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Development and Validation of Rp-Hplc Method for the Determination of Triamterene and Benzthiazide in Pharmaceutical Dosage Form

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ABSTRACT:
A simple and selective RP-HPLC method is described for the determination of Triamtereneand Benzthiazide in pharmaceutical dosage forms. Chromatographic separation was achieved on a c_{18} column using mobile phase consisting of a mixture of 60 volumes of triethylamine buffer,40 volumes of acetonitrile with detection of 245 nm. Linearity was observed in the range 12.5-37.5 µg/ml for Triamtereneoxalate (r^2 =0.992) and 6.25-18.75 µg /ml for Benzthiazide (r^2 =0.991) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim.

The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

KEYWORDS Ttiamterene,Benzthiazide,RP-HPLC,Validity

I. INTRODUCTION

DIURETICS

Diuretics, also called water pills, these are medications designed to increase the amount of water and salt expelled from the body as urine. They are often prescribed to help treat high blood pressure.

- They help the kidneys remove water from the body. They are used to treat oedema which is a build-up of fluid in the body. This may occur in many situations, for instance during treatment with steroids, with liver and kidney problems or with heart[26,32]

CLASSIFICATION OF DIURETICS

- Carbonic Anhydrase Inhibitors
- Osmotic Diuretic
- Loop Diuretics
- Thiazide Diuretics
- Potassium Sparing Diuretics

Diuretics, in particular thiazide compounds, were the earliest forms of practical and well-tolerated antihypertensive drugs introduced in the modern therapeutic era. Numerous large randomized clinical trials have demonstrated the value of these agents in reducing morbidity and mortality in the hypertensive patient. Despite questions raised concerning the metabolic effects of thiazide diuretics, newer trials and guidelines have reaffirmed their primacy in the safe and effective initial management of high BP and as an element of combination therapy in more complex or difficult cases. Contemporary strict BP treatment goals will require that diuretics continue to play a major role in hypertension therapy.[10,26]

HYPERTENSION:

Hypertension (HTN) is a worldwide

- health problem and a major preventable risk factor for cardiovascular (CV) events.
- Achieving an optimal blood pressure (BP) target for patients with HTN will often require more than one BP-lowering drug [26]

COMBIINATION THERAPY

Combination therapy is not only needed, but also confers many advantages such as better efficacy and a better tolerability. A better compliance and simplicity of treatment is noted with the single-pill combination. Available data suggest that at least 75% of patients will require combination therapy to achieve contemporary BP targets, and increasing emphasis is being placed on the practical tasks involved in consistently achieving and maintaining goal BP in clinical practice.

Potassium sparing diuretics are often prescribed with another diuretic because, while it spares

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potassium, it does not control blood pressure as well as thiazide diuretics do.

Thiazide diuretics reduces the amount of salt and water in the body. It is also the only type of diuretic that widens the blood vessels to lower blood pressure.[10,12]

Initially, when diuretics are given plasma volume decreases—lowering cardiac output and arterial BP. later, volume rises toward normal and cardiac output normalizes. However, BP remains low, with reduced peripheral vascular resistance.4 This effect is attributable to direct arterial relaxation

Side effects

Administration of the diuretic medicine Ditide (Benzthiazide/Triamterene IP) could trigger the occurrence of side effects, including:

- Gout
- Mouth ulcers
- Muscular cramping
- Weakness or vertigo
- Thirst or dryness in the mouth

Exercise caution during treatment and make your doctor aware of any side effects that occur. Other side effects could occur, including fever, headaches, bruising .[32]

Uses:

Ditide (Benzthiazide/Triamterene IP) is a combination diuretic medicine made up of two active ingredients. It works by increasing the volume of water that the kidneys remove from the body and is used in the treatment of oedema (fluid retention). Some patients have also been given it for high blood pressure (hypertension). Oedema is a condition in which fluid builds up in the body.

By causing the body to increase its production of urine, excess salt and water is removed from the body. This in turn helps to relieve symptoms of fluid retention. .[20]

EPIDEMIOLOGY-Hypertension is an epidemic affecting one billion people and is the commonest risk factor for death throughout the world. World health statistics 2012 has estimated the prevalence of hypertension to be 29.2% in males and 24.8% in females. Approximately 90 percent for men and women who are non hypertensive at 55 or 65 years will develop hypertension by the age of 80–85. Out of total 58.8 million deaths worldwide in year 2004, high blood pressure was responsible for 12.8% (7.5 million deaths). World over hypertension is responsible for 51% of cerebrovascular disease and 45% of ischemic heart disease deaths. Unlike the popular belief that hypertension is more important for high-income countries, people in low- and middle-income countries have more than double the risk of dying of hypertension. [22]

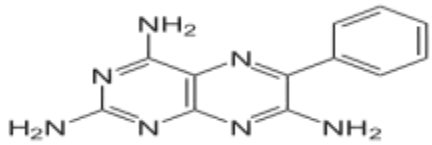
Understanding epidemiology of hypertension will significantly help in decreasing the burden of associated morbidity and mortality. Since 1972, age-adjusted death rates from stroke have decreased by about 60% and that by coronary heart disease has decreased by about 50 percent. Recent WHO initiative on non communicable diseases is expected to decrease hypertension related mortality and morbidity globally.[19]

1.2.DRUG PROFILE

1.2.1. Triamterene: Triamtereneis a potassium-sparing diuretic used in combination with thiazide diuretics for the treatment of hypertension and edema. It is commonly used in the treatment of hypertension in combination with a thiazide diuretic. [9,32]

A pteridine that is used as a mild diuretic.

Structure:



IUPAC name : 6-phenylpteridine-2,4,7-triamine

Category : Cardiovascular Agents

Molecular weight : 253.2626

Chemical formula : $C_{12}H_{11}N_7$[9]

Mechanism of Action-Triamterene inhibits the epithelial sodium channels on principal cells in the late distal convoluted tubule and collecting tubule, which are responsible for 1-2% of total sodium reabsorption. As sodium reabsorption is inhibited, this increases the osmolarity in the nephron lumen and decreases the osmolarity of the interstitium. Since sodium concentration is the main driving force for water reabsorption, triamterene can achieve a modest amount of diuresis by decreasing the osmotic gradient necessary for water reabsorption from lumen to interstitium. Triamterene also has a potassium-sparing effect. Normally, the process of potassium excretion is driven by the electrochemical gradient produced by sodium reabsorption. As sodium is reabsorbed, it leaves a negative potential in the lumen, while producing a positive potential in the principal cell. This potential promotes potassium excretion through apical potassium channels. By inhibiting

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sodium reabsorption, triamterene also inhibits potassium excretion. [6,32]

Pharmacodynamics:

Triamterene, a relatively weak, potassium-sparing diuretic and antihypertensive, is used in the management of hypokalemia. Triamterene is similar in action to amiloride but, unlike amiloride, increases the urinary excretion of magnesium.

Absorption: Rapidly absorbed, with somewhat less than 50% of the oral dose reaching the urine.

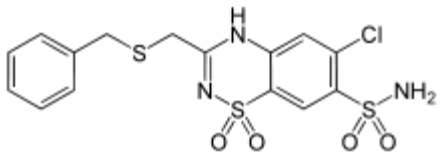
Protein binding: 55-67%

Indication: For the treatment of edema associated with congestive heart failure, cirrhosis of the liver, and the nephrotic syndrome; also in steroid-induced edema, idiopathic edema, and edema due to secondary hyperaldosteronism. [26]

1.2.2. Benzthiazide:

Benzthiazide is used to treat hypertension and edema. Like other thiazides, benzthiazidepromotes water loss from the body (diuretics). They inhibit Na+/Cl- reabsorption from the distal convoluted tubules in the kidneys. Thiazides also cause loss of potassium and an increase in serum uric acid. Thiazides are often used to treat hypertension, but their hypotensive effects are not necessarily due to their diuretic activity. Thiazides have been shown to prevent hypertension-related morbidity and mortality although the mechanism is not fully understood. Thiazides cause vasodilation by activating calcium-activated potassium channels (large conductance) in vascular smooth muscles and inhibiting various carbonic anhydrases in vascular tissue. [32]

Structure:



IUPAC NAME:

3-[(benzylsulfanyl) methyl]-6-chloro-1,1-dioxo-4H-1λ^6,2,4-benzothiadiazine-7-sulfonamide

CATEGORIES:

- Amides
- Heterocyclic Compounds
- Heterocyclic Compounds, 2-Ring
- Inorganic Chemicals
- Organic Chemicals
- Stimulants

CHEMICALFORMULA: $C_{15}H_{14}ClN_3O_4S_3$

MOLECULAR WEIGHT: 431.937 [8]

MECHANISM OF ACTION:

As a diuretic, benzthiazide inhibits active chloride reabsorption at the early distal tubule via the Na-Cl cotransporter, resulting in an increase in the excretion of sodium, chloride, and water. Thiazides like benzthiazide also inhibit sodium ion transport across the renal tubular epithelium through binding to the thiazide sensitive sodium-chloride transporter. This results in an increase in potassium excretion via the sodium-potassium exchange mechanism. The antihypertensive mechanism of benzthiazide is less well understood although it may be mediated through its action on carbonic anhydrases in the smooth muscle or through its action on the large-conductance calcium-activated potassium (KCa) channel, also found in the smooth muscle.

ABSORPTION:

Absorbed in the digestive tract.

INDICATION:

For the treatment of high blood pressure and management of edema.[10,32]

1.3. IMPORTANCE OF ANALYTICAL METHODS:

Quality is important in every product or service, but it is vital in medicine as it involves life. Unlike other consumer goods, there can be and there is no second quality. Therefore analytical methods which are a measure of quality of the drugs play a very comprehensive role in drug development and follow up activities, to assure that a drug product meets the established standard, is a stable and will continue to meet purported quality throughout its shelf life.

These methods should be selective and sensitive to monitor the known and unknown impurities, have to be written in a format such that they can be produced over a period of time and from laboratory to laboratory, i.e. these methods should be validated.[5,7]

1.4.INTRODUCTION TO ANALYTICAL METHODS:

Analytical methods are required to characterize drug substance and drug product composition during all phases of pharmaceutical development. Early phase methods must support changes in synthetic routes and dosage form and elucidate the structures and levels of impurities. In later phases, goals change to the development of rapid and robust methods for release and stability evaluation.

Analysis includes a wide range of simple and instrumental analytical methods, but the most widely most used analytical methods for quality assurance are spectroscopy and chromatography

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based. Most quantitative analysis requires, measuring specified components in the presence of sample matrix and /or related substances, therefore isolation or separation of the components are required preceding quantitative analysis. In such cases chromatographic techniques are used for quantitative analysis. In cases where matrix interference is not observed quantitative measurements are made using spectroscopic or titration methods directly.[7]

Chromatographic techniques:

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases (mobile phase and stationary phase).In chromatographic methods, separation is based on variation in the distribution of different Compounds between two dissimilar phases -a stationary phase and a mobile phase. .

Table 1.1: Types of Chromatographic techniques

Type	Stationary phase	Mobile Phase	Technique
Solid-Liqud	Solid	Liqud	Adsorption column Chromatography, Thin layer Chromatography (TLC), High performance liquid chromatography (HPLC) and Ion exchange chromatography
Solid-Gas	Solid	Gas	Gas chromatography(GC)
Liquid-Liquid	Liquid	Liquid	Partition chromatography, Paper chromatography and Thin layer Chromatography (TLC)
Liquid-Gas	Liquid	Gas	Gas chromatography(GC)

High Performance Liquid Chromatography (HPLC):

Introduction

High-performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems. In liquid chromatography, a mixture of molecules dissolved in a solution (mobile phase) is separated into its constituent parts by passing through a column of tightly packed solid particles (stationary phase). The separation occurs because each component in the mixture interacts differently with the stationary phase. Molecules that interact strongly with the stationary phase will move slowly through the column, while the molecules that interact less strongly will move rapidly through the column. This differential rate of migration facilitates the separation of the molecules.

Principle:

The principle of separation in normal mode and reverse mode is adsorption. When mixture of components is introduced into the HPLC column, they travel according to their affinities towards the stationary phase. The component which has more affinity towards adsorbent it elutes slower.

The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.

Table.1.2: Types of HPLC techniques

Based on modes of chromatography	Normal Phase Chromatography Reverse phase chromatography
Based on principle of separation	Adsorption chromatography Partition chromatography

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	Ion exchange chromatography Size exclusion chromatography Ion pair chromatography Chiral phase chromatography
Based on elution technique	Isocratic, Gradient
Based on scale of operation	Analytical, Preparative
Based on types of analysis	Qualitative, Quantitative

1.5 REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

About 75% of current HPLC analysis is performed using the reverse phase. In reversed phase chromatography the stationary phase is mainly silica chemically bonded through a siloxane (Si-o-Si-C) linkage to a low polar function group. These phases are prepared by treating the surface silanol groups of silica with an organochlorosilane reagent. The polarity of the column can be changed by varying the alkyl chain length in R.

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase. Elution can proceed either by isocratic conditions where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity.

Thus reversed phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. With these stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily.

Retention time can be increased by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent.[1,11]

Advantages of HPLC

- It offers high separation efficiency and speed of analysis.
- High degree of selectivity for specific analysis of components due to availability of wide variety of solvents and column packing.
- It requires less amount of sample and allows continuous monitoring of the column effluent.
- It allows complete separation of components from the mixture for further analysis and characterization.
- The results obtained are precise, accurate and reproducible.

Disadvantages of HPLC

- Maintenance cost of the equipment is high.
- Preparation of the sample is laborious and time consuming.
- Pretreatment procedures like degassing and filtration are required for solvents.
- Skilled and well trained personnel are required for carrying out the procedure.
- Large amounts of solvent (mobile phase) is required for separation.

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Instrumentation of HPLC

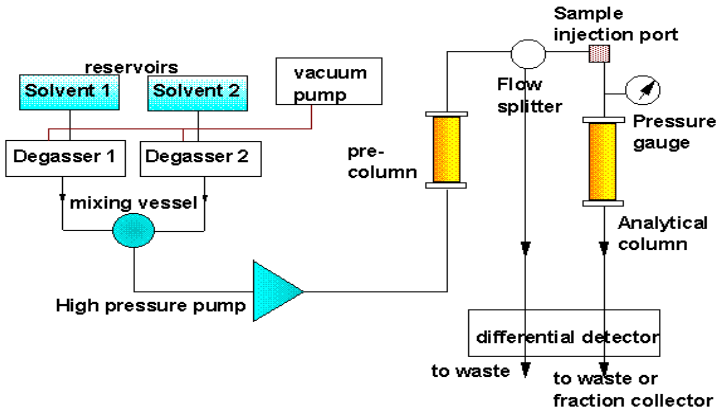

Figure 1.1: Diagrammatic representation of HPLC instrumentation

i. A solvent reservoir and mixing system
ii. A solvent degassing system
iii. A pump to move the eluent and sample through the system
iv. An injection device to allow sample introduction
v. A column(s) to provide solute separation
vi. A detector to visualize the separated components[30,34]

Chromatographic Separation Mechanisms

HPLC separations involve both the mobile phase (a liquid) and the stationary phase (usually materials of varying hydrophobicity chemically bonded to a solid support). In contrast, GC uses the mobile phase only to carry the analyte through the column. As an illustration, the amount of water in an HPLC mobile phase will determine how strongly a hydrophobic analyte is repelled into the stationary phase, and how well it is retained. The chemical nature of the stationary phase will also govern how strongly the analyte is retained. For this reason, HPLC is a technique that is driven by the 'selectivity' achieved using two interacting phases.

The Liquid Chromatograph

In HPLC, several instrument and column chemistry parameters need to be optimized in order to generate a satisfactory separation.

Each of the following parameters need to be optimized in order to generate a chromatogram that is suitable for qualitative or quantitative purposes.

Mobile phase compositionλ
Bonded phase chemistryλ
Column and packing dimensionsλ
Injection volumeλ
Sample pre-treatment and concentrationλ
Mobile phase flow rateλ
Column temperatureλ
Detector parametersλ

Where:

1: The mobile phase composition (usually water and an organic solvent plus other additives) needs to be optimized in order to gain good separation.

2: Degassers are often used to remove air from the mobile phase, leading to better chromatographic baselines.

3: The detector conditions are chosen to give the best response to the analytes of interest and to achieve good sensitivity.

4: The column dimensions and stationary phase chemistry are chosen and optimized to give separations of the quality required.

5: The autosampler introduces a plug of sample into the mobile phase flow which is then swept onto the column.

6: Dual reciprocating pumps are used to deliver the mobile phase at back pressures of up to 400 bar. A steady stream of liquid delivered at a constant flow rate is important.

