A VALIDATED STABILITY-INDICATING HPLC METHOD FOR ACETAZOLAMIDE SR CAPSULE IN THE PRESENCE OF DEGRADATION PRODUCTS AND ITS PROCESS-RELATED IMPURITY

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MASTER OF PHARMACY

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Submitted by

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This is to certify that the dissertation work entitled "**A VALIDATED STABILITY-INDICATING HPLC METHOD FOR ACETAZOLAMIDE SR CAPSULE IN THE PRESENCE OF DEGRADATION PRODUCTS AND ITS PROCESS-RELATED IMPURITY**" is a bonafide work of **Mr.DIBIN.E** carried out and in SHASUN PHARMACEUTICALS Ltd, PONDICHERRY under my guidance and has completed to my fullest satisfaction for partial fulfillment of the award of degree of **Master of Pharmacy in Pharmaceutical Analysis**, R.V.S college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

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INTERNAL EXAMINER **EXTERNAL EXAMINER**

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Date: **Date:**

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As required by university regulation, I wish to state that this work embodied in this thesis titled "**A VALIDATED STABILITY-INDICATING HPLC METHOD FOR ACETAZOLAMIDE SR CAPSULE IN THE PRESENCE OF DEGRADATION PRODUCTS AND ITS PROCESS-RELATED IMPURITY.**" Forms my own contribution to the research work carried out under the guidance of **Mr.R.SIVAKUMAR** and **Dr.G.RADHAKRISHNA REDDY**. This work has not been submitted for any other degree of this or any other university. Whenever references have been made to previous work of others, it has been clearly indicated as such and included in the bibliography.

Signature of the candidate

(Dibin.E)

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"The act of thanks giving does not exhibit ones sense of gratitude, but the true tendency of lending a helping hand during emergency and the fact that every work has thousands of hands behind".

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CONTENTS

LIST OF ABBREVATION

S.NO	UNITS	ABBREVATIONS
1	mg	Milli gram
2	ml	Milli litters
3	g	Gram
4	ppm	Parts per million
5	HPLC	High performance liquid chromatography
6	UV	Ultra violet
7	min	Minutes
8	RSD	Relative standard deviation
Q	SD	Standard deviation
10	RT	Retention time
11	RS	Resolution
12	RRT	Relative retention time
13	RRF	Relative Response Factor

1. INTRODUCTION

An impurity in a drug substance as defined by the international conference on Harmonization (ICH) Guidelines[1] is any component of the drug substance that is not the chemical entity defined as the drug substance and affects the purity of active ingredient or drug product [2]. Therefore any extraneous material present in the drug substance has to be considered an impurity even if it is totally inert or has superior pharmacological properties. The impurity profile of pharmaceuticals is of increasing importance as drug safety receives more and more attention from the public and from the media. Several recent books [3,4] and journal reviews[5] address this topic and guidelines are available from US and international authorities. Most active pharmaceutical ingredients (API) are produced by organic chemical synthesis. Various components, including residual solvents, trace amounts of inorganic, and organic components can be generated during such a process. Those components remaining in the final API are considered as impurities. The sources and routes of formation of impurities in generics are starting materials, by products and residual solvents from the API synthesis; degradants formed during the process and long term storage; contaminants from packaging components and other drug products manufactured in the same facility. Impurities could be forming from the impact of heat, light, and oxidants (including air) on the drug product and might be catalyzed or accelerated by trace metal impurities, changes in the pH of the formulation interactions with packaging components, excipients and other active ingredients, in the case of combination products. Therefore, identification, quantification, and control of impurities in the drug substance and drug product, are an important part of drug development and regulatory assessment.

1.1. Classification of impurities

Impurities in drug substance can be classified into the following categories[4]

- **Organic Impurities**

Organic impurities can arise during the manufacturing process and / or storage of the drug substance. They can be identified or unidentified, volatile, voltaic or nonvolatile

- **Starting materials or intermediates**

These are the most common impurities found in every API unless a proper care is taken in every step involved throughout the multi-step synthesis. Although the end products are always washed with solvents, there are always chances of having the residual unreacted starting materials may remain unless the manufacturers are very careful about the impurities.

- **Degradation products**

Impurities can also be formed by degradation of the end product during manufacturing of bulk drugs. However, degradation products resulting from storage, formulation to different dosage forms and aging are common impurities in the medicines.

- **Reagents, ligands, and catalysts**

These chemicals are less commonly found in APIs; however, in some cases they may pose a problem as impurities.

- **Enantiomeric impurities**

The single enantiomeric form of a chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favorable adverse reaction.

- **Inorganic impurities**

Inorganic impurities may also derive from the manufacturing processes used for bulk drugs. They are normally known and identified and include the following

- **Reagents, ligands, and catalysts**

The chances of having these impurities are rare: however, in some processes, these could create a problem unless the manufacturers take proper care during production.

- **Heavy metals**

The main sources of heavy metals are the water used in the processes and the reactors (if stainless steel reactors are used) where acidification or acid hydrolysis takes place. These impurities of heavy metals can easily be avoided using demineralized water and glass-lined reactors

- **Other materials (eg.filter aids, charcoal)**

The filters or filtering aids such as centrifuge bags are routinely used in the bulk drugs manufacturing plants, and, in many cases, activated carbon is also used

The regular monitoring of fibers and black particles in the bulk drugs is essential to avoid these contaminations.

- **Organic volatile impurities**

Organic volatile impurities relates to residual solvents that may be found in the drug substance.

- **Method related**

During the production process of bulk active pharmaceutical ingredients, many opportunities for the generation of impurities may arise. In cases such as this, the impurities often result from "primary" impurities in raw materials, which are carried through the manufacturing process.[6]

- **Environmental related**

The primary environmental factors that can reduce stability of drug so these impurities know as environmental related impurity include the following.

- **Exposures to adverse temperatures**

There are many APIs that are labile to heat or tropical temperatures. For example, vitamins as drug substances are very heat – sensitive and degradation frequently leads to loss of potency in vitamin products, especially in liquid formulations.

- **Light-especially UV light**

Several studies have reported that ergometrine as well as methyl ergometrine injection is unstable under tropical conditions such as light and heat, and a very low level of active ingredient was found in many field samples.

- **Humidity**

For hygroscopic products, humidity is considered detrimental to both bulk powder and formulated solid dosage forms. Aspirin and ranitidine are classical examples.

- **Dosage form factors related**

Due do the nature of dosage form these impurities formed. Fluocinonide Topical solution USP, 0.05% (Teva pharmaceuticals USA, Inc., Sellersville, Pennsylvania) in 60 mL bottles, was recalled in the United States because of degradation / impurities leading to sub-potency. In general, liquid dosage forms are very much susceptible to both degradation and microbiological contamination. In this regard, water content, Ph of the solution /suspension, compatibility of anions and

cations, mutual interactions of ingredients, and the primary container are critical factors.

- **Mutual interaction amongst ingredients**

In this case impurities are formed due to mutual amongst ingredients in the formulation. Most vitamins are very labile and on aging they pose a problem of instability in different dosage forms, especially in liquid dosage forms. Because of mutual interaction, the presence of nicotinamide in a formulation containing 4 vitamins (nicotinamide, pyridoxine, riboflavin, and thiamine) causes the degradation of thiamine to a sub-standard level within a 1-year shelf-life of vitamin B-complex injections.

- **Functional group-related typical degradation**

a) Hydrolysis

Hydrolysis is a chemical reaction during which molecules of water (H_2O) are split into hydrogen cations and hydroxide anions (OH) in the process of a chemical mechanism. Examples include benzylpenicillin, barbital, chloramphenicol, chlordiazepoxide, lincomycin, and oxazepam.

b) Oxidative degradation

The chemical process of removing electrons from an element or compound is known as oxidative degradation. Hydrocortisone, methotrexate, adinazolam, hydroxyl group directly bonded to an aromatic ring (e.g., phenol derivatives such as catecholamines and morphine), conjugated diens (e.g., vitaminA and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (e.g., flavorings) are all susceptible to oxidative degradation.

c) Photolytic cleavage

The chemical decomposition of matter due to absorption of incident light is known as photolytic cleavage. Pharmaceutical products are exposed to light while being manufactured as a solid or solution, packaged, held in pharmacy shops or hospitals pending use, or held by the consumer pending use, ergometrine, nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo – oxidation.

