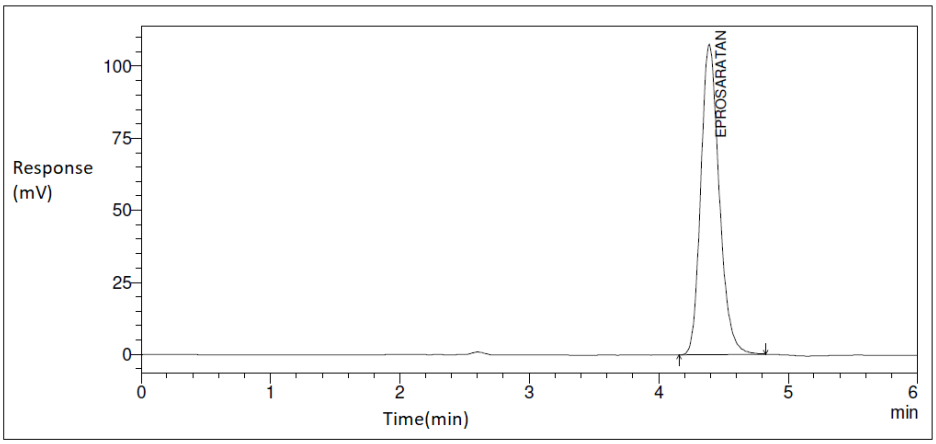


**Figure 10: Chromatogram of degradation studies of Eprosartan - Acidic Stress.**

### 3.2.12 Photolytic stress degradation

Shown in Figure 11 & 12.

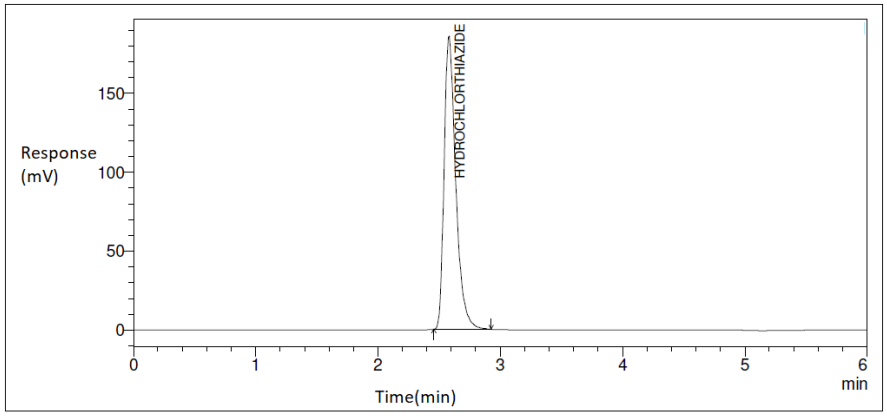


**Figure 11: Chromatogram of degradation studies of Hydrochlorothiazide - Photolytic Stress.**

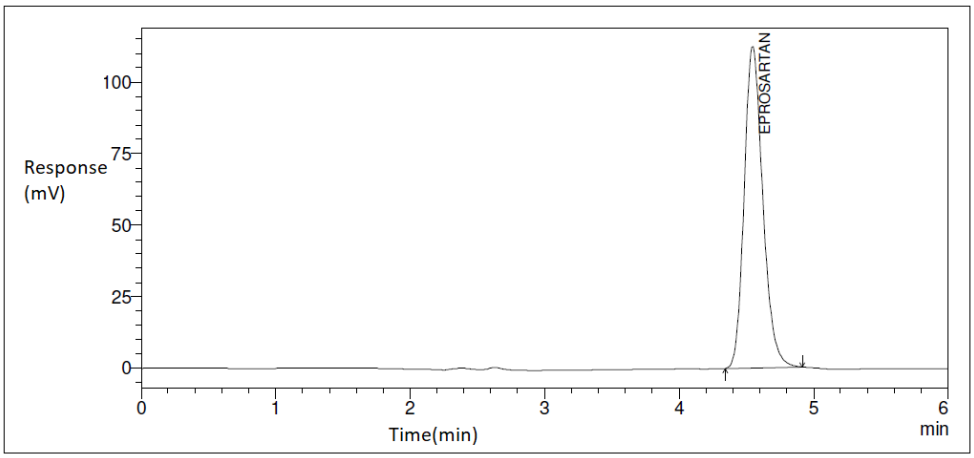


**Figure 12: Chromatogram of degradation studies of Eprosartan - Photolytic Stress.**

## 4.0 SUMMARY

For routine analytical purpose it is desirable to establish methods capable of analysing huge number of samples in a less time period which is accurate, precise and with good robustness without any prior separation step. HPLC method generates large amount of quality data, which serves as highly powerful and convenient analytical tool. Hydrochlorothiazide and Eprosartan were freely soluble in ethanol, methanol and sparingly soluble in water. Methanol and phosphate buffer was chosen as the mobile phase. The run time of the HPLC procedure was 7 minutes. A new sensitive, accurate and specific method was developed and validated for the simultaneous estimation of Hydrochlorothiazide and Eprosartan. The mobile phase was composed with 50% of methanol and 50% of 0.1% orthophosphoric acid with the flow rate of 0.8 mL/min, and the detection wavelength of 232 nm. The retention times of Hydrochlorothiazide and Eprosartan are 2.5 and 4.3 minutes respectively. The column used is Phenomenex 150 X 4.6 mm C18 column with the particle size of 5 µm. The proposed method was validated and all the system suitability parameters are in acceptable limit, hence it was concluded that the system is suitable to perform the assay. The developed method has given accurate and precise results in the concentration range of 5.01 - 50.16 µg/mL and 5.03 - 50.31 µg/mL. As there was no interference of extra peaks with the drug peak due to the excipients and mobile phase, the method was said to be specific. The results obtained from the analysis are good enough by changing the flow rate and mobile phase composition separately and analysis was performed by different analysts. Hence the method is said to be robust and rugged. The drugs are tested for the stress conditions like Photostability, acid stability, alkaline and oxidation conditions for 24 hours. The drugs are stable and did not show any signs of degradation under stress conditions except in alkaline medium. Hence the proposed method is said to be stable and it is successful in the identification of the medium that in which selected drug is going to degrade. This method also succeeded in the clear separation of degradation peak from the respective drug peak. Good agreement was seen in the results of combined dosage form by developed method. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of Hydrochlorothiazide and Eprosartan in combined dosage form.

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