The Liquid Chromatographic Process

The mobile phase is continuously pumped at a fixed flow rate through the system and mixed (if required) by the pump. The injector is used to introduce a plug of sample into the mobile phase without having to stop the mobile phase flow, and without introducing air into the system.

The mixture of components is carried in a narrow band to the top of the column. Some compounds in the sample mixture will have a greater preference for the stationary phase than the mobile phase and will be retained in the column longer. Those components that are not retained as strongly are carried by the mobile phase down the column. The longer the column, the more opportunities for

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interaction with the stationary phase, and the greater the separation.

The detector is then used to respond to a physicochemical property of the analyte. This response is digitally amplified and sent to a data system where it is recorded as the 'chromatogram'.

The Chromatogram:

As the components elute from the column they pass into a detector – where some physicochemical property of the analyte produces a response from the detector. This response is amplified and plotted against time – giving rise to a 'chromatogram'.

Components (such as the injection solvent) that are not retained within the column elute at the 'dead time' or 'hold up time' t_0.

Modes of Chromatography

The 'mode' of chromatography is usually defined by a combination of a certain stationary phase type with a certain mobile phase type. For example, 'Normal Phase' chromatography has a non-polar mobile phase with a more polar stationary phase. Reversed phase HPLC uses a system in which the mobile phase is more polar than the stationary phase.[11]

Applications of HPLC in pharmaceutical research[2]

Separation: The extent or degree of separation is determined by the choice of stationary phase and mobile phase along with parameters like flow, temperature and gradient programme.

Identification: For this purpose a clean peak of known sample has to be observed from the chromatogram. Selection of column mobile phase and flow rate matter to certain level in this process. Identification is generally done by comparing with reference compound based on retention time and also based on UV-Vis spectra in some cases.

Quantification: Analyte concentrations are estimated by measuring the responses (peak areas) known reference standards followed by unknown samples. Quantification of known and unknown components are done by various methods like - area normalization method, internal standard method, external standard method and diluted standard method along with relative response factors.

Isolation: It refers to the process of isolation and purification of compounds using analytical scale or preparative scale HPLC. Volatile buffers and solvents are preferred choice as mobile phases as it reduces the effort on purification. Solute purity and throughput is the key challenge in isolation and purification processes.

1.6. Introduction to Analytical Method Development

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of newtoxicities , development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs. Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B). [13,14]

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines.[21]

1.6.1 Need for the Development of a New Method

Several reasons are available for the development of a new method of analysis.

There may not be a suitable method for a particular analyte in the specific sample matrix.

- Existing methods may be too erroneous, artefact and/or contamination prone, or they may be unreliable (having poor accuracy or precision).
- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for

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improved methods, including analyte identification or detection limits, greater accuracy or precision, or better return on investment.

- There may be a need for alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods. [24]

1.6.2 Method Development Using HPLC

In method development, an attempt to select the best chromatographic conditions like the best column, the best mobile phase, the detection wavelength etc. to be used for routine analysis of any drug is done. For the method development by HPLC method some information about the sample is very essential i.e. number of components present in the sample, pKa values of different components, UV-Visible Spectra of each analyte, solubility in different solvents, concentration range of each component, nature of sample etc. Prior to method development there must be some technical information i.e. chromatography method selection according to the sample properties, the sample when analyzed with HPLC, the condition where all compounds elute in a reasonable time, optimization of HPLC method with regard to analysis time, resolution, selectivity and sensitivity. [21,24]

1.6.3 Analyte Standard Characterization

All the information about the analyte is gathered regarding the structure, physical and chemical properties, toxicity, purity, hygroscopicity, solubility and stability. The availability of the 100% pure standard analyte is determined along with its storage and disposal information. If multiple components are to be analyzed in a sample matrix, the number of components to be analyzed is noted and the availability of the standard for each component is checked.

1.6.4. Choosing a Suitable Method

Using the available literature and previous methodology, the methods are adapted and modified. Sample preparation and instrument conditions are adopted to make use of the latest methods and instrumentation.

If no previous methods exist for the analyte in the literature, work from analogy to investigate compounds that are similar in structure and properties. Usually a compound with analytical method exists that is similar to the analyte of interest.

1.6.5 Optimization

a) Choice of method

The most commonly used chromatographic methods are normal phase chromatography, reverse phase chromatography, reverse phase ion-pair chromatography and ion-exchange chromatography. In the selection of suitable chromatographic method for organic compounds, first reversed phase should be tried, if not successful, normal phase should be tried, then reverse phase ion-pair chromatography should be tried, ion-exchange chromatography at the end.

b) Choice of Mobile Phase

In reversed phase chromatography the selection of mobile phase is very important for the analysis of the drug. We can use acetonitrile frequently as it is suitable for the entire UV range, methanol and Isopropanol are not suitable below wavelength of 210nm, acetic acid is suitable above a wavelength of 240 nm, for the preparation of buffers, both $K_2HPO_4$and KH_2PO_4can be used in entire UV range, freshly distilled THF is suitable for HPLC above a wave length of 240nm, TEA is suitable above 240 nm, ammonium acetate can be used above 215 nm, EDTA can be used in entire UV range, sodium phosphate is suitable above 210 nm.

Changing the polarity of the mobile phase can alter the elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in un-dissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase in 5% steps of the organic phase concentrations is needed.

When separating acid or bases, buffered mobile phase is required to maintain consistency in retention time and selectivity. Buffered salts reduce peak tailing for basiccompounds by effectively masking silanol groups and also reduce potential ion-exchangeinteractions with a protonated silanol groups. As potassium is a stronger counter ion thansodium, it provides improved results compared to sodium (Na^+). Potassium phosphateis used for preparation of buffers of various pH. If band tailing is observed for basicamphoteric compounds few drops of diluted triethylamine or ammonium

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acetate is added,for acidic or amphoteric compounds, few drops of diluted triethylamine or ammoniumacetate is tried. For neutralcompounds, the aqueous eluent used in method development is water, for weak to medium acidic compounds, 100 mM H_3PO_4 buffer of pH 2.3, for weak to medium basic or acidic compounds in ionized form 100mM H_3PO_4 buffer of pH 4.0, 50 mM H_3PO_4buffer of pH 7.5 are used. Unknown sample should be analyzed first with water, then with an acidic and a neutral buffer.

c) Choice of Column Columns being the heart of HPLC for optimum separation, Stable, high performance column with good selectivity, efficiency is essential requirement for rugged and reproducible method. These characteristics are dependent on the columns manufacturer's production of good quality columns and packing materials.

Column length

- Longer columns are chosen for increased resolution.
- Shorter columns are chosen for shorter analysis time, lower back pressure, fast equilibration and less solvent consumption.

Column internal diameter

- Wider diameter columns are chosen for greater sample loading.
- Narrow columns are chosen for more sensitivity and reduced mobile phase consumption.

Particle shape

- Columns with spherical particle shapes are preferred when lower back pressure column stability and greater efficiency is required.
- Columns with irregular particle shapes are preferred when large surface area and high capacity is required.

Particle size

- Columns with small particle size of 3 - 4 µm are preferred for complex mixtures with similar components. Combination of a short column (10- 50 mm) with small particle size is used for fast, high resolution separations.
- Columns with larger particle size of 5 – 10 µm are preferred for structurally different compounds.
- Columns with large particle of 15 – 20 µm are used for preparative separations.

Surface area

- Columns with high surface area packing are selected for more capacity, greater resolution and longer retention.
- Columns with low surface area packing are selected for quicker equilibration time.

Carbon load

- Columns with high carbon load are chosen for greater column capacities and resolution.
- Columns with low carbon load for faster analysis time.

End capping

- Columns with end capped packing are selected to eliminate unpredictable secondary interactions with base material
- Columns with non-end capped packing are selected for selectivity differences for polar compounds by controlling secondary interactions. [11,24]

d) Choice of Detector

Detectors are eyes of the liquid chromatography system and measure the compounds after their separation on the column. Selected detector should be capable of responding to change in concentrations of all the components in the sample with adequate sensitivity even to measure trace substances. The detectors must have certain characteristics i.e. high sensitivity, higher linear dynamic range, application to most of the solutes, does not contribute to band broadening, non-destructive, faster response.[28]

1.6.6. Further Optimization

After the selection of a suitable method, mobile phase, column and detector, further optimization can be done to obtain a well developed method.

For shorter analysis time

- Change to isocratic method. The suitable mobile phase composition is estimated from the gradient run.
- Use of shorter column, if proper resolution is obtained.

For better resolution

- Use of longer column.
- Use of stationary phase with smaller particles (3 – 4 µm).

For better selectivity and sensitivity

- Other stationary phases e.g. phenyl, CN etc.
- pH control with ion-forming compounds
- Use of methanol or THF instead of acetonitrile.
- Detection at the absorption maximum of the substance
- All factors which leads to narrower and higher peaks as gradient elution, smaller particle, micro bore columns. [24]

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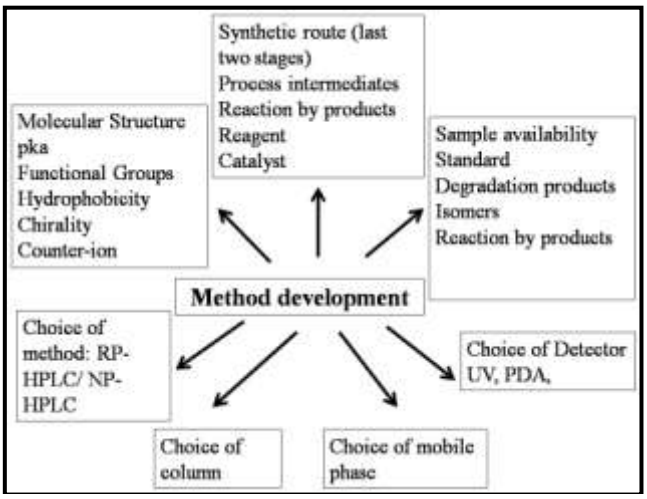

Fig.1.2: Outlineof the process involved in method development

1.6.7. Parameters Affecting Changes in Chromatograph

The various parameters affecting the changes in chromatographic conditions are

1. Flow rate
2. Temperature
3. pH
4. Ion pair reagent
5. Column efficiency
6. Capacity factor
7. Resolution
8. Retention time
9. Peak asymmetry

1.6.7.1 Effect of Flow Rate The efficiency of a HPLC column varies with flow rate. It is sometimes useful and readily utilized to increase the resolution. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation (less band broadening). However, the maximum flow rate is limited because a finite time is required for analyte to equilibrate between stationary phase and mobile phase. A slower flow rate will decrease the column back pressure and a corresponding increase in the run time is observed.

Table 1.3: Flow rates for column with different internal diameter.

Internal diameter of column (mm)	Standard flow rate (µl/ min)
4.6	1000
2.1	200
1.0	50
0.30	4

1.6.7.2 Effect of Temperature

Elevated temperatures decrease viscosity and increase solubility and diffusivity. Retention, peak shape, column efficiency, and total analysis time are affected by temperature because both the thermodynamics and kinetics of adsorption processes are functions of temperature. Additionally, temperature control results in improved reproducibility. In liquid chromatography temperature is used in the optimization of a separation. At higher temperature, peaks will be sharper and elute earlier. System pressure is affected by temperature. The viscosity of the mobile phase decreases with increasing temperature.

1.6.7.3 Role of pH

For some preparations the effect of changing pH is minimal. However for acids and bases, a small change in pH is significant. Changing the pH changes the degree of ionization of molecules in solution, affecting polarity of the solution thereby changing the retention times in an HPLC separation. In a sample mixture, the retention time of the components of the mixture are also changed to different extents. Hence it affects

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the degree of selectivity, where the peaks become further apart or at a particular pH they may co-elute and then the peak elution order will change. Selection of a proper buffer pH is necessary to reproducibly separate ionizable compounds by RP-HPLC. Selection of an improper pH for ionizableanalytes leads to asymmetric peaks that are broad, tall or split.

$$pH = pKa + \log([A^-]/[HA])$$

During the selection of a buffer, pKa of the analyte should be considered. A buffer with 2 pH units above or below pKa of the analyte is recommended for a good peak shape. From Henderson- Hasselback equation,

Itcan be determined that 99% of the analyte is in a single form, Good peak shape is possible only when ananalyte is in a single form. [11.28]

1.6.7.4 Role of Ion-Pair Reagent

Most ofthese compounds are ionic or polar; hence the use of reversedphase-high performance liquid chromatography (RP-HPLC) is somewhat restricted. Initially when deciding to select RP-HPLC or RP-HPLC with ion-pairing, the nature of the analyte of interest is considered. If the sample is neutral, RP-HPLC is used first; and if the sample is ionic, RP-HPLC with ion pairing is used. Thus RP-HPLC and RP-HPLC with ion pairing are similar except that the latter consists of an ion-pair reagent in the mobile phase to improve the selectively of ionic samples. The ionic pair reagents are large ionic molecules having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ioncombines with the ions of the eluent, becoming ion pairs in the stationary phase. This results in different retention, thus facilitating separation of analytes.

1.6.7.5 Column Efficiency (N)

The efficiency of a chromatographic column is given terms of number of theoretical plates (plate number), N

$$N = 16\left(\frac{t_r}{w}\right)^2$$

Where, t_r- retention time measured from the time of injection, w- peak width peak width obtained by drawing tangents to the sides of the Gaussian curve at the inflection points and extrapolating the tangents to intercept the baseline as in fig.1.3.

Efficiency of the column is also expressed as height equivalent to theoretical plate, (or plate height) HETP (or h)

$$h = \frac{L}{N}$$

Where, L- length of thecolumn,
N- number of theoretical plates.

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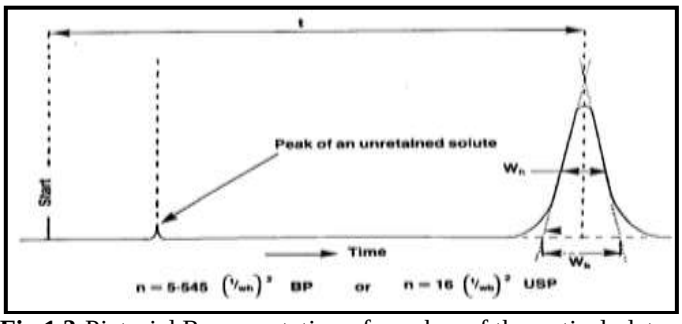

Fig.1.3: Pictorial Representation of number of theoretical plates.

1.6.7.6 Capacity Factor (k')

It is measure of the position of a sample peak in the chromatogram, being specific for a given compound, a parameter which specifies the extent of delay of substance to be separated.

$$k' = \frac{t_r - t_m}{t_m}$$

Where, t_r– retention time of the solute,t_m – retention time of the unretained compound by the column packing.

Fig 1.4 shows capacity factor of a solute.

k' depends at stationary phase, mobile phase, temperature and quality of column packing. For good chromatographic performance with isocratic separation, k' value should be in the range of 1-10. If k' < 1.0, the bands are inadequatelyseparated from excessively unretained material, if k' > 10 separation takes too long andbands broadened, if k' > 30, satisfactory isocratic separation usingpresent column and mobile phase is not obtained and gradient elution should be tried.

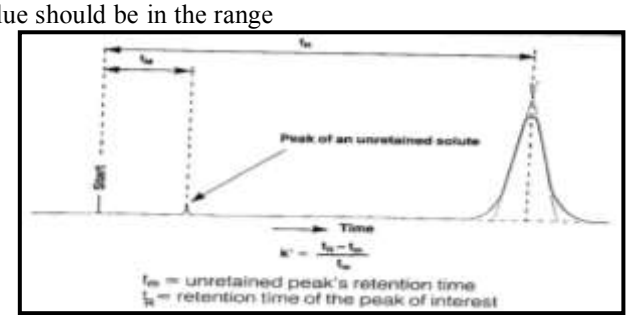

Fig.1.4: Pictorial representation of capacity factor.

1.6.7.7 Resolution(R_s)

The ability of the column to separate two solutes. In a chromatogram it is the distance of separation of two peaks.

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w_1 - w_2)}$$

Where,t_{r1},t_{r2}– retention time of two immediately adjacent peaks,
w_1, w_2 – peak widths of two immediately adjacent peaks as shown in fig1.5

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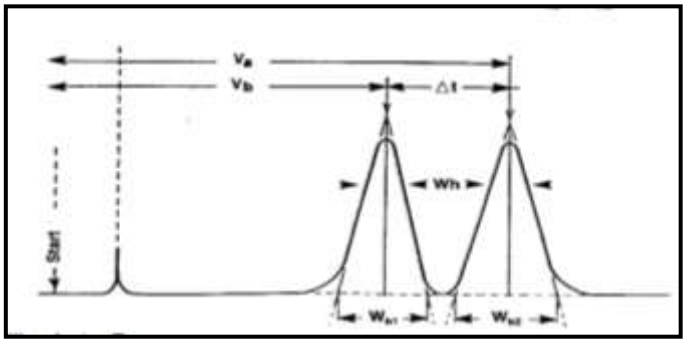

Fig1.5: Pictorial Representation of Resolution.