1.2. Importance of impurity profiling in regulatory requirements

There is an ever increasing interest in impurities present in API`s.Recently, not only purity prile but also impurity profile has become essential as per various regulatory requirements. In the pharmaceutical world, an impurity is considered as

any other organic material, besides the drug substance, or ingredients, arise out of synthesis or unwanted chemicals that remains with API`s. The impurity may be developed either during formulation, or upon aging of both API`s and formulated API`s medicines. A good illustration of this definition may be identification of impurity in API`s like 1-(1, 2, 3, 4, 5, 6, 7-hexahydro-s-indacen-4-yl)-3-4[-1-hydroxy-1-methyl-ethyl)-furan-2-sulphonylurea using Multidisciplinary approach. The presence of these unwanted chemicals, even in small amount, may influence the efficacy and safety of the pharmaceutical products. Impurity profiling (i.e., the identity as well as the quantity of impurity in the pharmaceuticals), is now gaining critical attention from regulatory authorities. The different pharmacopoeias, such as the British Pharmacopoeia (BP), United States Pharmacopeia (USP), and Indian Pharmacopoeia (IP) are slowly incorporating limits to allowable levels of impurities present in the API`s or formulation. The international conference on Harmonization of Technical Requirements for registration of Pharmaceuticals for Human Use (ICH) has also published guidelines for validation of methods for analyzing impurities in new drug substances, products, residual solvents and microbiological impurities [7,8,9,10]

1.3. Forced Degradation study

According to an ICH guidance document, a stability-indicating method is "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating method accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities". The demonstration of specificity and the ability of the method to monitor a change in the chemical properties of the drug over time, a forced degradation (stress testing) study to be done on the drug substance and drug product. Forced degradation on the drug substance and product will (in addition to establishing specificity) also provide the following information[11]:

1) Determination of degradation pathways of of drug substances and drug products
2) Discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances (eg,excipients)
3) Structure elucidation of degradation products
4) Determination of the intrinsic stability of a drug substance molecule in solution and sold state

5) Reveal the thermolytic, hydrolytic, oxidative, and photolytic degradation mechanism of the drug substance and drug product.

Experimental design

In designing forced degradation studies , it must be remembered that more stenuous conditions than those used for accelerated studies (25^{O} C/60% RH or 40^{o} C/75% RH) should be used. At a minimum , the following conditions should be investigated: (1) acid and base hydrolysis, (2) hydrolysis at various Ph, (3)thermal degradation, (4)photolysis, and (5) oxidation. For the drug substance and drug product. The initial experiments should be focused on determining the conditions that degrade the drug by approximately 10% (care should be taken to avoid under stressing or unduly over-stressing the drug substance or product, for this may lead to aberrant and non-representative results. A degradation level of approximately 10% of the drug substance should be optimal for method optimization). However, some scientists have found it practical to begin at extreme conditions (80^{o}C or even higher, 0.5N NaOH, 0.5N HCl, 3% H2O2) and testing at shorter (2, 5, 8, and 24 hrs, etc) multiple time points, thus allowing for a rough evaluation of rates of degradation.

Forced degradation is only considered complete after the manufacturing process is finalized, formulation established, and test procedures developed and qualified. Temperatures potentially generate a variety of impurities that may differ from that of the photolysis or pH conditions. The degraded drug products provide information into the potential impurities of the drug that may be generated during stability testing. Forced degradation studies can also be used to quickly assess packaging material compatibility or sensitivities [11]

1.4. Thresholds for degradation products in new drug products

Reporting thresholds

Table-1

≥1g	0.1%
> 1g	0.05%

Identification thresholds

Table-2

Maximum daily dose	**Threshold**
< 1mg	1.0% or 5 µg TDI, whichever is lower
1mg – 10 mg	0.5% or 20 µg TDI, whichever is lower
> 10 mg – 2g	0.2% or 3mg TDI , whichever is lower
> 2 g	0.15%

Qualification thresholds

Table-3

Maximum daily dose	**Threshold**
<10mg	1.0% or 50 µg TDI, whichever is lower
10mg – 100 mg	0.5% or 20µg TDI, whichever is lower
>100mg -2g	0.2% or 3mg TDI, whichever is lower
>2g	0.15%

1. Thresholds for degradation products are expressed either as a percentage of the drug substance or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation product is unusually toxic.
2. Higher thresholds should be scientifically justified. [12,13]

1.5. Importance of analytical method for impurity quantification

Use of analytical method is almost importance all phase of synthetic research and related areas. The analytical activities related to the estimation of impurity profiles do not come to an end after the R&D phase of the introduction of a new

drug. It is essential to ensure that no new impurities appear in the course of scaling up procedure and the quantity of the impurities in the bulk drug identified during the synthetic research phase remain below the specification limits. For this reason the analytical control of all steps of scaling up procedure is of key importance. More or less the same applies to the cooperation or drug analysts in the course of production of bulk drug in the routine scale. The identification, structural elucidation, quantification of impurities and degradation products are of prime importance in the course of all the phase of research and development and production of drug formulation. The pharmaceutical analyst should have a clear picture about impurity profile of bulk drug used for the development of the formulation in order to able to differentiate between synthesis related impurities and degradation products. In such a way stability indicating nature of method established. These studies indicate which of the impurities in the bulk drug are degradation products types. The increase of these is expected during the stability studies while the synthesis related impurities are likely to remain constant. A number of articles have stated guidelines and designed approaches for isolation and identification of process-related impurities and degradation products, using Mass spectrometry (MS), Nuclear Magnetic Resonance (NMR), High performance Liquid Chromatography (HPLC), Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS), and Tandem Mass Spectrometry for pharmaceutical substances. [14]

1.6 Instruments used to analyze the impurities

1. Ultra Violet Spectroscopy
2. IR Spectroscopy
3. NMR Spectroscopy
4. Mass Spectrometry
5. Gas Chromatography
6. HPLC

1.7. Chromatography

Chromatography is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a “mobile phase” through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture.

1.7.1. High performance liquid chromatography

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitative the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion [ppt] may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.

1.7.2 Types of HPLC techniques

a) Normal phase chromatography

b) Reversed phase chromatography

a) Normal – Phase HPLC

Normal phase uses a polar normal – phase HPLC (NP-HPLC), or adsorption chromatography, this method separates analytes based on adsorption to a stationary surface chemistry and by polarity NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time.

b) Reversed – Phase HPLC

Reversed phase mode has a non- polar stationary phase and polar mobile phase. With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily.To increase retention time by adding polar mobile phase, thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to more hydrophilic mobile phase. Similarly, Decrease retention time by adding organic solvent to the eluent.

1.7.2.1. Instrumentation

Fig-1

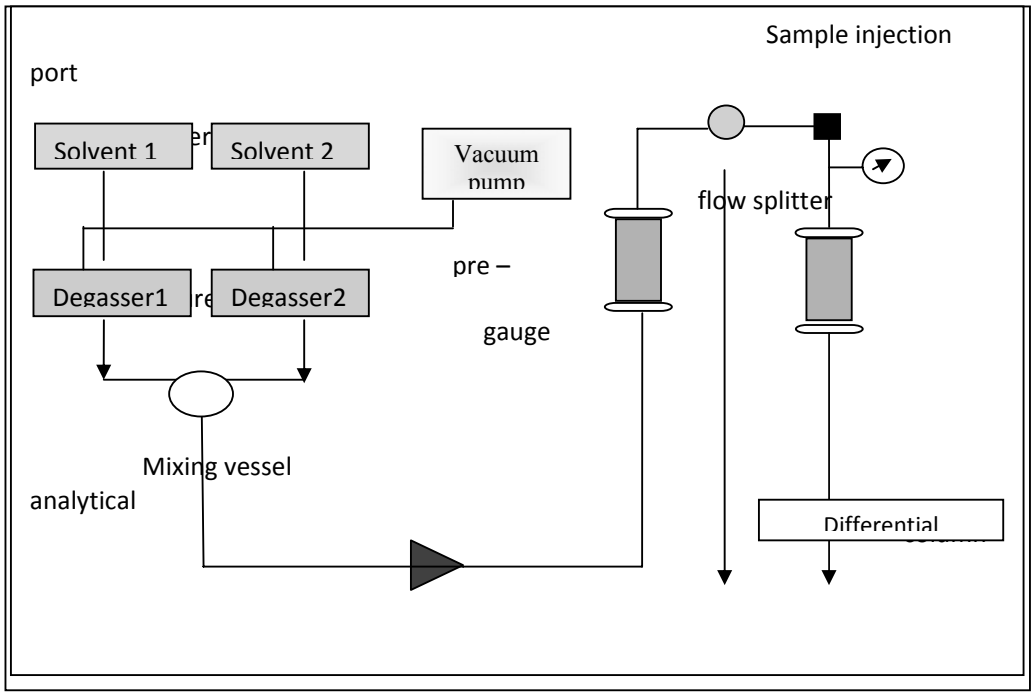


Figure 1. Schematic representation of HPLC

i) Solvent delivery system

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents.

ii) Pump

High pressure pumps are required to force the solvent through packed stationary phase beds. An ideal pump should generate pressure up to 6000 psi (400

bar) with a pulse-free output giving a flow rate from 10mL/min to less than 1µl/min. these types of pumps are available.

They are

- ❖ Reciprocating pumps
- ❖ Displacement pumps
- ❖ Pneumatic pumps

iii) Sample introduction system

A sample introduction system is required to deliver the sample to the head of the HPLC column.

There are two types of injectors

- ➢ Manual injectors
- ➢ Auto samplers

iv) Column

In order to achieve high efficiency of separation, the column material (micro particles, 5-10 µm size) packed in such a way that highest numbers of theoretical plates are possible. Silica ($SiO_2X\ H_2\ O$) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing inter connecting pores. The most popular material is octadecyl – silica), which contains C_{18} chains, but materials with $C_{2,}$ C_6, C_8 and C_{22} chains are also available. During manufacture, such materials may be reacted with a small mono functional silane (e.g.trimethyl chloro silene) to reduce further the number of silanol groups remaining on the surface (end-capping)

v) **Detectors**

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram.

Type of detectors

- ➢ UV Detector
- ➢ Refractive index Detector
- ➢ Fluorometric detection
- ➢ Atomic spectroscopic Techniques
- ➢ Ampherometric Detection
- ➢ Conductivity Detector

1.8. ANALYTICAL METHOD DEVELOPMENT

HPLC method development and validation is important for the analyzing of drugs in my formulation. Whatever method is used for quantitation or identification of drug the method should be a validated one. The method must be able to detect or quantitative the particular drug in presence of other components.

The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. While there are a number of HPLC methods available to the development chemist, perhaps the most commonly applied method is reversed phase chromatography method. The water soluble drug is further differentiated as ionic or nonionic which can be separated by reverse phase. Similarly, the organic soluble drugs can be classed as polar and non polar and equally separated by reverse phase. In some cases the non polar API any have to be separated using adsorption or normal phase HPLC, in which mobile phase would be non polar organic solvent. Samples like proteins, peptides nucleic acids and synthetic polymers are analyzed by using some special columns or ion pair reagents (i.e. 0.1% TFA)[15]

1.8.1 General conditions to initiate HPLC method development

Either isocratic or gradient mode may be used to determine the initial conditions of the separation, following the suggested experimental conditions given in the Table-4.

Selection of initial HPLC chromatographic conditions

Table -4

Chromatographic variables	**Neutral compounds**	**Ionic – acidic compounds (carboxylic acids)**	**Ionic – basic compound (amines)**
Column dimension (length, i.d)	250x4.6 mm.	250x4.6 mm.	250x4.6 mm
Packing material	C_{18} or C_8	C_{18} or C_8	C_{18} or C_8
Particle size	5µm	10µm or 5µm	10µm or 5 µm
Mobile phase Solvents A and B	Water – acetonitrile	Buffer - acetonitrile	Buffer – acetonitrile
Ratio	50:50	20:80	20:80
Buffer and pH	No buffer required	Phosphate 25Mm 3.0& 7.5	Phosphate 25Mm 3.0& 7.5
Peak modifier	Do not use initially	1% v/v acetic acid	0.1% v/v triethylamine
Flow rate	1.0 to 2.0 ml/min	1.0 to 2.0 ml/min	1.0 to 2.0 ml/min
Temperature	Ambient	Ambient	Ambient
Injection volume	10µl to 25 µl	10µl to 25 µl	10µl to 25 µl
Sample concentration	< 100 µg	< 100 µg	< 100 µg

Column & mobile phase selection shall be done as per the table given below

Table -5

Method /Description/Column	**When the method preferred**
Reverse – Phase HPLC	
Water / organic mobile phase	For neutral and non ionized compounds that dissolve in water / organic mixtures.
Column: C18, C8, Phenyl, cyano, and Trimethylsilyl (TMS) Columns.	
Normal Phase HPLC	
Mixture of organic solvents as mobile phase	For samples that do not dissolve in water / organic mixture.
Column: Silica, Cyano and Amino columns	

1.8.2. Selection of mobile phase

The selection of the mobile phase mainly based on the solubility and polarity of the compound. Usually, in RP-HPLC method water and organic solvents are used as the mobile phase. In NP-HPLC method non polar solvents like Hexane and THF were used. If the sample contains ionic or ionizable compounds, then use of a buffered mobile phase to ensure the reproducible results. Under unfavorable circumstances, pH changes as little as 0.1 pH units can have a significant effect on the separation. On the other hand properly used buffer allows controlling the ph easily. Buffer works best at the pKa values of its acid. At this pH, the concentration of the acidic form and the basic form of the buffering species are equal, and the buffering capacity is maximum. Phosphate has three pKa values in the range of interest for silica based chromatography at pH 2, pH 7 and pH 12.32. The pKa of acidic buffer is 4.75. Citrate has three pKa values 3.08, 4.77 and 6.40. Between citrate and phosphate buffers, the entire pH range useful for silica chromatography can be covered. In many cases, a silanophilic interaction causes tailin, mainly for the basic compounds due to ion-exchange interaction. This can usually be reduced or suppressed by the use of mobile phases modifies (0.1% v/v triethylamine for basic analyte or 1% v/v glacial acetic acid for the acidic analyte), or a combination thereof. Whenever buffers or other mobile phase activities are used, check the solubility in mobile phase. This is especially true for gradient applications. Acetonitrile is the preferred organic modifier

in reversed phase chromatograpy. Acetonitrile base mobile phases can give up to two fold lower pressure drop that can methanol based mobile phases at equal flow rate. This means that column efficiency is higher. The elution strength increases in the order methanol, acetonitrile and tetrahydrofuran. The retention changes by roughly 1% for every 1% change in the concentration of organic modifier.

<-----Non-polar-----Moderately polar----Polar----->

C18<C8<C6<Phenyl<Amino<Cyano<Silica.

Experiments are to be conducted using different columns with different mobile phase to achieve best separation in chromatography. A column which gives separation of all the individual impurities and degradants from each other and from API peak and which is rugged for variation in mobile phase shall be selected.

1.8.3 Selection of buffer

In reversed phase chromatography mobile phase pH values are usually between 2.0 and 7.5 buffers are needed when analyte is ionizable under reversed phase conditions or the sample solution is outside this pH range. Analyte ionisible under reverse phase conditions often have amine or acid functional group with pKa between 1.0 and 11.0. A form, whether ionic or neutral. If the sample solution is at pH damaging to the column, the buffer will quickly bring the pH of the injected solution to a less harmful pH.

If the analyte contain only amine functional group buffer selection is easier. Most amine will be in cationic form at pH values less than 9.0, so any buffer effective at pH 7.0 or lower will work. Buffer at pH 7.0 are used, even though pH of water is 7.0, because amine retention and peak shapes are pH dependent. As pH is lowered amine retention time shortens and peak shape sharpens as the buffer protonates the acid silanols on silica surface. Any buffer with pKa less than 7.0 is suitable, but we have found potassium phosphate at pH 3.0 is best for amines. In both condition (acidic and alkaline) potassium phosphate buffer pH 3.0 works well in general is an excellent buffer for analyte that contains acidic and amine functional groups. The potassium salt works better than the sodium salt for amines. It is important to use the buffers with suitable strength to cope up for the injection load on the column otherwise peak tailing may arise during chromatography. Therefore, strength of the buffer should be suitable enough to take injection load on the column so that peak tailing is avoided.

The retention time also depends on the molar strength is inversely proportional to the retention times. Ideally strength of the buffers shall be between 0.05M to 0.20M. The selection of buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be increased, if necessary, to achieve the required separation. But it is to be ensured that the higher buffer strengths shall not result in precipitants and turbidities either in mobile phase or in standard and test solution while allowed to stand in bench top or refrigerator. Experiments shall be conducted using different strengths to obtain the required separations.The buffers having a particular strength which gives separation of all individual impurities and degradation product.

1.8.4. Selection of pH

pH is another factor in the resolution that will affect the selectively of the separation in reversed-phase HPLC. In reverse –phase chromatography sample retention(k`) increases when analyte is more hydrophobic (nonpolar).Sample retention (k`) decreases when the analyte is more hydrophilic (polar). Thus when an acid or base undergoes ionization it becomes more hydrophilic and less interacting with column binding sites. When the pH value of the value of the mobile phase equal to the pKa value of the analyte, it is said to be half ionized, i.e. the concentration of the ionized and unionized species are equal. As mostly all of the pH caused changes in the retention occur within ±2.0 pH unit of pKa value, it is the best to adjust the mobile phase to pH value at least ±2.0 pH unit above or below the pKa to ensure practically 100% ionization of analyte for retention purpose. Generally at low pH peak tailing is minimized and method ruggedness is maximized. On the other hand, operating in the intermediate pH offers an advantage in increased analyte retention and selectivity. pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Drug molecules retention time depending on the pKa value for example Acids shows an increase in retention as the pH is reduced, while base a show a decrease, experiments shall be conducted using buffers having different pH to obtain the required separations. It is importants to maintain the pH of the mobile phase in the range of 2.0 to 0.8 as most columns does not withstand to the pH which are out of range. This is due to the fact that the siloxane linkages are cleaved below pH 2.0, while at values above 8.0 silica

may dissolve. If a pH outside the range of 2.0 to 8.0 is found to be necessary, packing materials which can withstand to that ranges shall be chosen.

1.8.5 Selection of column

The HPLC column is the heart of the method. The column must posses the selectivity, efficiency and reproducibility to provide good separation. Commonly used reversed phase are cross linking Si-OH groups with alkyl chains like, C_8 (octylsilane), C_{18} (octadecyl silane) and nitrile groups (CN), phenyl groups ($-C_6H_6$) and amino groups ($-NH_2$). They are chemically different phases and demonstrate significant changes in the selectivity using the same mobile phase. For example, a C_8 phase (reverse phase) can provide a further time saving over a C_{18} as it doesn`t retain analyte as strongly as the C_{18} phase.

The following are the parameters of a chromatographic column which are to be considered while choosing a column for separation of impurities and degradants.