1.6.7.8 Selectivity factor (α) / Relative Retention

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks separation depends on the components interaction with the stationary phase.

$$\alpha = \frac{k'_b}{k'_a}$$

Where,

k'_a, k'_b– capacity factors of peak a and peak b respectively.

The value of the separation factor is always greater than unity. The separation factor is also identical to the ratio of the corresponding distribution constants. If the capacity factor is used, the separation factor should be consistent for a given column, mobile phase composition and specified temperature, regardless of the instrument used.

Fig 1.6.shows a pictorial representation of measurement of selectivity factor

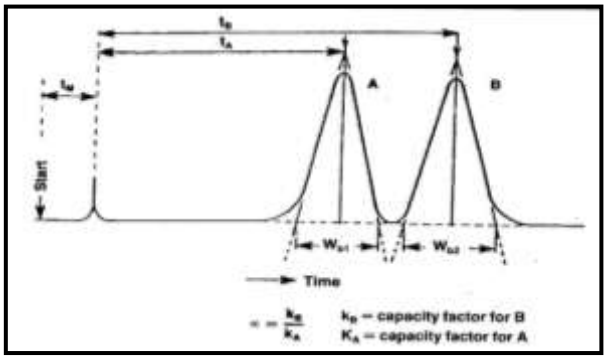

Fig 1.6: Pictorial representation of selectivity factor.

1.6.7.9 Peak Asymmetry (A_s)

It is also known as tailing factor. Theasymmetry factor for a peak can be calculated using the following formula.

$$A_s = \frac{W_{0.05}}{2f}$$

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Where, $W_{0.05}$– peak width at 5% height from the base line,
f – distance between maximum and leading edge of the peak as in Fig.1.7
It is also calculated from

$$A_s = \frac{b}{a}$$

Where, b - distance from the point at peak maxima to the trailing edge,
a – distance from the leading edge of the peak to the peak maxima (both measured at 10% height of the baseline) as shown in Fig.1.8

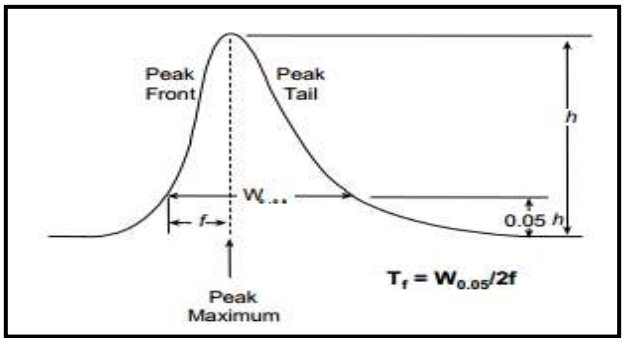

Fig.1.7: Pictorial representation ofpeak asymmetry.

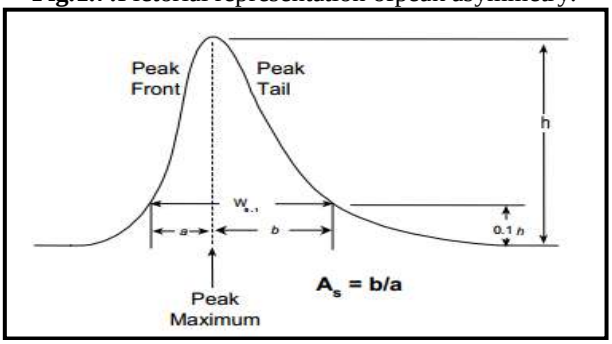

Fig.1.8: Pictorial representation ofpeak asymmetry.

1.7. Introduction to Method Validation

Validation is an integral part of quality assurance; it involves the systematic study of systems, facilities and processes aimed at determining whether they perform their intended functions adequately and consistently as specified. Validation in itself does not improve processes but confirms that the processes have been properly developed and are under control. [29]

Method validation is defined as the process of proving that an analytical method is acceptable for its intended use. To ensure compliance with quality and safety standards, the United States, Europe, Japan, and other countries have published compendia, or pharmacopeias, that describe official test methods for many marketed drug products. For example, analytical methods found in UnitedStates Pharmacopeia (USP) are legally recognized analytical procedures under section 501 (b) of the Federal Food, Drug, and Cosmetic Act. For these compendia methods, USP provides regulatory guidance for method validation. In addition, validation of analytical methods is covered by the United States Code of Federal Regulations (CFR). A great deal of effort has been devoted to the harmonization of pharmaceutical regulatory requirements in the United States, Europe, and Japan. As part of this initiative, the International Conference on Harmonization (ICH) has issued guidelines for

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analytical method validation. The recent FDA methods validation draft guidance documents as well as U.S. both refer to ICH guidelines.[14]

The required validation parameters, also termed analytical performancecharacteristics or analytical figs of merit. Methods should be validated or revalidated

- Before their introduction and routine use;
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.
- Wherever the method is changed and the change is outside the original scope of the method.

The validation of analytical procedures is directed to the four most common types of analytical procedures: Identification tests; Quantitative tests for impurities' content; Limit tests for the control of impurities; Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

1.7.1. Method Validation (ICH Guidelines) [11.29]

1. Accuracy,
2. Precision,
   - Repeatability,
   - Intermediate precision.
3. Specificity / Selectivity,
4. Limit of Detection,
5. Limit of Quantitation,
6. Linearity,
7. Range,
8. Robustness,
9. System Suitability.

The validation terminology in ICH guidelines differ from the validation given in USP with two exceptions. Ruggedness is not included in ICH guideline and treats system suitability as a part of method validation, whereas the USP considers it in a separate chapter.

1.7.1.1 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy should be established across the specified range of the analytical procedure. Accuracy is measured as the percentage of the analyte recovered by assay, spiking samples in a blind study.

Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guidelines for Submitting Samples and Analytical Data for Methods Validation[11,25]

1.7.1.2. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

The precisionn of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

1.7.1.3. Specificity

Specificity is the ability to assess accurately the analyte in the presence of components which may be expected to be present in the sample matrix. Typically these might include impurities, degradants, matrix, etc. it is a measure of the degree of interference from such other things such as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to a single component only. [27,29]

1.7.1.4. Limit of Detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. It is a limit test that specifies whether or not an analyte is above or below a certain value.

1.7.1.5. Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

1.7.1.6. Linearity and Range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line.

Range is the (inclusive) interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method.

1.7.1.7. Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated varying method parameters such as percent organic solvent, pH, ionic strength, or

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temperature and determining the effect (if any) on the results of the method.

1.7.1.8. System Suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as a whole.

System suitability is the checking of a system to ensure system performance before or drying the analysis unknowns. Parameters such as plate count, tailing factor, resolution and reproducibility (% RSD retention time and area for repetitive injections) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability sample that is a mixture of main components and expected by-products. [29]

Table 1.4: Acceptance Limits for System Suitability Test.[29,30]

Parameter	Limit
Capacity Factor	k'> 2
Injection precision	RSD < 1% for n ≥ 5
Resolution	R_s> 2
Tailing factor	$A_s \leq 2$
Theoretical plates	N> 2000

1.8Analytical Method Development for Pharmaceutical Formulations

Quality investigation plays a very important role in quality specification establishment of chemical drugs. The number of drugs introduced into the market every year .very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. Hence, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs. [28]

Basic criteria for new method development of drug analysis:

The drug or drug combination may not be official in any pharmacopoeias.

A proper analytical procedure for the drug may not be available in the literature due to patent regulations.

- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.Analytical method development provides the support to track the quality of the product from batch to batch.

Method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.[1,25,28]

Single dosage forms with combination of drugs are widely used today due to their advantages and their simultaneous estimation of individual component is a challenging task.

1.9. AIM AND OBJECTIVE

Aim of the work:-

To develop and validate new RP-HPLC method for the determination of Triamterene and Benzthiazide in pharmaceutical dosage form.

OBJECTIVE:-

To develop a method using RP-HPLC for the drugs determination in pharmaceutical dosage form

To validate the drug using different solvents, retention time and other parameters.

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Validate the developed method as per ICH guidelines.

II. CHAPTER 2. REVIEW OF LITERATURE

2.1 Literature Review of Analytical Methods Available For Triamterene and Benzthiazide

Shah .VN et al.,(2014)[29]

A specific, accurate, precise and reproducible RP-HPLC method has been developed and subsequently validated for the simultaneous determination of Triamterene and Benzthiazide in tablets. The proposed HPLC method utilizes BDS hypersil (Thermo scientific) C_{18} column (250 mm × 4.6 mm id, 5 µm particle size), and mobile phase consisting of phosphate buffer: methanol (70:30) and pH adjusted to 3.5 with sodium hydroxide and flow rate of 1.0 ml/min. Quantitation was achieved with UV detection at 245 nm based on peak area with linear calibration curves at concentration ranges 10-30 µg/ml for Triamterene and 5-15 µg/ml for Benzthiazide. The retention time of Triamterene and Benzthiazide were found to be 5.960 min and 3.493 min respectively.

The method was validated in terms of accuracy, precision, linearity, limit of detection, limits of quantitation and robustness. This method has been successively applied to tablet formulation and no interference from the formulation excipients was found.

AvinashRaskar et al.,(2015)[3]

RP-HPLC method has been developed and subsequently validated for the simultaneous determination of Triamterene and Benzthiazide in tablets. The proposed HPLC method utilizes BDS hypersil (Thermo scientific) C_{18} column (250 mm × 4.6 mm id, 5 µm particle size), and mobile phase consisting of acetonitrile and sodium dihydrogen phosphate buffer methanol (80:20)v/v and pH adjusted to 5.5 with sodium hydroxide and flow rate of 1.0 ml/min. Quantitation was achieved with UV detection at 245 nm based on peak area with linear calibration curves at concentration ranges 10-30 µg/ml for Triamterene and 5-15 µg/ml for Benzthiazide. The retention time of Triamterenewas found to be 9.8min and 3.493 min respectively. The method was validated in terms of accuracy, precision, linearity, limits of detection, limits of quantitation and robustness.

Asma Begum et al.,(2016)[4]

A specific, accurate, precise and reproducible RP-HPLC method has been developed and subsequently validated for the simultaneous determination of Triamterene and Benzthiazide in tablets. The proposed HPLC method utilizes BDS hypersil (Thermo scientific) C_{18} column (250 mm × 4.6 mm id, 5 µm particle size), UV detection at 245 nm based on peak area with linear calibration curves at concentration ranges 10-30 µg/ml for Triamterene and 5-15 µg/ml for Benzthiazide.The proposed HPLC method was applied for the determination of diuretics and validated as per ICH guidelines. the accuracy, reproducibility and high sensitivity of the proposed method is suitable for analytical method of diuretic agents in various pharmaceutical and biological samples with help of RP-HPLC.

Satish kumar. .A. Shetty et al.(2012)[27]

The proposed method involves zero order derivative and First order derivative spectroscopy method. A novel, simple and rapid UV Spectrophotometric determination method for Simultaneous estimation of Triamterene and Benzthiazide was successfully developed and validated in bulk and pharmaceutical formulation. First method is zero order derivative method where the solutions were scanned in the range from 400-200 nm and the peaks were observed at a λmax of 363 and 283nm for Triamterene and Benzthiazide respectively. Second method First order derivative spectroscopy method, which involved the measurement of absorbances of one drug at zero crossing point of other drug. 385 nm and 308 nm were selected for the estimation of Triamterene and Benzthiazide respectively in bulk drug and formulation. Both the methods showed linearity from 6 – 30 µg/ml and 3 -15 µg/ml for Triamterene and Benzthiazide respectively. Recovery studies showed that the method is accurate. Precision of the proposed methods were found to be within the acceptable limits. Thus the two proposed methods and results were validated according to ICH guidelines. So, the methods can be applied for routine analysis in bulk and pharmaceutical formulation.

Rachitajain et al.,(2017) [23]

The RP-HPLC method describes developed simple, rapid, specific, selective, accurate and precise method for the simultaneous estimation of Benzthiazide and Triamterene in tablet form. Benzthiazide falls under the category of Thiazide Diuretic and Antihypertensive RP-HPLC method for Benzthiazide and Triamterene were developed using mobile phase buffer (pH3.5):

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methanol (70:30v/v), flow rate 1mL/min, injection volume 20µL, temperature 25 ºC detection wavelength 222nm. Linearity range was found to be 5-15µg/ml for Benzthiazide and 10-30µg/ml for Triamterene. LOD and LOQ values were found to be 1.513 and 4.587 µg/ml for Benzthiazide and 0.695 and 2.108 µg/ml for Triamterene respectively. The assay result found to be 99.75 % and 99.97 % for Benzthiazide and triamterene respectively. The developed method was validated according to ICH guidelines.

RESEARCH ENVISAGED :The available literature is searched for all types of information related to the analyte. Availability of information regarding the synthesis, physical and chemical properties, solubility or relevant analytical methods is determined. Books, periodicals, regulatory agency compendia, such as IP, USP/NF, BP etc. Chemical Abstracts Service (CAS) automated/ computerized literature searches are also used for literature purpose.

- Literature survey reported that till now there are very few RP-HPLC methods for analysis of Triamterene and Benzthiazide in dosageform
- Hence an attempt has been made to develop a new method which is with more precise and accurate, increased sensitivity, linearity by RP-HPLC method.

2.2. PLAN OF WORK

1. Solubility determination of TriamtereneandBenzthiazide invarious solvents and buffers.
2. Analytical method development for assay of TriamtereneandBenzthiazide in pharmaceutical dosage form

A. Preparation of solutions
B. Determine the absorption maxima of both the drugs in UV–Visible region in different solvents/buffers.
C. Selection of chromatographic conditions
D. Selection of stationary phase and mobile phase
E. Optimization of the chromatographic variablesfor proper resolution and retention times.

3. System suitability studies.
4. Validation of developed RP-HPLC method as per ICH guidelines.

III. CHAPTER 3 MATERIALS AND METHODS

3.1 INSTRUMENTS AND CHEMICALS

Table 3.1: Instruments used

UV-Visible Spectrophotometer	Nicolet evolution 100
UV-Visible Spectrophotometer software	Vision Pro
HPLC software	Spin chrome (LC SOLUTIONS)
HPLC	Shimadzu(LC 20 AT VP)
Ultra sonicator	Citizen, Digital Ultrasonic Cleaner
pH meter	Global digital
Electronic balance	Shimadzu
Syringe	Hamilton
HPLC Column	Inertsil ODS 3V(250x4.6mm) 5µm

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Table 3.2: Reagents used

Water	HPLC Grade
Methanol	HPLC Grade
Acetonitrile	HPLC Grade
Triethyl amine	AR Grade
Orthophosphoric acid	AR Grade

Table3.3: Drugs used

Triamterene and Benzthiazide drugs	Gift Samples obtained from Lee Pharma labs, Hyd.
Triamterene and Benzthiazide **50mg and 25mg**	Obtained from local pharmacy

Marketed Dosage Form

Brand Name : Ditide
Active Ingredients : BENZTHIAZIDE
Manufacturer : GlaxoSmithKline
Country of Origin : India
Intended Patient : Unisex

Fig.: Marketed formulation of Ditide (Benzthiazide/Triamterene) - 25mg/50mg (10 Tablets)

3.2.ANALYTICAL METHOD DEVELOPMENT FOR ASSAY OF Triamtereneandbenzthiazide

3.2.1. Solubility Studies: These studies are carried out at 25 ^{0}C

Triamterene: Freely soluble in ethanoland methanol,and slightly soluble in acetone and isopropanol and very slightly soluble in water.

Benzthiazide: Freely soluble in methanol and water.

3.2.2. Determination ofWorking Wavelength (λmax)

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.

3.2.3 Mobile Phase:

A mixture of Triethylamine:ACN were prepared. The mobile phase was sonicated for 10min to remove gases and filtered through 0.45µ membrane filter for degassing of mobile phase.

3.2.4. Preparation of buffer:

5ml of triethylamine is pipetted out and dissolved in 1000ml of water. Adjust the pH to 3.5 with orthophosphoric acid(3ml in 10ml of water) drop wise.

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3.2.5. Preparation of standard stock solution of TRIAMTERENE

10 mg of TRIAMTERENE was weighed and transferred in to 100ml volumetric flask and dissolved in water and then make up to the mark with water and prepare 40µg /ml of solution by diluting 4ml to 10ml with water.