1. *Length and diameter of the column*

Column diameters employed in chromatography vary widely. In general, analytical columns have small diameter and preparative columns have wide diameters. Larger column (but in this case packed) having diameters between 0.5-2 mm are employed for similar purpose in LC to produce very fast separations and high efficiencies. These LC column range in length from 10 cm to 2 m, the longer columns being rather difficult to pack. Packed columns having diameters ranging from 2 to 6 mm ID are used in both GC and LC.

2. Packing material

Porous silica particles are the most common substract material used for HPLC column packing. Silica-based columns can withstand high pressures, are compatible with most organic and aqueous mobile-phase solvents, and come in a wide range of bonded phases. Silica-based columns are often used for separations of low molecular weight analytes using mobile phase solvents and samples with a pH range of 2 to 7.5.

Shape of the particles

Irregular Shape

The first available HPLC columns were packed using irregularly shaped silica particles. Because of this, many standard analytical methods are still based on these materials. Irregular particles are also used in large-scale preparative applications because of their high surface area, capacity and low cost.

Spherical Shape

The majority of new HPLC methods are performed on spherical shaped or spheroidal(Almost spherical) particles. Sphericals particles provide higher efficiency, better column stability and lower back – pressures compared to irregularly shaped particles.

3. Size of the particles

Particle size affects the back-pressure of the column and the separation efficiency. Column back-pressure and column efficiency are inversely proportional to square of the particles diameter. This means that as the particle size decreases, the column back – pressure and efficiency increase. Highly efficient, small-particle (3μm and 4 μm) columns are ideal for complex mixture with similar components. Fast high-resolution separations can be achieved with small particles packed in short(10-50 mm length) columns.

4. *Carbon loading.*

The carbon load is measure of the amount of bonded phase bound to the surface of the packing. High carbon loads provide greater column capacities and resolution. Low carbon loads produce less retentive packings and faster analysis times.

5. *Pore volume.*

Pore volume is a measurement of the empty space within a particle. Pore volume is a good indicator of the mechanical strength of a packing. Particles with large pore volumes are typically weaker than particles with small pore volumes. Pore volumes of 1.0 mL/g or less are recommended for most HPLC separations. Pore volumes of greater than 1.0mL/g are preferred for size-exclusion chromatography and useful for low-pressure methods.

6. Surface area

The physical structure of the particle substrate determines the surface area of the packing material. Surface area is determined by pore size. Pore size and surface area are inversely related. A packing material with a small pore size will have a large surface area, and vice versa. High surface area materials offer greater capacity and longer analyte retention times. Low surface area packings offer faster equilibration time and are often used for large molecular weight molecules.

7. *End capping.*

A reversed-phase HPLC column that is end-capped has gone through a secondary bonding step to cover unreacted silanols on the silica surface. End-capped packing materials eliminate unpredictable secondary interactions. Basic produce asymmetric tailed peaks on non end-capped columns, requiring the addition of modifiers to the mobile phase. Non end capped materials exhibit different selectivity than end-capped.

8. *Column dead volume*

An accurate estimation of the dead volume is very important when measuring retention data, particularly if the corrected retention volume is small and commensurate with dead volume. The dead volume comprises a number of different components a number of different components of the column volume, and the distribution of the total column volume into those parts of chromatographic significance is a little complicated. [16]

1.8.6. Selection of Column temperature

Temperature variation over the course of a day has quite significant effect on HPLC separations. This can even occur in air conditioned rooms. While temperature is a variable that can affect the selectivity, its effect is relatively small. Always it is preferable to optimize the chromatographic conditions with column temperature as ambient. However, if the peak is symmetry could not be achieved by any combination of column and mobile phase, then the column temperatures above ambient can be adopted. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions. When found necessary, the column temperatures between 30^{o}C and 80^{o}C shall be adopted. If a column temperature of above 80^{o}C is found to be necessary, packing materials which can withstand to that temperature shall be chosen.

1.8.7 Selection of flow rate

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure.

Flow rate shall be selected based on the following data.

1. Retention times
2. Column back pressures
3. Separation of impurities (resolution)
4. Peak symmetries

Preferably the flow rate shall be not more than 2.5 ml/min. Check the ruggedness of the method by varying the flow rate by = 0.2 ml from the selected flow rate. Select the flow rate which gives least retention tomes, good peak symmetries, least back pressures and better separation of impurities from each other and from API peak.

1.8.8. Selection of solvent delivery system

Chromatographic separation with a single eluent (isocratic elution) i.e.: All the constituents of the mobile phase is mixed and pumped together as single eluent is always preferable. Gradient elution is a powerful tool in achieving separation between closely eluting compounds or compounds having widely differing in polarities.The important feature of the gradient elution which makes it a powerful tool is that the polarity and ionic strength of the mobile phase can be changed (can be increased or decreased) during the run. Conduct experiments using different mobile phase combinations and different gradient programme to achieve separation of all the impurities and degradants from other and from API peak. In general, while running a gradient, two mobile phases having different composition is kept in different channels.

1.8.9. Selection of detector wavelength

Selection of detector wavelength is a critical step in finalization of the analytical method for impurities and degradants. Inject the impurity and API standard solutions into the chromatographic system with photodiode array detector and collect the spectra. Also conduct forced degradation studies and collect the UV spectra of all the major degradation products. Overlay the spectra of all the compounds and select a wavelength which is most common and gives higher responses for all compounds.

1.8.11. Selection of test concentration, injection volume

The test concentration is generally chosen based upon the response of API peak and impurities at the selected detector wavelength. The test concentration shall be finalized after it is proved that API is completely extractable at the selected test concentration. Generally an injection volume of 10 to 20µl is recommended for

estimation of impurities. If the extractions are found to be difficult, then the test concentrations can be kept low and the injection volume can be increase up to 50µl. But it is to be ensured that at the selected injection volume the column is not overloaded, resolution between individual impurities and the peak symmetries are not compromised. After the test concentration and the diluents is finalized, prepare a test solution and keep the filtered solution in closed condition in a stoppered flask on the bench top and observe for any precipitation or turbidity after 24 hours. The solution should not show any turbidity / precipitation [17,18]

1.9. Analytical Method Validation

Validation definition

Validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

Typical validation characteristics which should be considered are listed below.

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity
- Range
- Robustness
- Ruggedness

Furthermore revalidation may be necessary in the following circumstances

- Changes in the synthesis of the drug substance
- Changes in the composition of the finished product
- Changes in the analytical procedure

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well[13].

1.9.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

1.9.2 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. This is sometimes termed trueness.

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.

For the drug product, this is performed frequently by the addition of known amounts of drug by weight or volume to the placebo formulation working in the linear range of detection of the analyte

It is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with confidence intervals. It should be assessed using a minimum of three concentrations and three replicates of each concentration.

Acceptance criteria: based on statistical significance of the test of the null hypothesis that the slope is 1.0 is not an acceptable approach.

1.9.3. Precision

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. It is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurements. It can be sub divided into three categories.

A) Repeatability

B) Intermediate precision

C) Reproducibility

A) Repeatability

a) Injection Repeatability

It is measured by multiple injections of a homogeneous sample indicates the performance of the HPLC instrument under the chromatographic conditions and day tested.

Recommendations

i) As part of methods validation, a minimum of 10 injections with an RSD of $\leq$ 1 % is recommended.

b) Analysis repeatability

It consists of multiple measurements of a sample by the same analyst under the same analytical conditions. For practical purpose, it is often combined with accuracy and carried out as a single study.

B) Intermediate precision (part of ruggedness)

The long – term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method` intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these. The objective of validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

C) Reproducibility

The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts and by using operational and environmental conditions that may differ from but are still within the specified parameters of the method.

a) Typical variations affecting a method`s reproducibility
b) Differences in room temperature and humidity
c) Operators with different experience and thoroughness
d) Equipment with different characteristics
e) Variations in material and instrument conditions
f) Equipment and consumables of different ages
g) Columns from different suppliers or different batches
h) Solvents, reagents and other materials with different quality.

Acceptance criteria

1) % RSD should be less than 10.0% (For residual solvents)
2) % RSD should be less than 1.0% (For Assay)
3) %RSD should be less than 5.0% (For related substances)

1.9.4. Detection limit

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.

LOD = 3.3 σ /S

Where σ is the standard deviation of the response and S is the slop of the calibration curve (of the analyte).

1.9.6 Linearity

The linearity of an analytical procedure is ability to obtain test results which are directly proportional to the concentration of analyte in the sample. The goal is to have a model, whether linear or nonlinear, that describes closely the concentration-response relationship.

Linearity should be established across the range of the analytical procedure. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted.

For the linearity study, a minimum of five concentrations normally be used. The following minimum specified ranges should be considered.

a) Assay of a drug substance (finished product): from 80% to 120% of the test concentration.
b) Determination of an impurity: from 50% to 120% of the acceptance criterion.
c) For content uniformity: a minimum of 70% to 130% of the test concentration, unless a wider or more appropriate range based on the nature of the dosage form.

1.9.7. Range

The range of suitability of a given analytical procedure is the interval between minimum and maximum concentrations of compound to be determined in which.

1) The linearity is observed
2) The characteristics of repeatability fall within permissible limits
3) The accuracy is maintained at a sufficiently high level.