3.2.6. Preparation of standard stock solution of BENZTHIAZIDE

10mg of BENZTHIAZIDE was weighed in to 100ml volumetric flask and dissolved in water and then dilute up to the mark with water and prepare 30 µg /ml of solution by diluting 3ml to 10ml with water.

3.3.METHOD DEVELOPMENT OF TRIAMTERENE and BENZTHIAZIDE

Trial - 1

Chromatographic conditions

Mobile phase :20mM KH_2PO_4:ACNin 70:30(v/v)
pH : 4.0
Column : Inertsil ODS 3V (250mm×4.6mm× 5µm)
wavelength : 245 nm
Flow rate : 1ml/min

Preparation of mixed standard solution

weigh accurately 10mg of TRIAMTERENE and BENZTHIAZIDE in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase.From above stock solution 10 µg/ml of TRIAMTERENE and of BENZTHIAZIDE is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Trial- 2

Chromatographic conditions

Mobile phase : 20mM KH_2PO_4:ACN in 60:40 (v/v)
pH : 4.0
Column : Inertsil ODS 3V (250mm×4.6mm ×5µm)
wavelength : 245nm
Flow rate : 1ml/min

Preparation of mixed standard solution

weigh accurately 10mg of TRIAMTERENE and BENZTHIAZIDE in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10µg/ml of TRIAMTERENE andBENZTHIAZIDE is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Trial- 3:

Chromatographic conditions

Mobile phase : 20mM KH_2PO_4+ACN in55:45 (v/v)
pH : 3.5 adjusted with ortho phosphoric acid
Column : Inertsil ODS 3V, (250mm×4.6mm× 5µm)
Wavelength : 245nm
Flow rate : 1ml/min

Preparation of mixed standard solution

weigh accurately 10 mg of TRIAMTERENE and BENZTHIAZIDE in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of TRIAMTERENE andBENZTHIAZIDE is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Trial- 4:

Chromatographic conditions

Mobile phase : Ammonium acetate buffer + Acetonitrile in 30:70 (v/v)
pH : 6.0 adjusted with phosphoric acid
Column : InertsilODS , (250mm×4.6mm× 5µm)
Wavelength : 245nm
Flow rate : 1ml/min

Preparation of mixed standard solution: weigh accurately 10 mg of TRIAMTERENE andBENZTHIAZIDE in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase.From above stock solution 10 µg/ml of TRIAMTERENE and BENZTHIAZIDE is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram

Trial- 5:

Chromatographic conditions

Mobile phase : TEA+ACN in 60:40(v/v)
pH : 3.5 adjusted by using ortho phosphoric acid
Column : InertsilODS , (250mm×4.6mm× 5µm,)
Wavelength : 245 nm
Flow rate : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of TRIAMTERENE and BENZTHIAZIDE in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up

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the volume with mobile phase. From above stock solution 10µg/ml of TRIAMTERENE and BENZTHIAZIDE is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

3.4. Assay

3.4.1 Preparation of samples for Assay

Preparation of mixed standard solution: weigh accurately 10mg of Triamtereneand 10 mg of Benzthiazide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Triamtereneand Benzthiazideis prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample :10 tablets(each tablet contains Benzthiazide-25mgTriamterene -50 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of BENZTHIAZIDE and TRIAMTERENE (µg/ml) were prepared by dissolving weight equivalent to 10 mg of BENZTHIAZIDE and TRIAMTERENE and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 10ml with mobile phase. Further dilutions are epared in 5 replicates of 10µg/ml of BENZTHIAZIDE and TRIAMTERENE was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Calculation

The amount of BENZTHIAZIDE and TRIAMTERENE present in the formulation by using the formula given below, and results shown in above table:

$$\%\ \text{Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{\text{LC}} \times 100$$

Where,

AS: Average peak area due to standard preparation
AT: Peak area due to assay preparation
WS: Weight of BENZTHIAZIDE /TRIAMTERENE in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation

3.5. VALIDATION OF THE DEVELOPED RP-HPLC METHOD

3.5.1. System suitability

The system suitability studies can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision[11,24]

Procedure:

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

Acceptance criteria

1. The % RSD for the retention times of TRIAMTERENE and BENZTHIAZIDEPeaks from 6 replicate injections of each Standard solution should be not more than 2.0 %
2. The number of theoretical plates (N) for the TRIAMTERENE and BENZTHIAZIDEpeaks is not less than 2000.
3. The Tailing factor (T) for the TRIAMTERENE and BENZTHIAZIDE peak is not more than 2.0.

3.5.2. Specificity by Direct comparison method

There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their dosage form.

Preparation of mixed standard solution

weigh accurately 10mg of TRIAMTERENE and 10 mg of BENZTHIAZIDEin 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10µg/ml of TRIAMTERENE and BENZTHIAZIDEis prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains BENZTHIAZIDE– 25 mgTRIAMTERENE -50 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of BENZTHIAZIDE and TRIAMTERENE (µg/ml) were prepared by dissolving weight equivalent to 10 mg of BENZTHIAZIDEand 20 mg of TRIAMTERENE and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 10ml with mobile phase. Further dilutions are prepared in 5 replicates of 10µg/ml of BENZTHIAZIDEand TRIAMTERENE was made by adding 1 ml of stock solution to 10 ml of mobile phase.

3.5.3 Linearity and range

Preparation of standard stock solution

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Standard stock solutions of TRIAMTERENE and BENZTHIAZIDE(microgram/ml) were prepared by dissolving 10 mg of TRIAMTERENE and BENZTHIAZIDE dissolved in sufficient mobile phase and dilute to 100 ml with mobile phase.

Preparation of solution 1:

1.25ml of Triamterene and 0.625 ml of Benzthiazidestock solution is transferred to a 10ml volumetric flask and volume is made up with diluent to get a required concentration

Preparation of solution 2:

1.82ml of Triamterene and 0.937 ml of Benzthiazidestock solution is transferred to a 10ml volumetric flask and volume is made up with diluent to get a required concentration

Preparation of solution 3:

2.5ml of Triamterene and 1.25 ml of Benzthiazidestock solution is transferred to a 10ml volumetric flask and volume is made up with diluent to get a required concentration

Preparation of solution 4:

3.125ml of Triamterene and 1.56 ml of Benzthiazidestock solution is transferred to a 10ml volumetric flask and volume is made up with diluent to get a required concentration

Preparation of solution 5:

3.75ml of Triamterene and 1.87 ml of Benzthiazidestock solution is transferred to a 10ml volumetric flask and volume is made up with diluent to get a required concentration

Acceptance criteria: The relationship between the concentration of TRIAMTERENE and BENZTHIAZIDE and area of TRIAMTERENE and BENZTHIAZIDE should be linear in the specified range and the correlation should not be less than 0.99.

3.5.4. Accuracy

Accuracy of the method was determined by Recovery studies.. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 80%,100%, 120%.

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations /3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

Preparation of mixed standard solution

weigh accurately 10mg of TRIAMTERENE and 10 mg of BENZTHIAZIDEin 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase.From above stock solution 10µg/ml of TRIAMTERENE and BENZTHIAZIDEis prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Preparation Sample solutions:

For preparation of 80% solution (With respect to target Assay concentration):

10 tablets (each tablet contains BENZTHIAZIDE– 25 mg TRIAMTERENE -50 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of BENZTHIAZIDE and TRIAMTERENE (µg/ml) were prepared by dissolving weight equivalent to 10 mg of BENZTHIAZIDEand 20 mg of TRIAMTERENE and dissolved in sufficient mobile phase and dilute to 10ml with mobile phase. Further dilutions are prepared to get concentration of 10µg/ml of BENZTHIAZIDEand TRIAMTERENE was made by adding 1 ml of stock solution to 10 ml of mobile phase.

For preparation of 100% solution (With respect to target Assay concentration):

10 tablets (each tablet contains BENZTHIAZIDE– 25 mg TRIAMTERENE -50 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of BENZTHIAZIDE and TRIAMTERENE (µg/ml) were prepared by dissolving weight equivalent to 10 mg of BENZTHIAZIDEand 20 mg of TRIAMTERENE and dissolved in sufficient mobile phase and dilute to 10ml with mobile phase. Further dilutions are prepared to get concentration of 12µg/ml of BENZTHIAZIDEand TRIAMTERENE was made by adding 1.2 ml of stock solution to 10 ml of mobile phase.

For preparation of 120% solution (With respect to target Assay concentration):

10 tablets (each tablet contains BENZTHIAZIDE– 25 mg TRIAMTERENE -50 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of BENZTHIAZIDE and TRIAMTERENE (µg/ml) were prepared by dissolving weight equivalent to

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10 mg of BENZTHIAZIDEand 20 mg of TRIAMTERENE and dissolved in sufficient mobile phase and dilute to 10ml with mobile phase. Further dilutions are prepared to get concentration of 14µg/ml of BENZTHIAZIDEand TRIAMTERENE was made by adding 1.4 ml of stock solution to 10 ml of mobile phase.

Acceptance criteria: The % recovery of TRIAMTERENE and BENZTHIAZIDE should lie between 98% and 102%.

3.5.5. Precision

Method precision

Prepared sample preparations of BENZTHIAZIDEand TRIAMTERENE as per test method and injected 6 times in to the column.

Preparation of mixed standard solution

weigh accurately 10mg of TRIAMTERENE and 10 mg of BENZTHIAZIDEin 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase.From above stock solution 10µg/ml of TRIAMTERENE and BENZTHIAZIDEis prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Acceptance criteria

The % Relative standard deviation of Assay preparations of BENZTHIAZIDE and TRIAMTERENE should be not more than 2.0%.

3.5.6. Limit of Detection

Limit of detection is determined by standard deviation method. It is calculated by using the formula

$$\text{LOD} = \frac{3.3\sigma}{S}$$

Where,σ = the standard deviation of the response
S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

3.5.7 Limit of Quantification(LOQ):

Limit of quantification is determined by standard deviation method. It is calculated by using the formula

$$\text{LOQ} = \frac{10\sigma}{S}$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

3.5.8 Robustness

Chromatographic conditions variation

To demonstrate the robustness of the method, prepared solution as per test method and injected at different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision.[30]

Acceptance criteria

The system suitability should pass as per the test method at variable conditions.

3.5.9. Ruggedness

The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts

Acceptance criteria:

The % Relative standard deviation of Assay values between two analysts should be not more than 2.0%.

3.6. Definitions and Formulas Used in Method Validation

3.6.1. Mean/ Average (x_i)

The average result (ā) is calculated by summing the individual results and dividing the sum by the number (n) of individual values.

$$x_i = \frac{x_1 + x_2 + x_3 \ldots}{n}$$

Where, x_1, x_2, x_3.. = Values of individual results
n = Number of individual results

3.6.2 Standard Deviation (SD)

It is the root mean square deviation of values from their average.

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$$SD = \sqrt{\frac{\sum(x - x_i)}{n - 1}}$$

Where Σ = Sum of observations
x_i = Mean or arithmetic average (Σx / n)
x = Individual observed value
$x - x_i$ = Deviation of a value from the mean
n = Number of observations

3.6.3. Relative Standard Deviation (RSD)

It is defined as standard deviation expressed as the percentage of mean.

$$RSD = \frac{SD}{x_i} \times 100$$

Where SD = Standard deviation
x_i = Mean or arithmetic average (Σx / n)

3.6.4. Correlation Co-Efficient (R)

The correlation coefficient is used to indicate the relationship of two random variables. It provides a measure of the strength and direction of the correlation varying from -1 to +1. Positive values indicate that the two variables are positively correlated, meaning the two variables vary in the same direction. Negative values indicate that the two variables are negatively correlated, meaning the two variables vary in the contrary direction. Values close to +1 or -1 reveal the two variables are highly related.

$$R = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[\sum x^2 - (\sum x)^2]\,[[\sum y^2 - (\sum y)^2]}}$$

Where n = number of observations
x = first value
y = second value
Σxy = sum of products of first and second value
Σx = sum of first values
Σy = sum of second values
Σx^2 = sum of squares of first value
Σy^2 = sum of squares of second value

3.6.5. Linear Regression

A regression is a statistical analysis assessing the association between two variables. It is used to find the relationship between two variables. [13]

The equation of straight line is

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$$y = a + bx$$

Where b = slope
a = intercept

$$Slope\ (b) = [n(\Sigma xy) - (\Sigma x)(\Sigma y)] / [n(\Sigma x^2) - (\Sigma x)^2]$$

$$Intercept(a) = [(\Sigma y) - b(\Sigma x)] / n$$

Where n = number of observations
x = first value
y = second value
Σxy = sum of products of first and second value
Σx = sum of first values
Σy = sum of second values
Σx^2 = sum of squares of first value
Σy^2 = sum of squares of second value

IV. CHAPTER 4 RESULTSAND DISCUSSION

4.1.Determination of Working Wavelength (λmax)

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately

4.1.1. Results

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank.
The resulting spectra are shown in the fig. no. 4.1, and the isobestic point was found to be 245 nm for the combination.

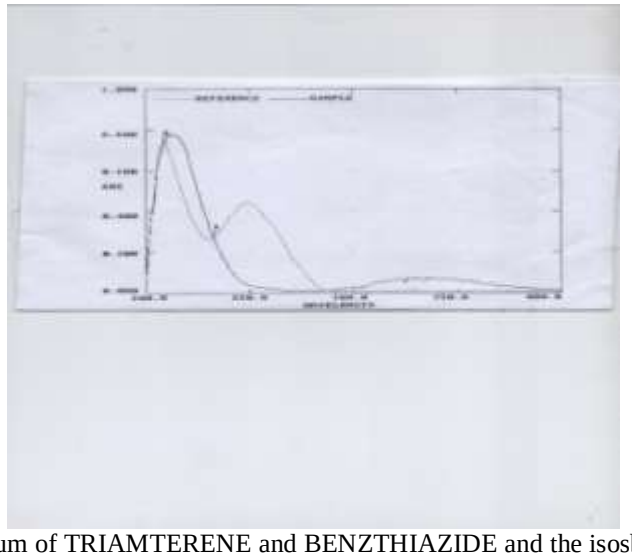

Fig. 4.1: UV-VIS spectrum of TRIAMTERENE and BENZTHIAZIDE and the isosbestic point was 245 nm

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Observation: The Isobestic point was found to be 245 nm for TRIAMTERENE and BENZTHIAZIDE in combination

4.2 METHOD DEVELOPMENT OF TRIAMTERENE ANDBENZTHIAZIDE

Trial - 1

Chromatographic conditions

Mobile phase :20mM KH2PO4:ACN
pH : 4.0
Ratio : 70:30(v/v)
Column : Inertsil ODS 3V (250mm×4.6mm× 5µm)
wavelength : 245 nm
Flow rate : 1ml/min

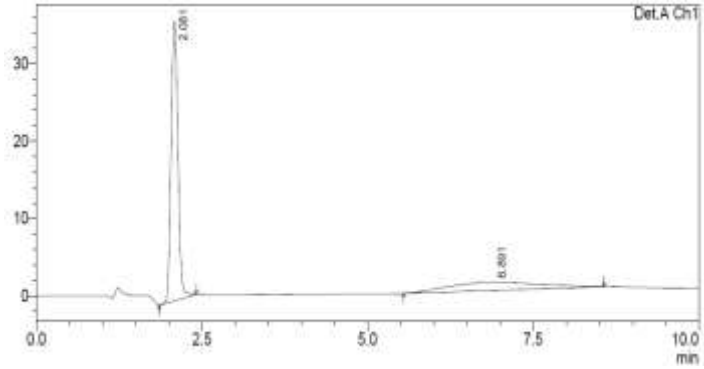

Fig. 4.2: Chromatogram of BENZTHIAZIDE and TRIAMTERENE by using mobile phase

Observation:

- Although the Efficiency was not satisfactory for BENZTHIAZIDE.
- The peak response of TRIAMTERENE was very less.
- Hence it was not taken for optimization. The details are given in figure 4.2.