This range could be determined using threshold RSD values obtained in the course of validation of the linearity and precision.

1.9.8. Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as “a measure of its capacity to remain unaffected by small, but deliberate

variations in method parameters". The most important aspect of robustness is to develop methods that allow for expected variations in the separation parameters.

1) The content of the organic solvent in the eluent (±2%)
2) The amount of additives (salts, ion-pair reagents etc) in the eluent (±10%)
3) pH of the buffer solution (±0.5)
4) HPLC column temperature (±5°c)
5) Time of extraction of the analyzed compound from a drug eluent (±20%)
6) Extractant composition (±5%)
7) Eluent concentration gradient (±2%)
8) Mobile phase flow rate
9) Column type and / or manufacturer

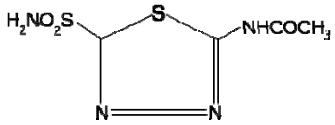
1.9.9. Ruggedness

The long – term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method`s intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these. The objective of validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.[19,20,21]

2. DRUG PROFILE

Acetazolamide extended release capsules are an inhibitor of the enzyme carbonic anhydrase.

Acetazolamide extended release capsules is a white to faintly yellowish white crystalline, odorless powder, weakly acidic, very slightly soluble in water and slightly soluble in alcohol. The chemical name for DIAMOX is N-(5- Slfamoyl-1,3,4-thiadiazol-2-yl) acetamide and has the following chemical structre:



Formula weight = 222.24

Introduction

Acetazolamide extended release capsules are extended release capsules, for oral administration, each containing 250 mg of acetazolamide and the following inactive ingredients:

Microcrystalline cellulose, sodium lauryl sulfate and talc.

The ingredients in the capsule shell are D&C red no. 28,D&C yellow no. 10,FD&C blue no. 1 aluminum lake, FD&C blue no 2aluminum lake, FD&C red no. 40 aluminum lake, pharmaceutical glaze, propylene glycol and synthetic iron oxide.[22,23,24]

DOSAGE AND ADMINISTRATION

Glaucoma

The recommended dosage is I capsule (250 mg) two times a day. Usually I capsule is administered in the morning and I capsule in the evening. It may be necessary to adjust the dose, but it has usually been found that dosage in excess of 2 capsules (1g) does not produce an increased effect. The dosage should be adjusted with careful individual attention both to symptomatology and intraocular tension. In all cases, continuous supervision by a physician is advisable.

In those unusual instances where adequate control is not obtained by the twice a day administration of Acetazolamide extended release capsules the desired control may be established by means of Acetazolamide extended release capsules. Use tablets or parenteral in accordance with the more frequent dosage schedules recommended for these dosage forms, such as 250 mg every four hours, or an initial dose of 500 mg followed by 250 mg or 125 mg every four hours, depending on the case in question.

Acute Mountain Sickness: Dosage is 250 mg to 500 mg daily, in divided doses using tablets or extended release capsules as appropriate. In circumstances of rapid ascent, such as in rescue or military operations, the higher dose level of 500 mg is recommended. It is preferable to initiate dosing 24 to 48 hours before ascent and to continue for 48 hours while at high altitude, or longer as necessary to control symptoms.

NDC 51285 – 754 – 02

Store at controlled room temperature 20 ^{o} to 25 ^{o} C (68 ^{o} to 77^{o} F)

SIDE EFFECTS

Body as whole:	headache, malaise, fatigue, fever, pain at injection site, flushing, growth retardation children ,flaccid paralysis, anaphylaxis.
Digestive:	gastrointestinal disturbances such as nausea, vomiting, diarrhea
Hematological / Lymphatic:	Blood dyscraias such as aplastic anemia, agranulocytosis, leucopenia, throm bocytopenic purpura, melena
Hepato-biliary disorders:	abnormal liver function, cholestatic jaundice, hepatic insufficiency, fulminant hepatic necrosis
Metabolic/nutritional:	metabolic acidosis, electrolyte imbalance, including hypokalemia, hyponatremia, osteomalacia with long – term phenytoin therapy, loss of appetite, taste alteration, hyper/hypoglycemia
Nervous:	Drowsiness, peresthsia (including numbness and tingling of extremities and face), depression, excitement, ataxia, confusion, convulsions dizziness.
Skin:	Allergic skin reactions including urticaria, photosensitivity, stevens-johnson syndrome, toxic epidermal necrolysis
Special senses:	hearing disturbances, tinnitus, transient myopia

Urogenital:	crystalluria, increased risk of nephrolithiasis with long term therapy, hematuria, glycosuria, renal failure polyuria.

DRUG INTERACTIONS

Acetazolamide extended release capsules modifies phenytoin metabolism with increased serum levels of phenytoin. This may increase or enhance the occurrence of osteomalacia in some patients receiving chronic phenytoin therapy. Caution is advised in patients receiving chronic concomitant therapy. By decreasing the gastrointestinal absorption of primidone, Acetazolamide extended release capsules may decrease serum concentrations of primidone and its metabolites,with a consequent possible decrease in anticonvulsant effect, caution is advised when beginning , discontinuing , or changing the dose of Acetazolamide extended release capsules in patients receiving primidone.

Because of possible additive effects with other carbonic anhydrase inhibitors, concomitant use is not advisable.

Acetazolamide may increase the effects of other folic acid antagonists.

Acetazolamide decreases urinary excretion of amphetamine and may enhance the magnitude and duration of their effect.

Acetazolamide reduces urinary excretion of quinidine and may enhance its effect. Acetazolamide may prevent the urinary antiseptic effect of methenamine acetazolamide increases lithium excretion and the lithium may be decreased.

Acetazolamide and sodium bicarbonate used concurrently increases the risk of renal calculus formation.

Acetazolamide may elevate cyclosporine levels.

PRECAUTIONS

General

Increasing the dose not increase the dieresis and may increase the incidence of drowsiness and /or pareshesia. Increasing the dose often results in a decrease in dieresis. Under certain circumstances, however, very large doses have been given in conjunction with other diuretics in order to secure dieresis in complete refractory failure.

Laboratory Tests

To monitor for hematologic reactions common to all sulfonamides, it is recommended that a baseline CBC and platelet count be obtained on patients prior to initiating therapy and at regular intervals during therapy. If significant changes occur, early discontinuance and institution of appropriate therapy are important. Periodic monitoring of serum electrolytes is recommended.

Carcinogenesis, mutagenesis, impairment of fertility

Long –term studies in animals to evaluate the carcinogenic potential of Acetazolamide extended release capsules have not been conducted in a bacterial mutagenicity assay, Acetazolamide extended release capsules was not mutagenic when evaluated with and without metabolic activation.

The drug had no effect on fertility when administered in the diet to male an female rats at a daily intake of up to 4 times the recommended human dose of 1000 mg in a 50 kg individual

Pregnancy: Teratogenic effects: Pregnancy Category C

Acetazolamide, administered orally or ally or parenterally, has been shown to be teratogenic (defects of the limbs) in mice, rats, hamsters, and rabbits. There are no adequate and well controlled studies in pregnant women. Acetazolamide should be used in pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing mothers

Because of the potential for serious adverse reactions in nursing infants from Acetazolamide extended release capsules a decision should be made whether to discontinue the drug taking into account the importance of the drug to the mother. Acetazolamide should only be used by nursing women if the potential benefit justifies the potential to the child

Pediatric Use

The safety and effectiveness of Acetazolamide extended release capsules in pediatric patients below the age of 12 years have not been established. Growth retardation has been reported in children receiving long term therapy, believed secondary to chronic acidosis.

Geriatric Use

Metabolic acidosis, which can be severe, may occur in the elderly with reduced renal function.

In general, dose selection for an elderly patent should be cautious, usually starting at the low end of the dosing range, reflecting the greater frequency of decreased hepatic, renal or cardiac function, and concomitant disease or other drug therapy.

Overdose

No specific antidote is known. Treatment should be symptomatic and supportive.

Electrolyte imbalance, development of an acidotic state, and central nervous system effects might be expected to occur, serum electrolyte levels (particularly potassium) and blood pH levels should be monitored.

Supportive measures are required to restore electrolyte and pH balance. The acidotic state can usually be corrected by the administration of bicarbonate.

Despite its high intraerythrocytic distribution and plasma protein binding properties. Acetazolamide extended release capsules may be dialyzable. This may be particularly important in the management of Acetazolamide extended release capsules overdosage when complicated by the presence of renal failure

Contraindications

Hypersensitivity to acetazolamide or any excipients in the formulation. Since acetazolamide is a sulfonamide derivative, cross sensitivity between acetazolamide, sulfonamides and other sulfonamide derivatives is possible.

Acetazolamide therapy is contraindicated in situations in which sodium and / or potassium blood serum levels are depressed, in cases of marked kidney and liver disease or dysfunction, in suprarenal gland faiure, and in hyperchloremic acidosis. It is contraindicated in patients with cirrhosis because of the risk of development of hepatic encephalopathy.

Long term administration of Acetazolamide extended release capsules IS contraindicated in patients with chronic non congestive angle closure glaucoma since it may permit organic closure of the angle to occur while the worsening glaucoma is masked by lowered intraocular pressure.