Trial- 2

Chromatographic conditions

Mobile phase : 20mM KH2PO4:ACN
pH : 4.0
Ratio : 60:40 (v/v)
Column : Inertsil ODS 3V (250mm×4.6mm ×5µm)
wavelength : 245nm
Flow rate : 1ml/min

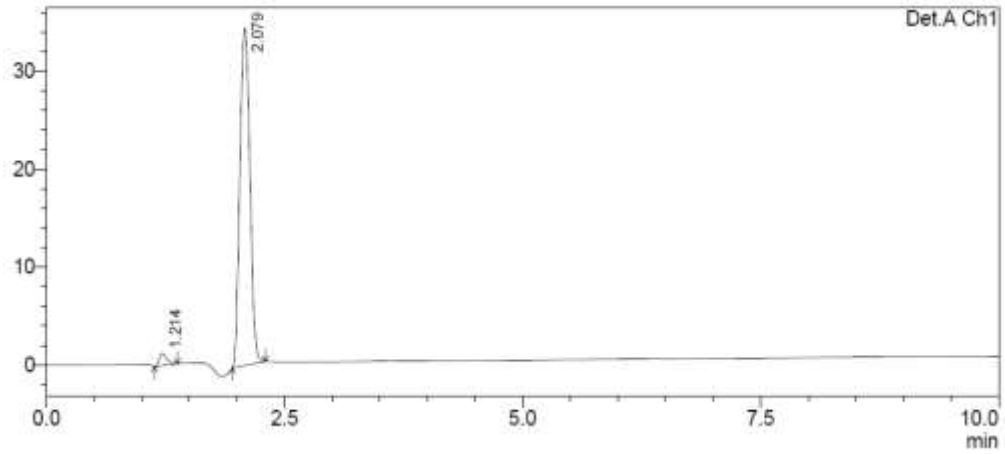

Fig. 4.3: Chromatogram of BENZTHIAZIDE and TRIAMTERENE by using Mobile phase

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Observation:

- Efficiency of both the drugs was good.
- The run time is very more.
- The peaks of TRIAMTERENE and BENZTHIAZIDE showed tailing.
- The details are given in the figure 4.3, Hence it was not taken for optimization.

Trial- 3:

Chromatographic conditions

Mobile phase : KH2PO4+ACN
pH : 3.5
Ratio : 55:45 (v/v)
Column : Inertsil ODS 3V, (250mm×4.6mm× 5µm)
Wavelength : 245nm
Flow rate : 1ml/min

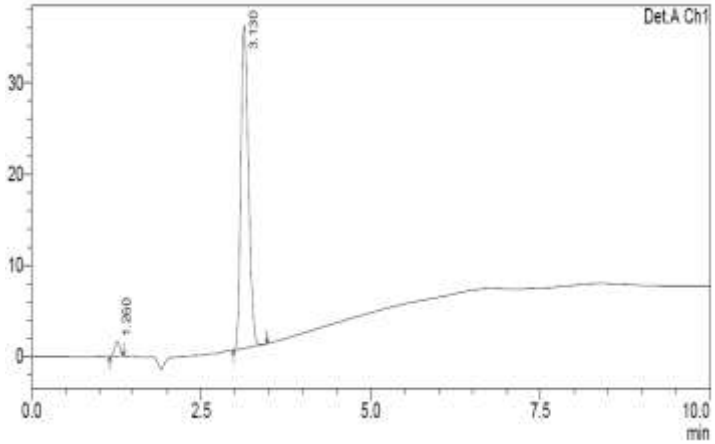

Fig. 4.4: Chromatogram of BENZTHIAZIDE and TRIAMTERENE by using mobile phase

Observation: Asymmetry factor for BENZTHIAZIDEdoes not meet the system suitability requirements.

- The run time is 11 minutes.
- The details are given in the table figure 4.4, hence it was not taken for optimization.

Trial- 4:

Chromatographic conditions

Mobile phase : Ammonium acetate buffer+ACN
pH : 6.0
Ratio : 30:70 (v/v)
Column :InertsilODS , (250mm×4.6mm× 5µm)
Wavelength : 245nm
Flow rate : 1ml/min

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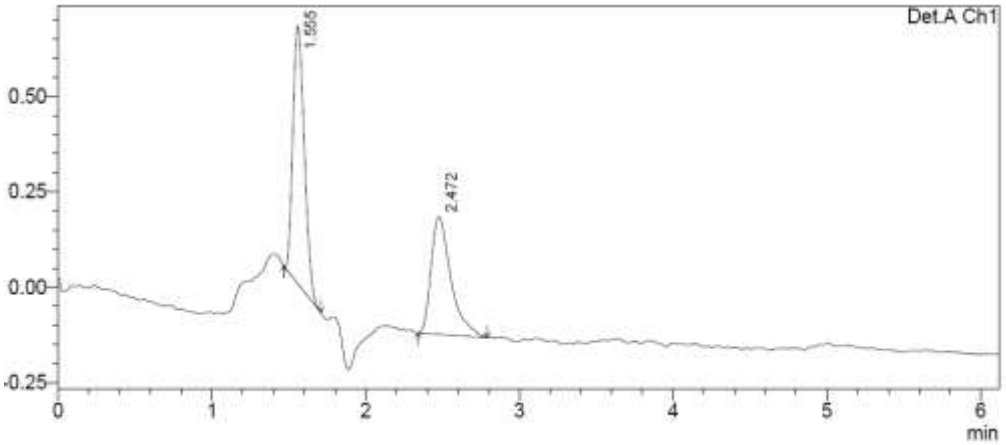

Fig. 4.5: Chromatogram of BENZTHIAZIDE and TRIAMTERENE by using mobile phase

Observation:

- Peak Asymmetry factor for TRIAMTERENE and BENZTHIAZIDE does not meet the system suitability requirements.
- The run time is very more.
- The details are given in the table figure 4.5, hence it was not taken for optimization.

Trial- 5:

Chromatographic conditions

Mobile phase : TEA+ACN
pH : 3.5
Ratio : 60:40(v/v)
Column : Inertsil ODS,(250mm×4.6mm× 5µm,)
Wavelength : 245 nm
Flow rate : 1ml/min

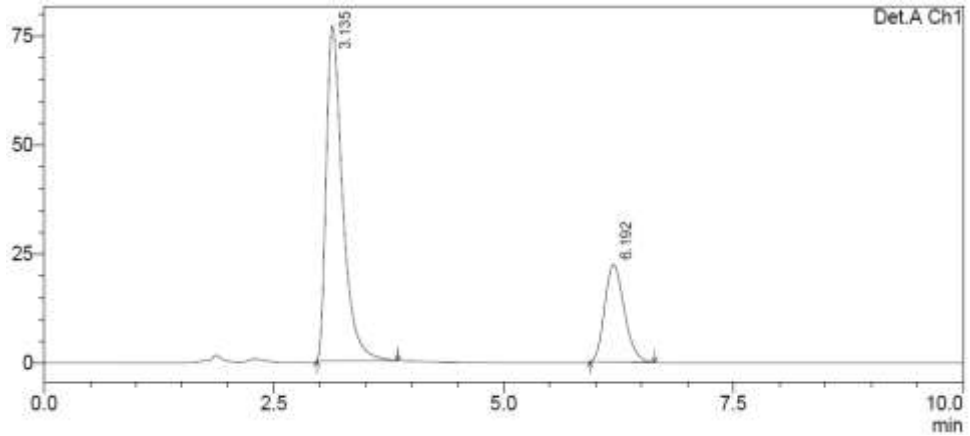

Fig. 4.6: Chromatogram of BENZTHIAZIDE and TRIAMTERENE by using mobile phase

Observation:

- All the system suitability requirements were met.
- The peak Asymmetry factor was less than 2 for both BENZTHIAZIDE and TRIAMTERENE .
- The efficiency was more than 2000 BENZTHIAZIDE and TRIAMTERENE .
- Resolution between two peaks >1.5.
- Thedetails are given in the figure 4.6, hence this method was for optimized.

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Table 4.1: Optimized chromatographic conditions

Mobile phase	TEA buffer+ ACN (60:40v/v)
Ph	3.5
Column	Inertsil ODS 3V column,C18(150mmx4.6mm ID) 5µm
Flow rate	1.0 ml/min
Column temperature	Room temperature(20±5°C)
Sample temperature	Room temperature(20±5°C)
Wavelength	245nm
Injection volume	20 µl
Run time	10 min
Retention time	About 3.135min for TRIAMTERENE and 6.192 min for BENZTHIAZIDE.

4.3. Assay

4.3.1. Preparation of samples for Assay

Preparation of mixed standard solution

Weigh accurately 10mg of TRIAMTERENE and 10 mg of BENZTHIAZIDEin 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of TRIAMTERENE and BENZTHIAZIDEis prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

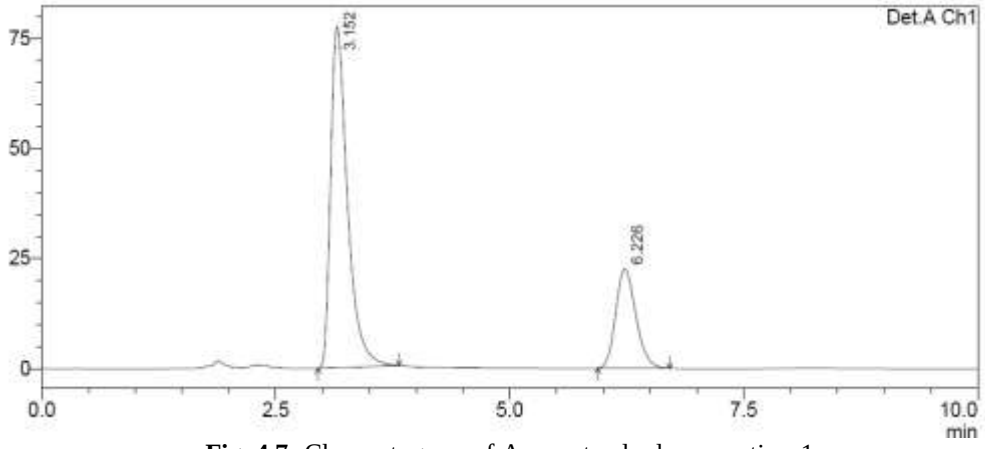

Fig. 4.7: Chromatogram of Assay standard preparation-1

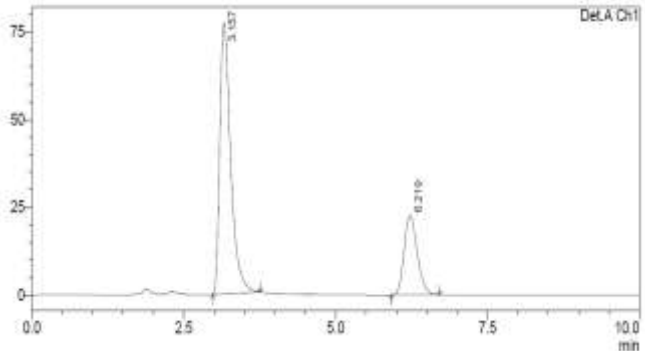

Fig. 4.8: Chromatogram of Assay standard preparation-2

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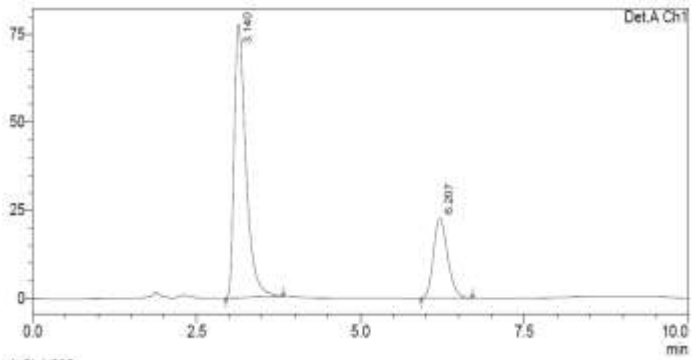

Fig. 4.9: Chromatogram of Assay standard preparation-3

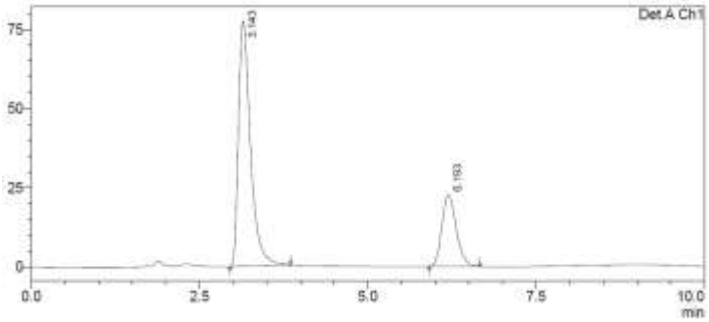

Fig. 4.10: Chromatogram of Assay standard preparation-4

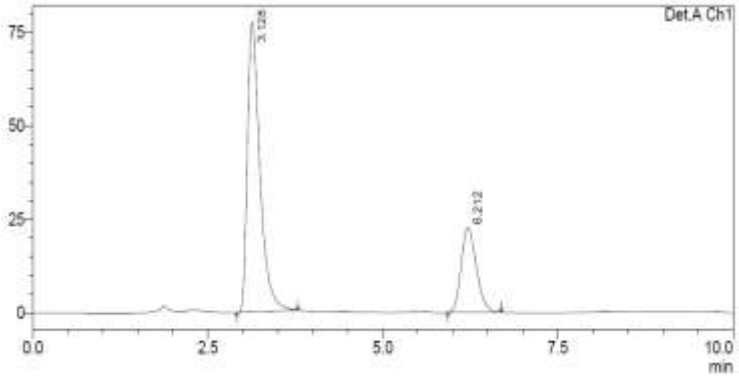

Fig. 4.11: Chromatogram of Assay standard preparation-5

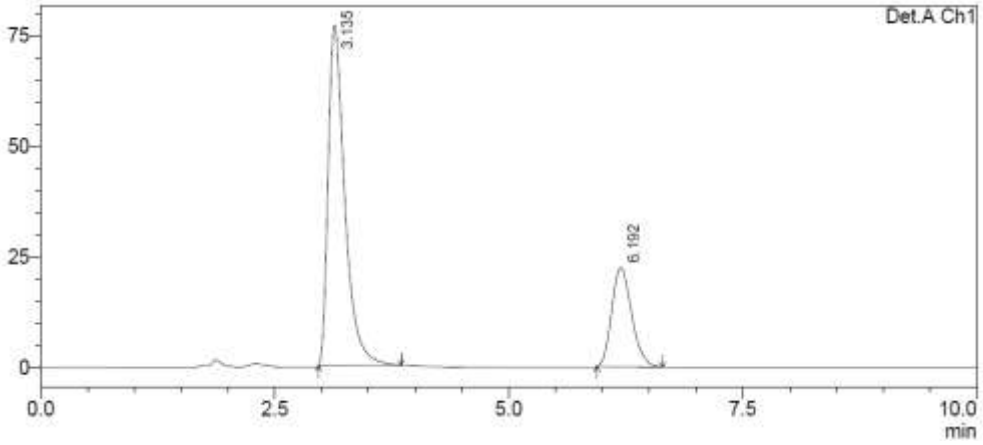

Fig. 4.12: Chromatogram of Assay sample preparation-1

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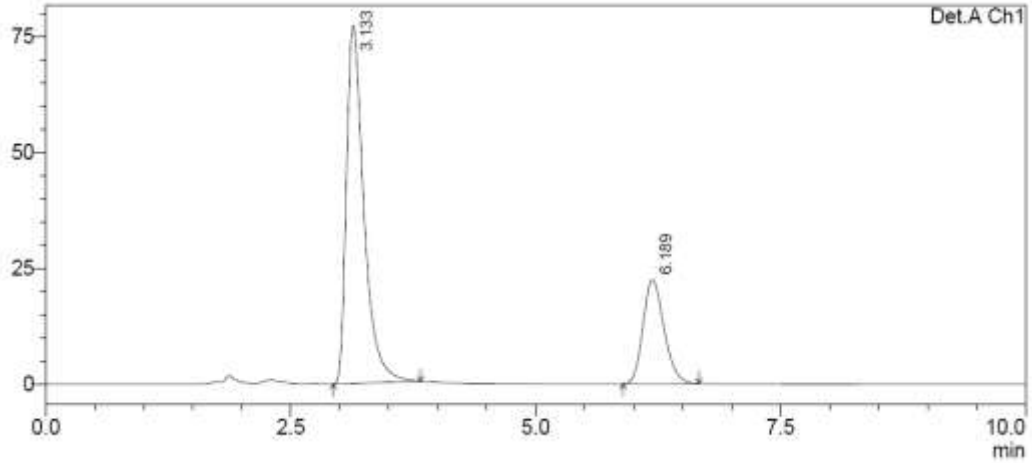

Fig. 4.13: Chromatogram of Assay sample preparation-2

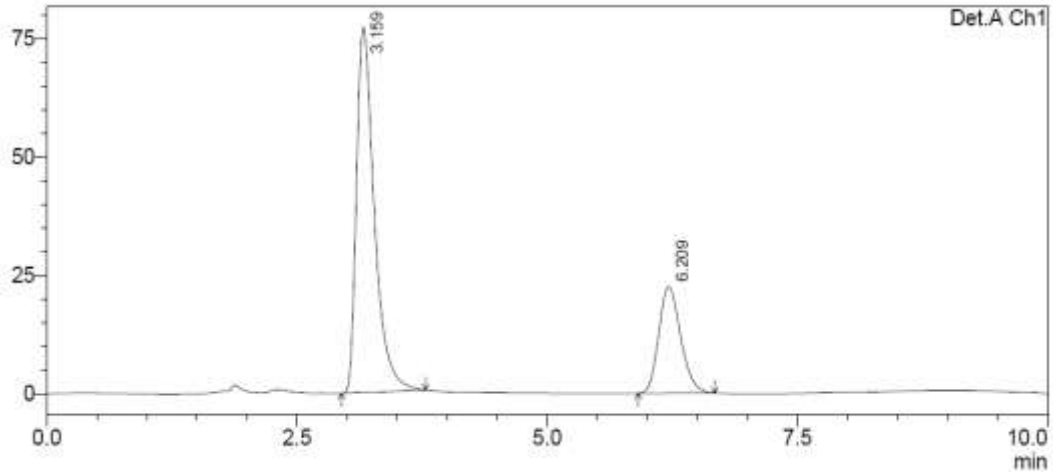

Fig. 4.14: Chromatogram of Assay sample preparation-3

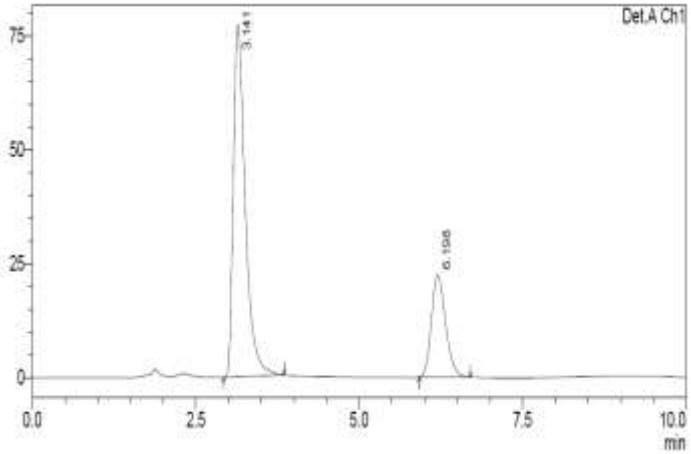

Fig. 4.15: Chromatogram of Assay sample preparation-4

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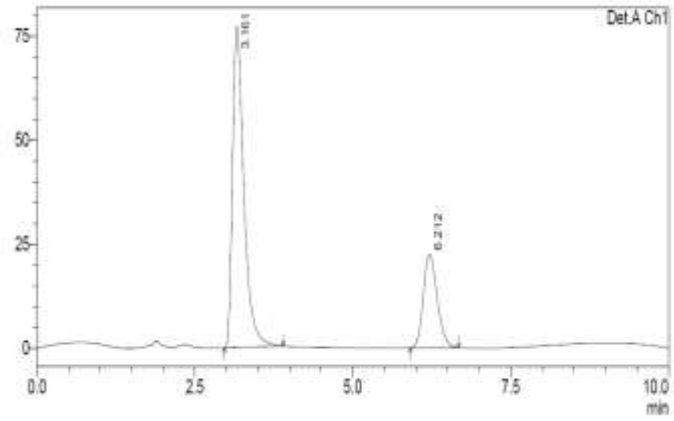

Fig. 4.16: Chromatogram of Assay sample preparation-5

Table No.4.2: Assay Results

TRIAMTERENE			**BENZTHIAZIDE**	
	Standard Area	**Sample Area**	**Standard Area**	**Sample Area**
Injection-1	970453	953219.000	338259	330792.000
Injection-2	962164.000	959560	340317.000	336648
Injection-3	962938	959938.000	338940	342812
Injection-4	963567	979280	338135	339796
Injection-5	962664.000	965957	343242.000	343112.000
Average Area	964357.200	963590.800	339778.6	338632
Assay(%purity)	**99.92**		**99.66**	

Observation

The amount of TRIAMTERENE and BENZTHIAZIDE present in the taken dosage form was found to be 99.92 % and 99.62 % respectively.

4.4. VALIDATION OF DEVELOPED RP-HPLC FOR ASSAY OF TRIAMTERENE AND BENZTHIAZIDE

4.4.1 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 asymmetric factor were evaluated.

Table 4.3: Results for system suitability of TRIAMTERENE

Injection	**Retention time (min)**	**Peak area**	**Theoretical plates (TP)**	**Tailingfactor (TF)**
1	3.133	986076.000	10291.503	1.698
2	3.128	972333.000	10564.055	1.682
3	3.146	974561.000	10341.239	1.679
4	3.151	962943.000	10540.745	1.685
5	3.144	971826.000	10468.515	1.673

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Mean	3.145	957998.000	-	-
SD	3.1412	970956.167	-	-
%RSD	0.0088	9760.957	-	-

Table 4.4: Results for system suitability of BENZTHIAZIDE

Injection	**Retention time (min)**	**Peak area**	**Theoretical plates**	**Tailing factor**
1	6.187	339373	25599.180	1.241
2	6.166	338536	25579.317	1.259
3	6.194	340815.000	26165.997	1.250
4	6.182	341269.000	25703.593	1.254
5	6.182	342995	25643.570	1.248
Mean	6.218	339066.000	-	-
SD	6.188	340342.333	-	-
%RSD	0.017	1670.344	-	-

Observation

The % RSD for the retention times and peak area of TRIAMTERENE and BENZTHIAZIDEwere found to be less than 2%. The plate count and tailing factor results were found to be satisfactory and are found to be within the limit.

4.4.2.Specificity by Direct comparison method:

There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their dosage form.

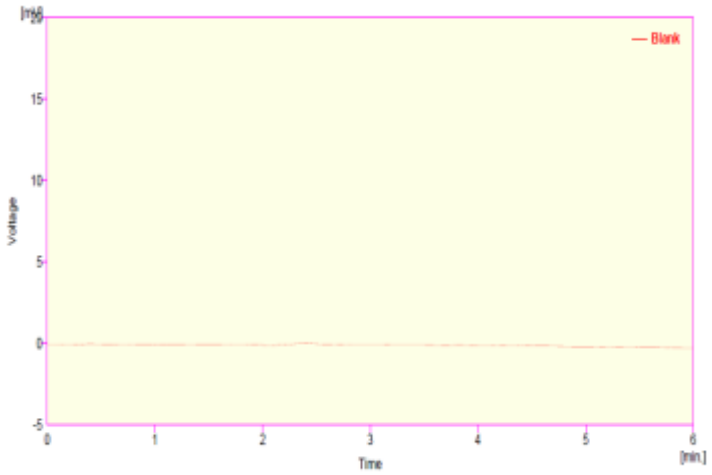

Fig. 4.17 : Blank chromatogram for specificity by using mobile phase

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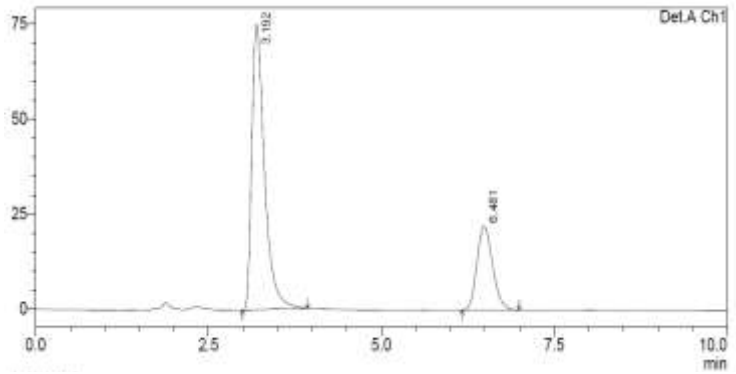

Fig. 4.18: Chromatogram for specificity of TRIAMTERENE and BENZTHIAZIDE sample

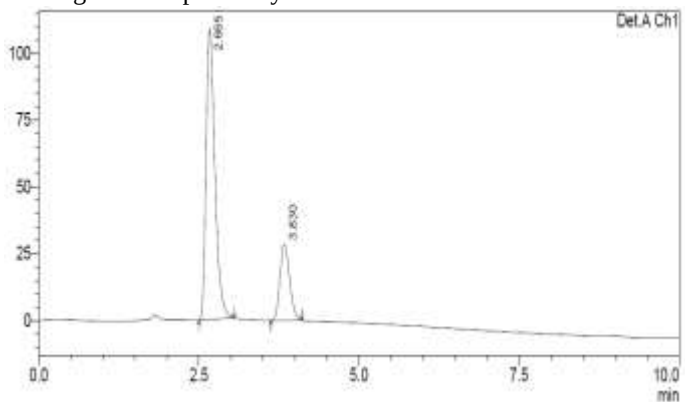

Fig. 4.19: Chromatogram for Specificity of TRIAMTERENE and BENZTHIAZIDE standard

Observation
It is observed from the above data, diluent or excipient peaks are not interfering with the TRIAMTERENE and BENZTHIAZIDE peaks.

4.4.3 Linearity and range
Preparation of standard stock solution
Standard stock solutions of TRIAMTERENE and BENZTHIAZIDE(microgram/ml) were prepared by dissolving 10 mg of TRIAMTERENE and BENZTHIAZIDE dissolved in sufficient mobile phase and dilute to 100 ml with mobile phase.
Further dilutions were given in the table No 4.5

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Table4.5: Linearity Preparations

Preparations	Volume from standard stock transferred in ml		Volume made up in ml (with mobile phase)	Concentration of solution(µg /ml)	
				TRIAMTERENE	BENZTHIAZIDE
Preparation 1	1.25	0.625	10	12.5	6.25
Preparation 2	1.82	0.937	10	18.75	9.37
Preparation 3	2.5	1.25	10	25	12.5
Preparation 4	3.125	1.56	10	31.25	15.625
Preparation 5	3.75	1.87	10	37.5	18.75

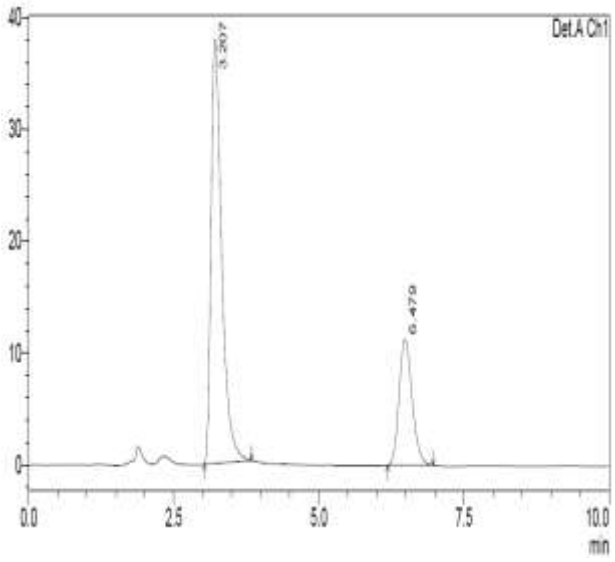

Fig. 4.20: Chromatogram of TRIAMTERENE and BENZTHIAZIDEpreparation-1

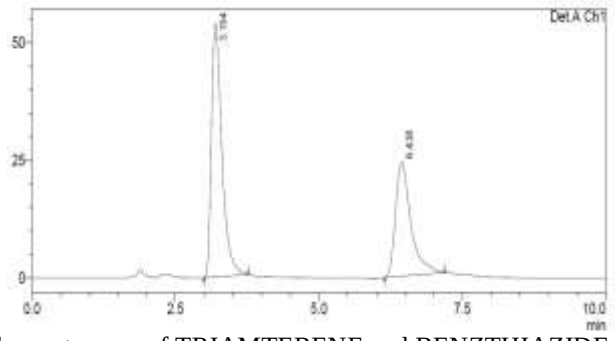

Fig. 4.21: Chromatogram of TRIAMTERENE and BENZTHIAZIDEpreparation-2

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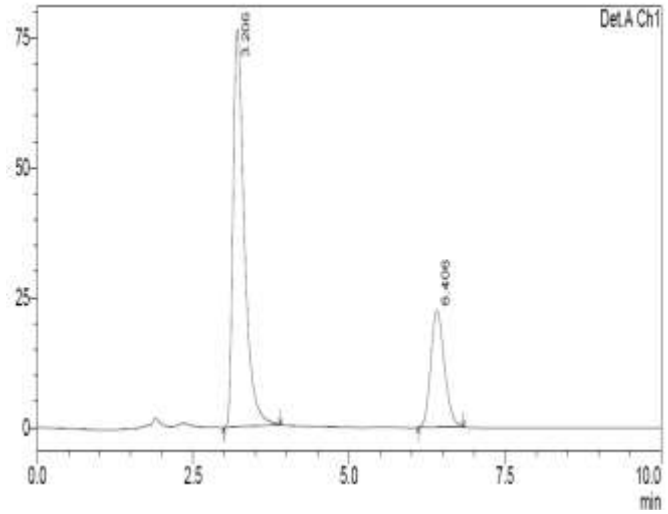

Fig. 4.22: Chromatogram of TRIAMTERENE and BENZTHIAZIDEpreparation-3

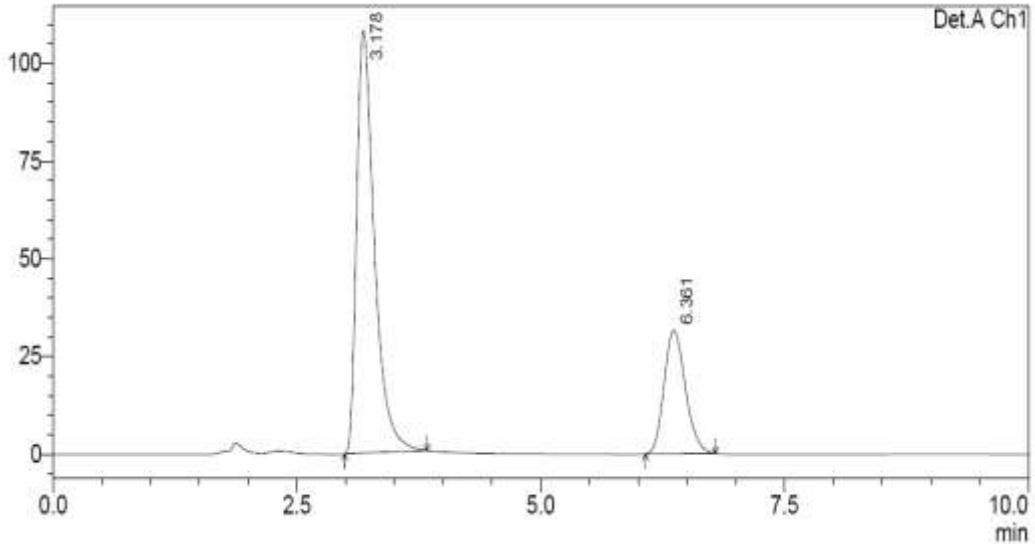

Fig.4.23: Chromatogram of TRIAMTERENE and BENZTHIAZIDE preparation-4

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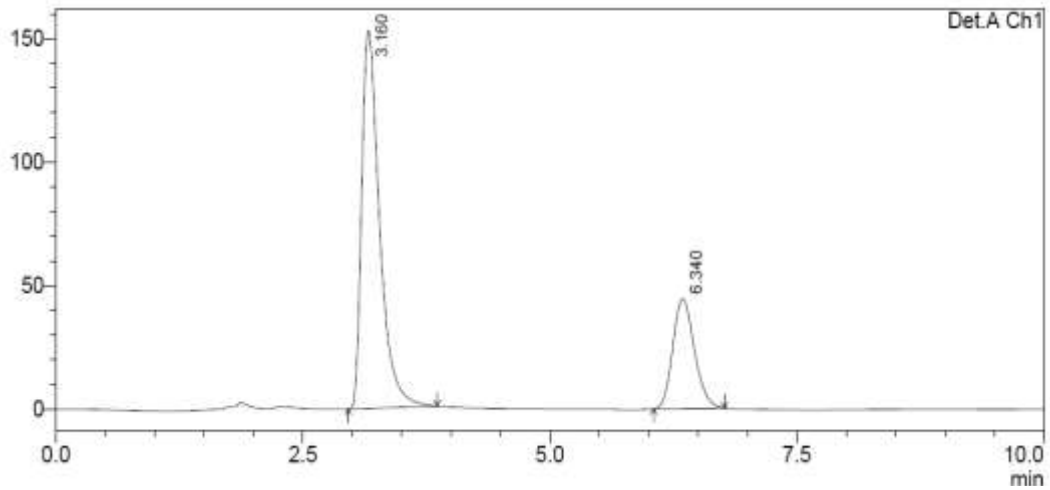

Fig. 4.24: Chromatogram of TRIAMTERENE and BENZTHIAZIDE for preparation-5

S.No.	Conc.(µg/ml)	Area
1	12.5	402854
2	18.75	680745
3	25	964865
4	31.25	1370938
5	37.5	1752793