Uses:

Acetazolamide is used to prevent and reduce the symptoms of altitude sickness. This medication can decrease headache, tiredness, nausea, dizziness, and shortness of breath that can occur when your climb quckly to high altitude (generally above 10,000 feet/3,048 meters) it is particularly useful in situations when you cannot make a slow ascent. The best ways to prevent altitude sickness are climbing slowly, stopping for 24 hours during the climb to allow the body to adjust to the new height, and taking it easy the first 1 to 2 days.

This drug is also used with other medications to treat a certain type of eye problem (open – angle glaucoma). Acetazolamide is a "water pill" (diuretic). It decreases the amont of fluid that can build up in the eye. It is also used to decrease a buildup of body fluids (edema) caused by congestive heart failure or certain medications. Acetazolamide can work less well over time, so it is usually used only for a short period.

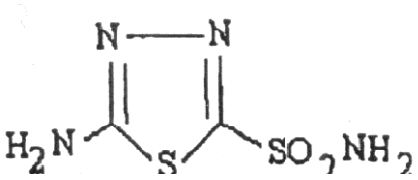
It has also been used with other medications to treat certain types of seizures.

Side effects

Dizziness, lightheadedness and an increased amount of urine may occur , Blurred vision, dry mouth , drowsiness, loss of appetite, stomach upset, headache , tiredness and allergic reaction.

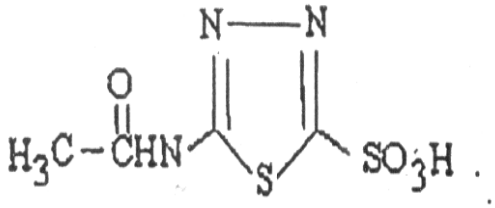
IMPURITIES:

Impurity – D (Process related degradation impurity)



(5 – amino, 1,34, thiadiazole -2, sulphonaminde (impurity – D)

Impurity-E (Forced degradation impurity)



5-acetamido-I,3,4-thiadiazole -2-sulphonic acid (Impurity-E)

Impurity limits as per finished product specification[6]

Table -6

Impurity Name	**Limit**
Impurity – D	NMT 0.15 %
Impurity – E	NMT 0.15%

3. LITERATURE REVIEW

B.Ihssane, et al., This paper describes the optimization and validation of an analytical method for HPLC analysis of acetazolamide in tablets using BoxBehnken design. This multivariate approach enables substantial improvement of chromatographic performance using fewer experiments, without additional cost of columns orth equipment. By use of quadratic regression analysis, equations were developed describing the behavior of the response as simultaneous functions of the selection independent variables. Accordingly, the optimum conditions were determined. Further quality control samples there were use of a C_{18} column with acetonitrile phosphoric acid buffer solution (0.01M, pH 7.1)15:85 (v/v) as mobile phase at a flow rate 0.1 mL min^{-1} Diode-arrey detection was performed at 266nm. The method was validated, in accordance with ICH guidelines, for accuracy, precision selectivity, and linearity. The method is simple, rapid, sensitive and accurate and the retention time is less than 4min. the method is therefore suitable for routine quality-control monitoring of acetazolamide in tablets[25].

B.Ramesh et al., A new quantitative densitometric High performance Thin Layer Chromatographic the analysis and stability indicating determination of acetazolamide, both in bulk and formulations. Acetazolamide from the formulations gel 60 F254 HPTLC plates with toluene: acetone: methanol (6:2:2 v/v), as mobile phase. Densitometric quantification was performed at I= 270 nm by refle resolved band for the main drug (Rf 0.53±0.02). Linear regression concentration analysis data for mean calibration plots showed good linear relationship in the coefficient, slope and intercept of 0.9991±0.00047, 11.28±0.351 and 131.32±1.52 respectively.The minimum amount of acetazolamide that could be authentically 40.23 ng/spot, respectively. The drug was subjected to acid, base, neutral hydrolysis, oxidation, photolysis and thermal decomposition to establish a validate drug undergoes degradation under acid, base, neutral and oxidative stress conditions. Also the degraded products were well separated from pure drug with proposed method was validated with respect to linearty, precision, accuracy, specificity and robustness. Further, the proposed HPTLC method was employed to and observed that the degradation process followed pseudo first order kinetics. Thus, the Arrhenius plot was constructed and activation energy was calculated[26].

Dennis J. Capron et al., A high – performance liquid chromatographic method for the determination of acetazolamid concentrations of acetazolamide were spiked with the internal standard, Sulfadiazine.Sam 25 s in boiling water. All samples were extracted with ethyl acetate; a phosphate buffer which was then washed with ether. Separation of acetazolamide and internal standard from acetonitrile- methanol-acetate buffer (pH 4.0). The eluant was monitored at 254 nm. All ca the method to human pharmacokinetic studies was demonstrated[27].

Shalini pachauria et al., A simple, precise, fast and gradient, high performance liquid chromatographic (HPLC) method was developed and validated for the determination of Aliskiren, Ramipril, Valsartan and Hydrochlorothiazide in solid dosage forms. The quantitative determination of analyte(s) was performed on a PUROSPHERE STAR rp 18e analytical column (250x4.6 mm) with 0.2 % v/v TEA buffer (pH: 3.0): CAN as mobile phase, at a flow rate of 1.0 ml min- 1. Detection was made by extracting PDA spectra at 215 nm respectively. During method validation, parameters such as precision, linearity, stability, Robustness, Ruggedness and specificity were evaluated, which remained within acceptable limits. The method has been successfully applied to assess the assay of solid dosage formulations[28].

M.I. Walash et al., A simple and sensitive spectrophotometric method was developed for the determination of acetazolamide (ACM) in pure form and pharmaceutical preparations. The proposed method is based on the complex formation of acetazolamide with Palladium (II) chloride in acetate buffer pH 5.4 and measuring the absorbance at 308 nm. The absorbance- concentration plot was rectilinear over the concentration range of 5-70 μg/ml with a minimum detection limit (LOD) of 0.98 μg/ml, limit of quantification (LOQ) of 2.96 μg/ml, and a molar absorptivity $\zeta = 2.7 \times 103$ L/mol.cm. The factors affecting the absorbance of the formed complex were carefully studied and optimized. The composition of the complex as well as its stability constant was also investigated. The proposed method was applied for the determination of acetazolamide in its tablets and the results obtained were favorably compared with those obtained using the official method. A proposal of the reaction pathway was postulated[29].

Morra V et al., An analytical procedure was developed for the fast screening of 16 diuretics (acetazolamide, althiazide, amiloride, bendroflumethiazide, bumetanide, canrenoic acid, chlorthalidone, chlorthiazide, clopamide, ethacrynic acid, furosemide, hydro chlorthiazide, hydroflumethiazide, indapamide, triamterene, trichlormethiazide) and a masking agent (probenecid) in human urine. The whole method involves three analytical steps, including (1) liquid/liquid extraction of the analytes from the matrix, (2) their reaction with methyl iodide at 70 degrees C for 2 h to form methyl derivatives, (3) analysis of the resulting mixture by fast gas chromatography/electron impact mass spectrometry (fast GC/EI-MS). The analytical method was validated by determining selectivity, linearity, accuracy, intra and inter assay precision, extraction efficiencies and signal to noise ratio (S/N) at the lowest calibration level (LCL) for all candidate analytes. The analytical performances of three extraction procedures and five combination of derivatization parameters were compared in order to probe the conditions for speeding up the sample preparation step. Limits of detection (LOD) were evaluated in both EI-MS and ECNI-MS (electron capture negative ionization mass spectrometry) modes, indicating better sensitivity for most of the analytes using the latter ionization technique. The use of short columns and high carrier gas velocity in fast GC/MS produced efficient separation of the analytes in less than 4 min, resulting in a drastic reduction of the analysis time, while a resolution comparable to that obtained from classic GC conditions is maintained. Fast quadrupole MS electronics allows high scan rates and effective data acquisition both in scan and selected ion monitoring modes[30].

Philip T. A simple, rapid and specific HPLC method has been developed to determine acetazolamideconcentrations in human plasma. The assay procedure requires only 250 µl of sample with direct injection of the organic supernatant after protein precipitation with acetonitrile. Chlorothiazide was used as an internal standard. A reversed-phase C18 µBondapak column was employed for the chromatographic separation. The eluent was monitored at 265 nm using a UV variable wavelength detector. The retention times for acetazolamide (ACZ) and chlorothiazide (CTZ) were 6 and 8 min respectively. A linear relationship (r).995) was obtained over the 1-20 µg/ml concentration range. The limit of sensitivity for ACZ was 0.5 µg/ml,with greater than 85% recovery of ACZ and internal standard. The method was

applied to human plasma samples obtained after administration of a 250 mg acetazolamide tablet[31].

V. Das Gupta Stability-indicating reverse phase high-pressure liquid chromatography methods to quantify acetazolamide in pharmaceutical dosage forms have been developed. The methods are accurate and precise with percent relative standard deviations based on six injections of 0.9 with a semipolar column and 0.4 with a nonpolar column. No preliminary extraction procedure is required to assay the acetazolamide sodium in vials and a very simple extraction is needed to extract acetazolamide from the tablets or contents of the capsules. Acetazolamide appears to be very unstable in the presence of 0.1N NaOH with a half-life of about 14 days. It underwent hydrolysis to acetic acid and 5-amino-1, 3, 4-thiadiazole-2 sulfonamide. The hydrolysis followed first-order law with a K value of 0.0495 day-1 at 25°[32].