Table 4.6: linearity of TRIAMTERENE

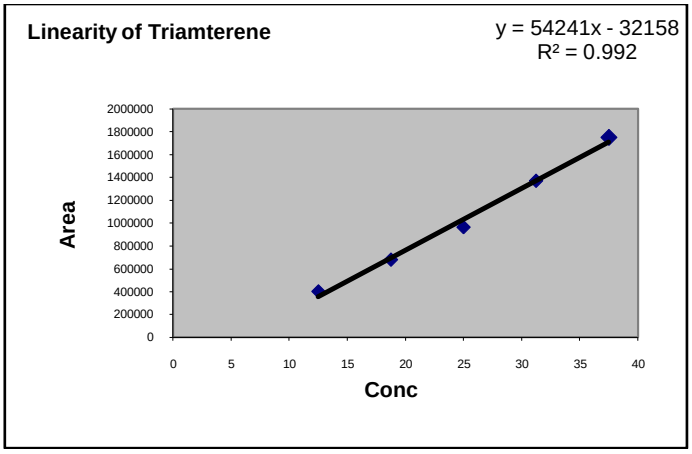

Fig.4.25: Linearity graph of TRIAMTERENE

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S.No.	Conc.(µg/ml)	Area
1	6.25	185902
2	9.37	332516
3	12.5	410967
4	15.625	529989
5	18.75	675347

Table 4.7: linearity of BENZTHIAZIDE

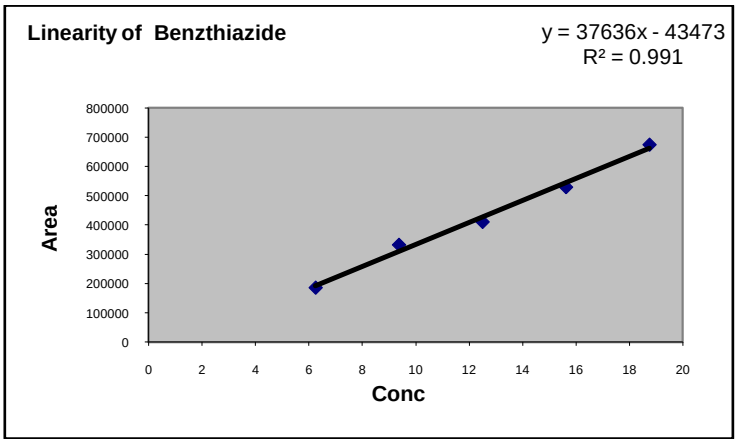

Fig. 4.26: Linearity graph of BENZTHIAZIDE

Observation

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of TRIAMTERENE and BENZTHIAZIDE is 0.999 and 0.996. The relationship between the concentration of TRIAMTERENE and BENZTHIAZIDE and area of TRIAMTERENE and BENZTHIAZIDE is linear in the range examined since all points lie in a straight line and the correlation coefficient is well within limits.

4.4.4. Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 80%,100%, 120%.

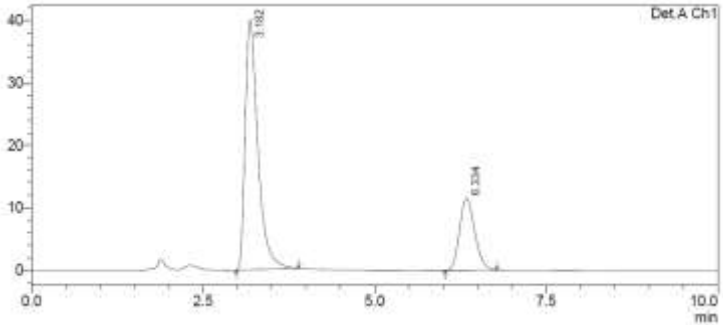

Fig.4.27: Chromatogram of 80% recovery (injection 1)

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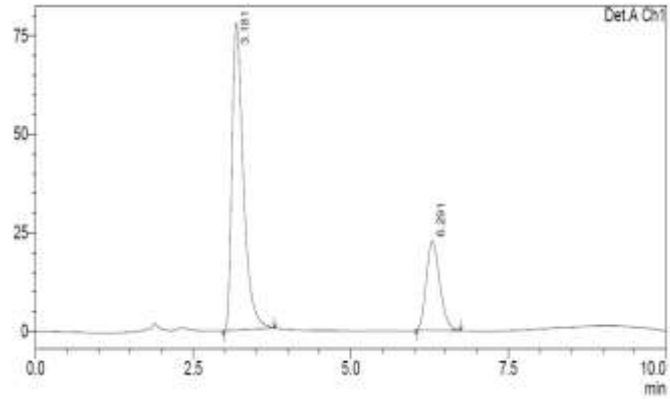

Fig. 4.28: Chromatogram of 100% recovery (injection 2)

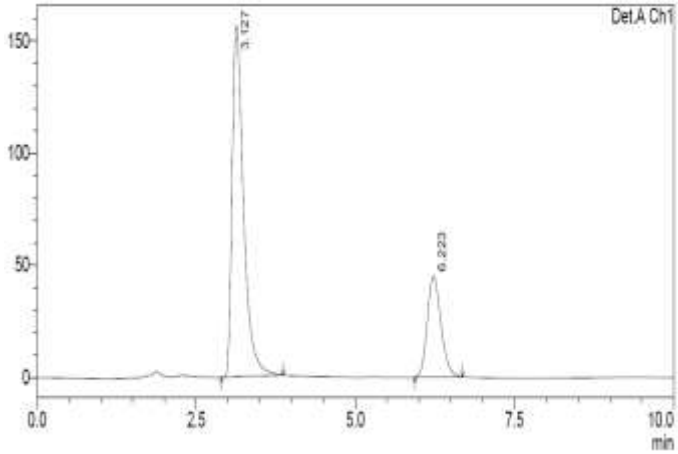

Fig. 4.29: Chromatogram of 120% recovery (injection 3)

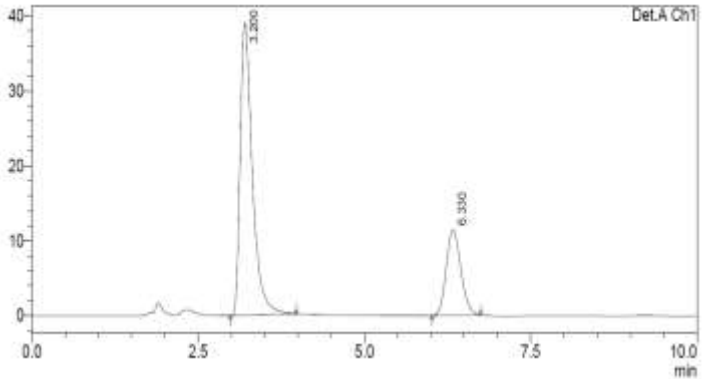

Fig. 4.30: Chromatogram of 80% recovery (injection 1)

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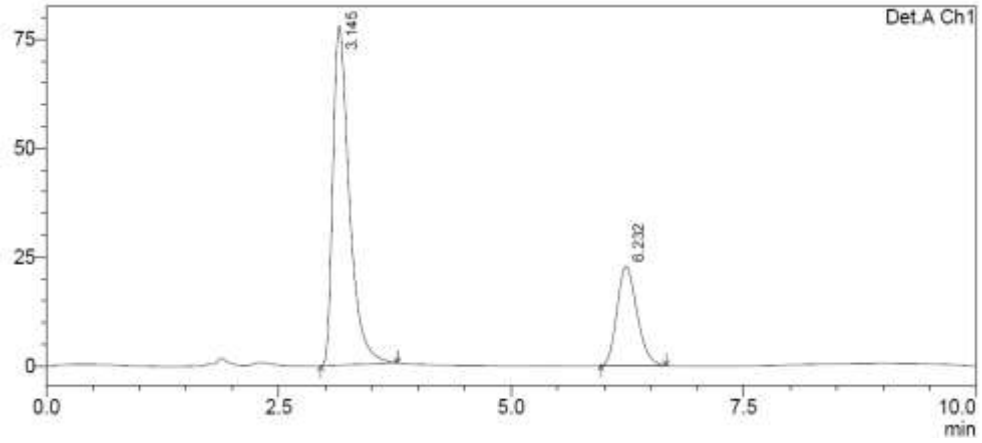

Fig.4.31: Chromatogram of 100% recovery (injection 2)

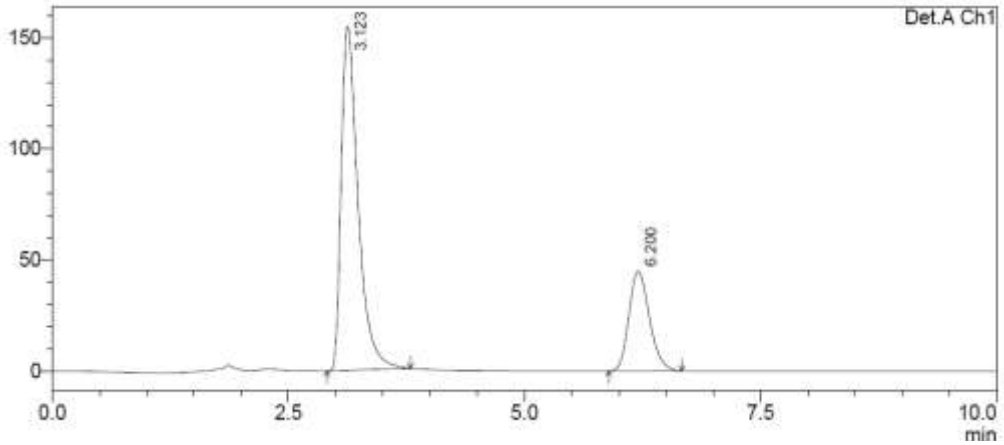

Fig. 4.32: Chromatogram of 120% recovery (injection 3)

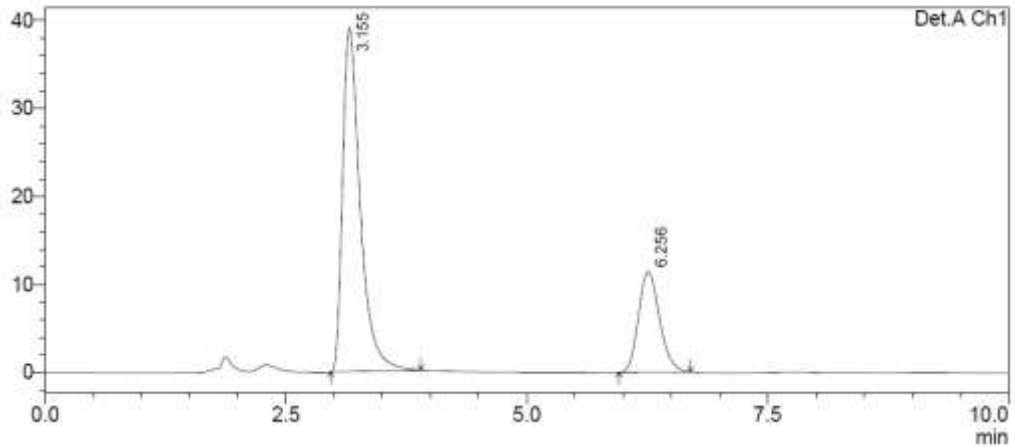

Fig. 4.33: Chromatogram of 80% recovery (injection 1)

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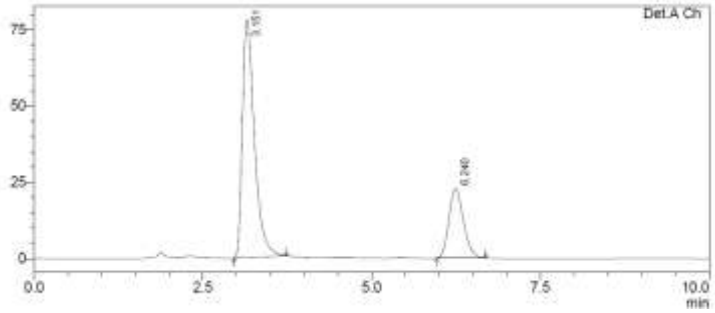

Fig. 4.34: Chromatogram of 100% recovery (injection 2)

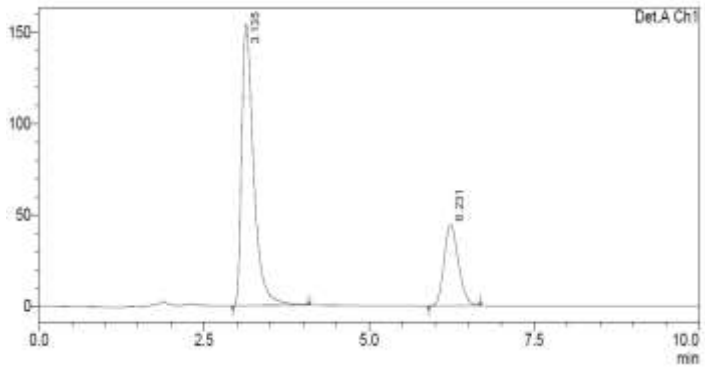

Fig.4.35: Chromatogram of 120% recovery (injection 3)

Acceptance criteria: The % recovery of TRIAMTERENE and BENZTHIAZIDE should lie between 98% and 102%.

Table 4.8: Recovery results for Triamterene

Recovery level	**Accuracyof Triamterene**		
	Amount taken (µg/ml)	Area	Average % recovery
80	**20**	**514338**	102.427
	20	**496912**	
	20	**495101**	
100	**25**	**974907**	99.66750982
	25	**958744**	
	25	**969532**	99.96656216
120	**29**	**1955911**	
	29	**1917639**	
	29	**1950238**	

Table 4.9: Recovery results for BENZTHIAZIDE

Recovery level	**Accuracy BENZTHIAZIDE**		
	Amount taken (**µg**/ml)	Area	Average % Recovery
80	10.4	**176747**	

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	10.4	**175164**	**102.83**
	10.4	**173049**	
100	12.5	**335603**	**99.36**
	12.5	**338962**	
	12.5	**339944**	
120	14.5	**674267**	**99.103**
	14.5	**677848**	
	14.5	**671627**	

.

Observation

The percentage mean recovery of TRIAMTERENE and BENZTHIAZIDE is 100.68 % and 100.43 % respectively

4.4.5. Precision

Method precision

Prepared sample preparations of BENZTHIAZIDE and TRIAMTERENE as per test method and injected 6 times in to the column.

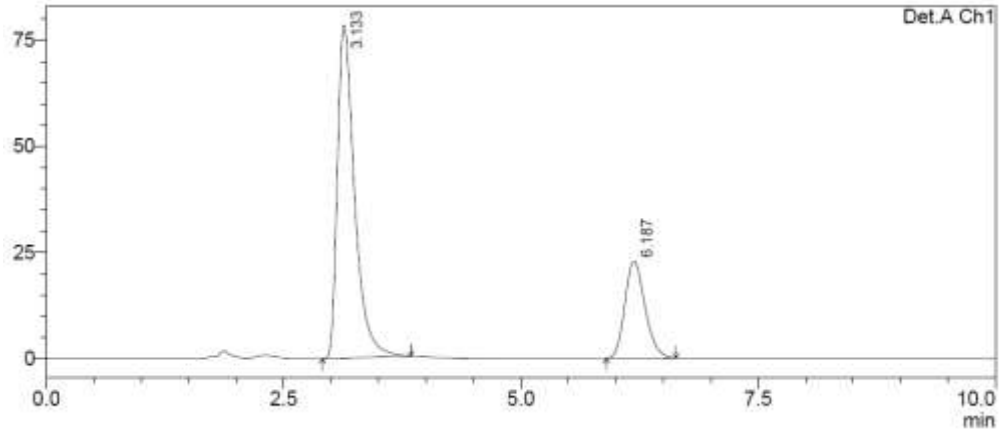

Fig. 4.36: Chromatogram of precision injection 1

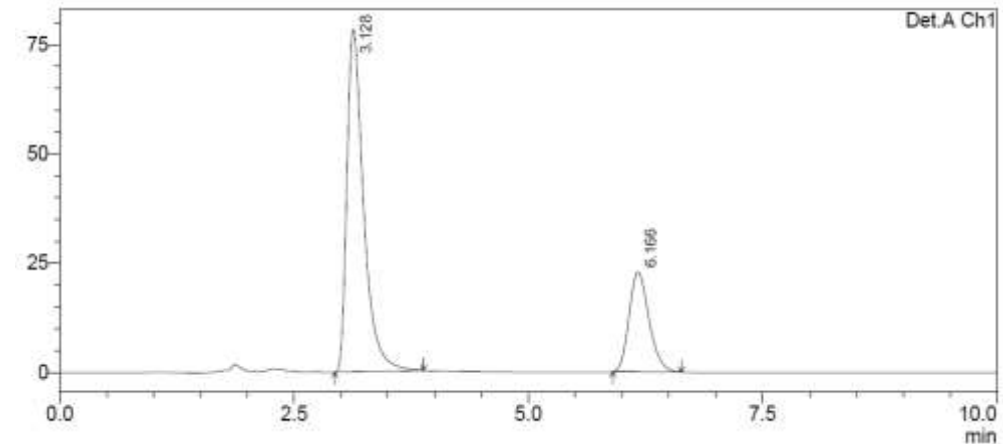

Fig. 4.37: Chromatogram of precision injection 2

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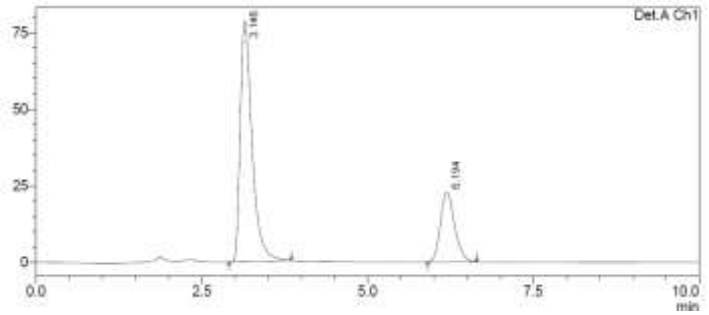

Fig. 4.38: Chromatogram of precision injection 3

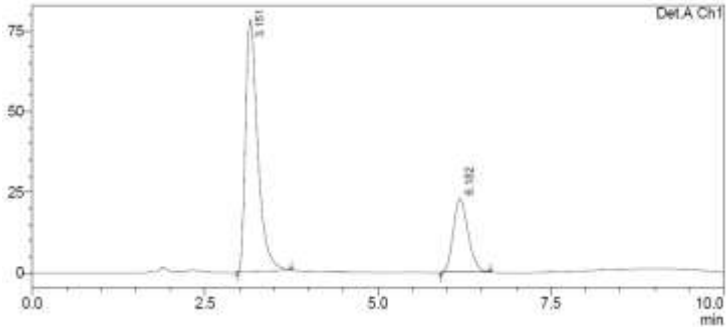

Fig. 4.39: Chromatogram of precision injection 4

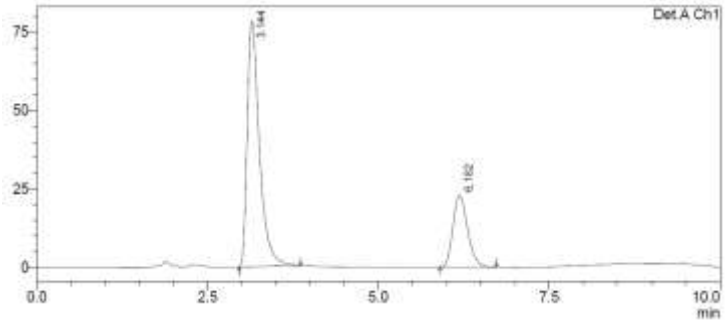

Fig.4.40: Chromatogram of precision injection 5

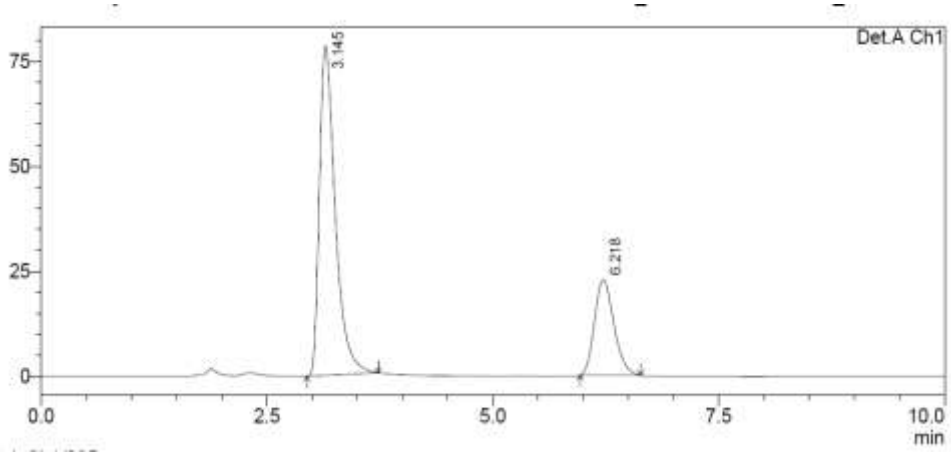

Fig. 4.41: Chromatogram of precision injection 6

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Table 4.10: Results for Method precision of TRIAMTERENE and BENZTHIAZIDE

TRIAMTERENE		
S.No.	**Retention time** (min)	Area
1	3.145	978370.000
2	3.165	962064.000
3	3.151	967422.000
4	3.148	955774.000
5	3.126	951906.000
6	3.116	962532.000
Average	3.1418	963011.333
Standard deviation	0.0178	9297.067
%RSD	**0.57**	**0.97**

BENZTHIAZIDE		
S.No.	**Retention time (**min)	Area
1	6.211	340457
2	6.224	341907
3	6.212	339323.000
4	6.194	339473.000
5	6.168	339074
6	6.170	340503.000
Average	6.197	340122.833
Standard deviation	0.023	1058.443
%RSD	**0.38**	**0.31**

Observation

Test results for BENZTHIAZIDE and TRIAMTERENE are showing that the %RSD of Assay results are within limits. The results were shown in table Table4.12.

4.4.6. Limit of Detection(LOD):

Limit of detection is determined by standard deviation method. It is calculated by using the formula

$$\text{LOD} = \frac{3.3\sigma}{S}$$

Where, σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

Observation:

The LOD for this method was found to be 4.61µg/ml and 14.0 µg/ml for BENZTHIAZIDE

4.4.7 Limit of Quantification(LOQ):

Limit of quantification is determined by standard deviation method. It is calculated by using the formula

$$\text{LOQ} = \frac{10\sigma}{S}$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

Observation:

The LOQ for this method was found to be 0.21µg/ml for TRIAMTERENE and 0.63µg/ml for BENZTHIAZIDE.

4.4.8 Robustness

Chromatographic conditions variation

To demonstrate the robustness of the method, prepared solution as per test method and injected at different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision.

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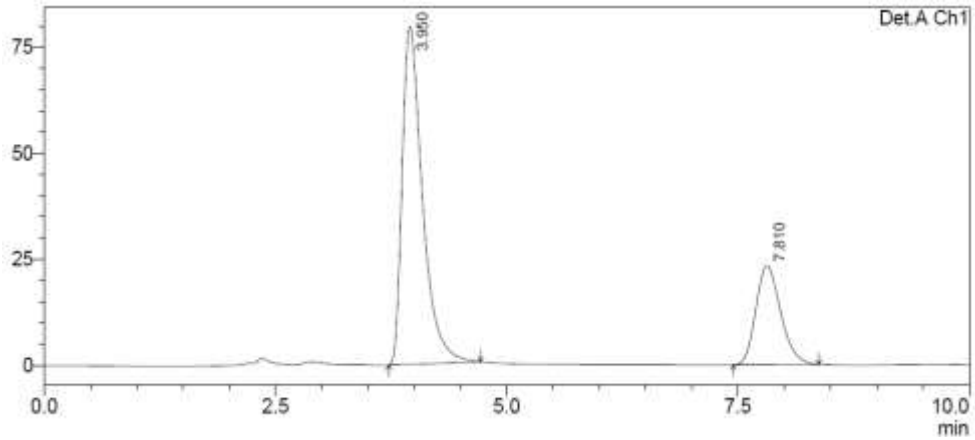

Fig. 4.42: Chromatogram of TRIAMTERENE and BENZTHIAZIDE Robustness (0.8 ml/min)

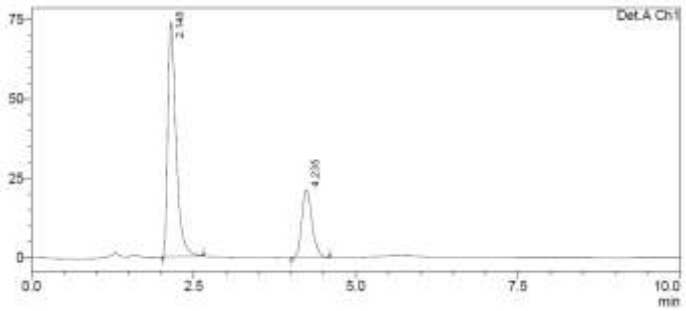

Fig.4.43: Chromatogram of TRIAMTERENE and BENZTHIAZIDE Robustness (1.2 ml/min)

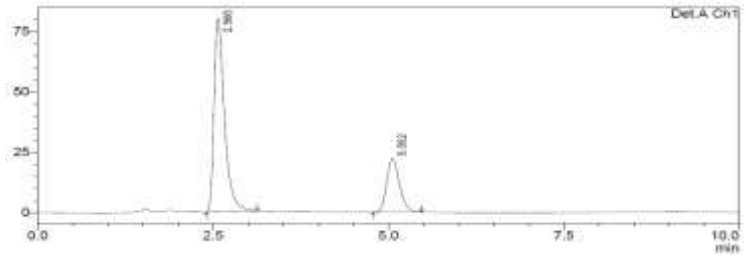

Fig. 4.44: Chromatogram of TRIAMTERENE and BENZTHIAZIDE for Robustness (243nm)

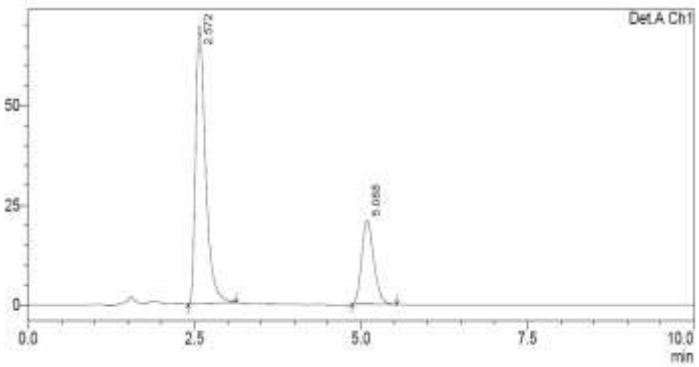

Fig. 4.45: Chromatogram of TRIAMTERENE and BENZTHIAZIDE for Robustness (247nm)

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Table 4.11: Result of Robustness study

Parameter	TRIAMTERENE		BENZTHIAZIDE	
	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor
Flow Rate **0.8 ml/min** **1.2 ml/min**	 3.950 2.148	 1.697 1.678	 7.810 4.235	 1.256 1.264
Wavelength **243nm** **247nm**	 2.566 2.572	 1.687 1.676	 5.052 5.088	 1.262 1.269

Observation

From the observation it was found that the system suitability parameters were within limit at all variable conditions.

4.4.9. Ruggedness

The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts

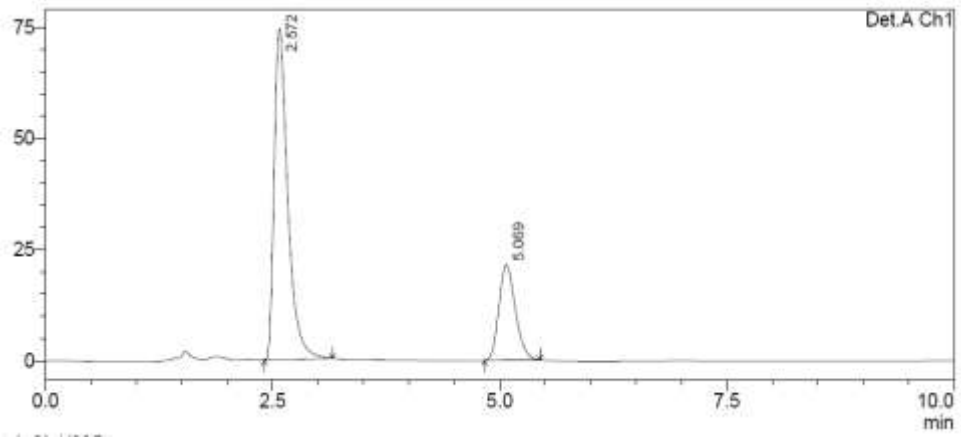

Fig. 4.46: Chromatogram of Analyst 01 standard preparation

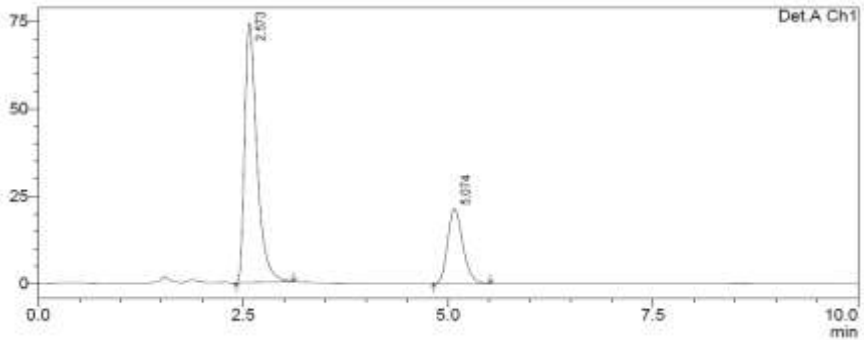

Fig. 4.47: Chromatogram of Analyst 01 sample preparation

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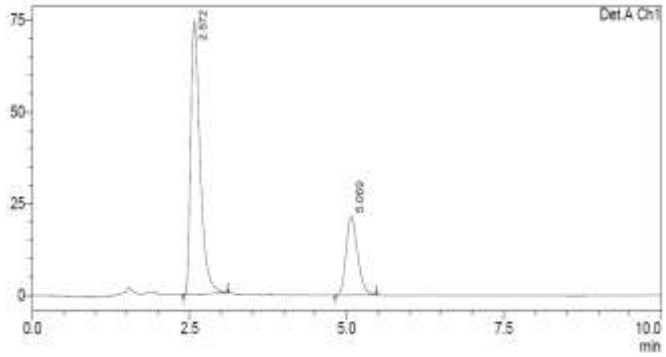

Fig. 4.48: Chromatogram of Analyst 02 standard preparation

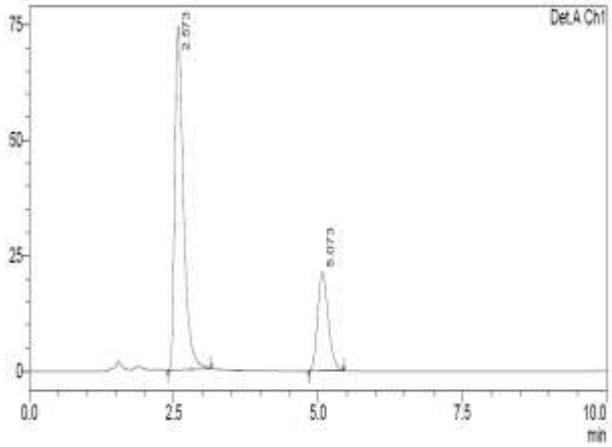

Fig. 4.49: Chromatogram of Analyst 02 sample preparation

Table 4.12: Results for Ruggedness

TRIAMTERENE	%Assay	BENZTHIAZIDE	%Assay
Analyst 01	99.4	**Analyst 01**	101.0
Anaylst 02	100.4	**Anaylst 02**	99.3

Observation
From the observation the between two analysts Assay values not greater than 2.0%, hence the method was rugged.

V. CONCLUSION

- A simple and selective HPLC method is described for the determination of Triamtereneand Benzthiazide in tablet dosage forms. Chromatographic separation was achieved on a c_{18} column using mobile phase consisting of a mixture of 60 volumes of triethylamine ,40 volumes of acetonitrile with detection of 245 nm.
- The diluent or excipient peaks are not interfering with the TRIAMTERENE and BENZTHIAZIDE peaks.
- Linearity was observed in the range 12.5-37.5 µg/ml for Triamterene(r^2 =0.992) and 6.25-18.75 µg /ml for Benzthiazide (r^2 =0.991) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim.
- From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation Triamtereneand Benzthiazide was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective

VI. FUTURE SCOPE

- From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous

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estimation Triamtereneand Benzthiazide was found to be , more acceptable and cost effective

- There may be scope to develop HPLC, UPLC, GC and MS methods for isolation and quantification of impurities present in the drug and pharmaceutical formulation.
- Bioanalytical methods may be developed for the estimation of Triamterene and Benzthiazide in pharmaceutical dosage form.
- It can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories studies in near future.

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