W. F. Bayne* A method for the analysis of acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, sensitive to 25 ng/ml in plasma, was developed. After extraction of acetazolamide and its propionyl analog, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide, the internal standard, from plasma with ethyl acetate and removal of lipids from the residue of the ethyl acetate extract with methylene chloride, the sulfonamides were chromatographed on an octadecyl trichlorosilane bonded phase using high-pressure liquid chromatography. The method was developed to study plasma level profiles of different dosage forms of acetazolamide[33].

Gomaa ZS . A high performance liquid chromatographic assay for the quantitation of acetazolamide in both tablet and injection form is described. Acetazolamideis extracted with 0.005 M NaOH solution containing 0.3 mg/mL sulphadiazine (internal standard). A commercially available mu-Bondapak C18 cartridge column was used for the separation together with a mobile phase made of acetonitrile, methanol and sodium acetate buffer mixture (10:2:88) (pH 4) at a flow-rate of 4 mL/min. Retention times of about 2.50 and 3.36 min were obtained for the drug and the internal standard, respectively[34].

4. AIM AND OBJECTIVE

Acetazolamide is a carbonic anhydrase inhibitor, mainly used to reduce intraocular pressure in the treatment of long term management of glaucoma

A few chromatographic method have appeared in the literature for the quantification of Acetazolamide in human and rat plasma. Several other method have been published for quantification of Acetazolamide in human urine

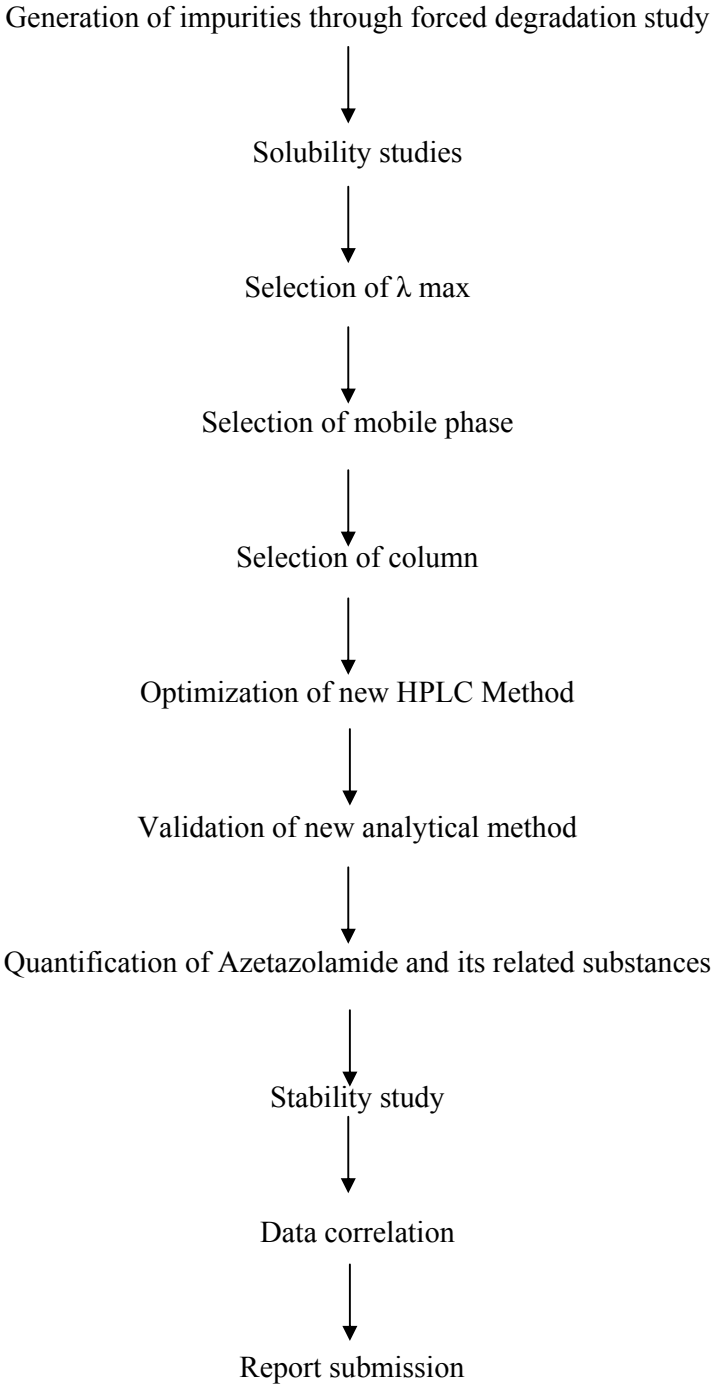
The aim of the current study was to develop a validated, specific and stability indicating reverse phase liquid chromatographic method for the quantitative determination of Acetazolamide and its related substances.

This study is designed to develop a novel method for the estimation of Acetazolamide SR capsules and its related substance like impurity –D (process related degradation impurity) and impurity–E (Forced Degradation impurity).

Objective

1. To develop a novel stability indicating method for the estimation of Acetazolamide and its related substance.
2. To validate the developed method for the estimation of selected drug and its related substances in sustained release formulation as per ICH guidelines

5. PLAN OF WORK



6. MATERIALS AND METHODS

MATERIALS

1. **Chromatographic system**

   Instrument : High Performance Liquid Chromatography system(HPLC)

   Instrument ID : QHPL 4012, QHPL 4013,QHPL4009

   Make : Waters

2. **Anaylytical balance (2mg-200g)**

   Instrument ID : IEWS4034,IEWS4038, IEWS4037

   Make : Satorius

3. **Acetazolamide Standard**

   Batch No : AWS –A03-00

   Potency : 99.7%

4. **Reagents**

   Potassium dihydrogen orthophosphate: Merck

   Acetoniltrile : HPLC Grade

   Water : Milli-Q

5. **Column**

   Synergi polar RP Phenomenex C-18 (150x4.6mm,4.0µm

6. **Mobile phase**

   Acetonitrile : Buffer (100:900)

METHOD DEVELOPMENT AND VALIDATION

Selection of Solvent:

Acetazolamide is readily soluble in potassium dihydrogen orthophosphate buffer. So mobile phase was used as a solvent of choice for the drug.

Optimization of mobile phase:

After many trials being done on the basis of pKa, solubility study results of the drug and review of literature survey potassium dihydrogen ortho phosphate and was choosen for buffer preparation. (**fig-3.1 to fig-3.8**)

Selection of column:

Experiments with deferent C-18 columns were conducted to achieve best separation of analyte peak with other blank and placebo peaks. C18 columns such as waters x-terra C-18 , inertsil C-18 , waters symmetry C-18, Hypersil BDS C-18,

Hypresil ODS C-18, Synergi polar RP phenomenex C-18 e.t.c were tried. (**fig-2.1 to fig-2.7**)

Detection and selection of wavelength:

Known concentration of Acetazolamide working standard was taken and dissolved in mobile phase such that the standard solution contains about 0.8ppm. placebo and blank solutions also prepared. All these solution were scanned between 200-400 nm using UV Visible Spectrophotometer then these are subjected to chromatograph containing PDA detector to determine accurate and optimized wavelength. After reviewing chromatograms and peak purity, a wavelength of 265nm is selected as the wavelength for this drug.

Selection of Flow Rate

Select the flow rate which gives least retention time, good peak symmetries , least back pressures and better separation of impurities from each other and from API peak.

After reviewing the result it was found that the flow rate having a 1.0ml /min. which have given least retention time , good peak symmetry , and better separation of impurities from each other and from API peak. (**fig-4.1 to fig-4.3**)

Preparation of buffer solution:

Weigh accurately and dissolve about 6.8g of potassium dihydrogen orthophosphate in 1000 mL of water and mix well. Filter through 0.45 µm nylon membrane filter and degas. pH of the buffer solution is 4.5.

Preparation of mobile phase:

Prepare a mixture of 900 mL of buffer solution and 100 mL of acetoniltrile and degas.

Preparation of diluent:

Mobile phase is used as diluents.

Impurity Stock Solutions:

Weigh accurately and transfer about 2 mg of impurity –E, 2mg of impurity –D , into a 25 mL volumetric flask. Add about 15 mL of methanol and sonicate to dissolve. Make the volume up to the mark with methanol and mix.

Preparation of standard solution:

Weigh accurately and transfer about 40.0 mg of Acetazolamide working standard in to a 100mL volumetric flask. Add About 60mL mobile phase and sonicate to dissolve. Dilute to volume with mobile phase and mix. Transfer 1mL of this

solution in to a 100mL volumetric flask and make the volume up to the mark with mobile phase. Further dilute 2mL of this solution in to 10mL with mobile phase and mix. (conc.**0.8 PPM**)

Preparation of sample solution

Weigh not less than 20 capsules and empty the contents and weigh the empty capsules. Calculate the average fill weight. Weigh accurately pellets equivalent to 40 mg of Acetazolamide and transfer in to a 100 mL volumetric flask. Add about 60mL of mobile phase and sonicate for 15 minutes with intermittent shaking. Cool the solution to room temperature and make the volume up to the mark with mobile phase and mix (conc:**0.4mg/mL**). Filter through 0.45 µ nylon syringe filter.

Note:

1. Prepare sample solution in duplicate
2. Filtered sample solution stable for 48 hours at ambient condition

Placebo preparation:

Weigh accurately and transfer of each strength equivalent to 40mg of Acetazolamide (substract the 40mg from the obtained equivalent weight) into a 100mL volumetric flask. Add about 60mL of mobile phase and sonicate for 15minutes with intermittent shaking. Cool the solution to room temperature and make up the volume up to the mark with mobile phase and mix (conc:**0.4mg /mL**). Filter through 0.45 µ nylon syringe filter.

Preparation of impurity spiked sample solution

Weigh not less than 20 capsules and empty the contents and weigh the empty capsules. Calculate the average fill weight. Weigh accurately pellets equivalent to 40mg of Acetazolamide and transfer into a 100mL volumetric flask. From the above impurity stock solution pipette out 0.75 mL into the volumetric flask add about 60mL of mobile phase and sonicate for 15min with intermediate shaking. Cool the solution to room temperature and make up the volume upto the mark with mobile phase and mix.

Evaluation of system suitability

1. Resolution between impurity –E and Impurity-D in resolution solution should not be less than 2.0
2. The % RSD of the areas of Acetazolamide peak obtained from the chromatograms of six replicate injection s of standard solution should not be more than 5.0

Note

The retention time for Acetazolamide peak is about 7.5 minutes.

RRT and RRF table:

Table -7

S.No	Name of Impurity	RRT	RRF
1	Impurity –D	0.40	0.64
2	Impurity –E	0.34	-

CALCULATION :

Calculate the % content of impurity D by the below formula

$$\frac{ATI}{AS} \times \frac{WS}{100} \times \frac{1}{100} \times \frac{2}{10} \times \frac{100}{WT} \times \frac{\text{Avg.Fill wt}}{LC} \times \frac{1}{RRF} \times P$$

Calculate the % content of unknown impurities by the below formula;

$$\frac{AT2}{AS} \times \frac{WS}{100} \times \frac{1}{100} \times \frac{2}{10} \times \frac{100}{WT} \times \frac{\text{Avg.Fill wt}}{LC} \times \frac{1}{RRF} \times P$$

Calculate the % content of Acetazolamide SR Capsule by the below formula;

$$\frac{AT3}{AS} \times \frac{WS}{100} \times \frac{1}{100} \times \frac{2}{10} \times \frac{100}{WT} \times \frac{\text{Avg.Fill wt}}{LC} \times \frac{1}{RRF} \times P$$

Where,

AT1= Area of the impurity –D in the sample chromatogram

AT2 = Area of the unknown impurity in the sample Chromatogram

AS = Average area obtained from the chromatograms of six replicate injections of standard solution

WS = Weight of standard taken in mg.

WT = Weight of sample taken in mg.

Avg. Fill wt= Average fill weight of Acetazoamide capsule in mg.

LC = Label claim in mg.

P = Potency of the working / reference standard

RRF = Relative retention factor.

Preparation Of Impurity Spiked Solution For Solution Stability

The percentage RSD of the Acetazoamide and impurities was calculated for the mobile phase and solution stability experiment. The solution stability of Acetazoamide and its impurity in the related substance method was carried out by living a spiked sample solution in a tightly capped volumetric flask at room temperature for 24 hrs. The content of impurity-D and Impurity –E was determined at 6hrs intervals up to the study period. The mobile phase stability was also investigated for 24hrs by injecting the freshly prepared sample solution for every 6 hrs interval. The content of impurity-D and Impurity – E was determined in the test solution. The prepared mobile phase remain constant during the study period. Cumulative percentage RSD was NMT 2% for peak area of Acetazoamide in standard.

Cumulative percentage RSD was NMT 5% for peak are of known impurities in sample solution.

Preparation of filter interference study solution

Filter interference study was performed by analysis of centrifuged sample solution (spiked with known impurity at specification level) and sample solution filter to 0.45 µm nylon filter. The percentage of known impurity and total impurity were calculated and compare the result with centrifuged sample results.

Preparation of Linearity Solutions

Linearity stock solution were prepared and inject solutions of different concentration of impurity –D at different linearity level ie; LOQ 25% , 50%, 75%, 100%,125% and 150%.

Limit of Quantification And Limit Of Detection

The LOD and LOQ determined by signal to noise ratio method.

LOQ level was verified by giving six replicate injections of standard solution containing Acetazolamide and impurity –D at about LOQ level.

Acceptance Criteria

Signal to noise ratio 10:1 at the level of LOQ and 2 or 3:1 at the level of LOD

The percentage RSD for peak areas at LOQ level is not more than 10.0.

Precision

The precision of a method is the extent to which the individual test results of multiple injections of a serious of standards agree. The ICH requires precision from at least 6 replications to be measured at 100 percent of the test target concentration or from at least 9 replications covering the complete specified range. For example, the results can be obtained at 3concentration with 3 injections at each concentration.

Accuracy

This was performed by the addition of known amounts of the formulation by weigh or volume to the placebo formulation working in the linear range of detection of the analyte. The test evaluated the specificity of the method in the presence of excipients under chromatographic condition used for the analysis of the formulation.

The accuracy was demonstrated by preparing recovery sample (spiking placebo with known quantities of API) at the level of LOQ 50% ,100% ,150% of the targeted concentration . Prepared each level in the triplicates and the average value taken to calculate the recovery .The RSD of the replicates will provide the analysis variation or how precise the method was.

Ruggedness

Six samples were analyzed as per test method with the spiking of known impurities in the specification level with different analyst, different day, different instrument and different column. The percentage of known impurities and total impurities six samples were calculate.

Robustness

To determined the Robustness of the developed method, the experimental conditions were slightly altered and the resolution between Acetazolamide and impurity – D was evaluated

Forced Degradation

The method was found to be specific as demonstrated by forced degradation studies in that all degradation impurities are resolved from the analyte peak. In addition, photodiode array detector (PDA) was use to demonstrate the peak spectral homogencity with the aid of peak purity results.

The replicated substance method has been demonstrated as suitable for monitoring the long term stability of Acetazolamide capsule.

Unstressed sample

Acid hydrolysis

Peroxide oxidation

Thermal degradation

Photo degradation (controlled)

Photo degradation (uncontrolled)

Preparation of samples/ placebo for forced degradation:

1. **Acid treated**

   5mg equivalent sample/ placebo + 2ml of 5N HCl---- Heated for 10min at 80°C----------→Cooled to room temperature and added 2ml of 5N NaOH to neutralize. Finally, diluted to 20mLwith diluents.

2. **Alkali treated**

   5mg equivalent sample /placebo + 2mL of 5N NaOH --- Heated for 10min at 80°C----------→Cooled to room temperature and added 2ml of 5N HCl to neutralize. Finally, diluted to 20mL with diluents.

3. **Hydrogen peroxide(H_2O_2) treated**

   5mg equivalent sample/ placebo + 2ml of H_2O_2 solution(30%)- Heated for 10min at 80°C ----------→ Cooled to room temperature and

Finally, diluted to 20mL with diluent. All the degraded samples and placebo were injected to the chromatographic system.

7. RESULTS AND DISCUSSION

HPLC method development and optimization

Selection of diluent

Acetazolamide is readily soluble in potassium dihydrogen ortho phosphate buffer. So mobile phase was used as a solvent(Diluent) of choice for the drug.

Wavelength Selection

Standard and impurities were scanned on PDA detector between 200 to 800 nm and 265 nm was selected as an optimum wavelength for analysis were even Acetazolamide and its impurities have a good response.

Selection of Column And Column Temperature

Experiments with different C_{18} columns were conducted to achieve best separation of analyte peak with other blank and placebo peaks. C_{18} columns such as waters x-terra C-18, inertsil C-18, waters symmetry C-18, Hypresil ODS C-18, Hypresil BDS C-18, synergi polar RP phenomenex C-18 etc were tried.

As usually initial stage column temperature is maintained at ambian and then increased slowly to obtain good peak shape , retention time , peak symmetry , better separation .

After reviewing the results it was found that the peak shape, retention time, resolution , tailing factor , column efficiency are good with synergi polar RP phenomenox C-18 (150 x4.6mm,4.0µm) and hence synergi polar RP phenomenox C-18 column and column temperature 25^{o}C was selected

Selection of Buffer

Ideally the strength of the buffers shall be between 0.05m to 0.20m. The selection of buffer and strength is done always in combination with selection of organic phase composition in mobile phase.

Experiments conducted using different buffer strength to obtain the requires separations of all individual impurities and degradents. The selected buffer strength and effect of variation studied.

After reviewing the results potassium dihydrogen ortho phosphate buffer is selected and it give better resolution than other buffers.

Selection of Flow Rate

Select the flow rate which gives least retention time, good peak symmetries , least back pressures and better separation of impurities from each other and from API peak.

After reviewing the result it was found that the flow rate having a 1.0ml /min. which have given least retention time , good peak symmetry , and better separation of impurities from each other and from API peak.

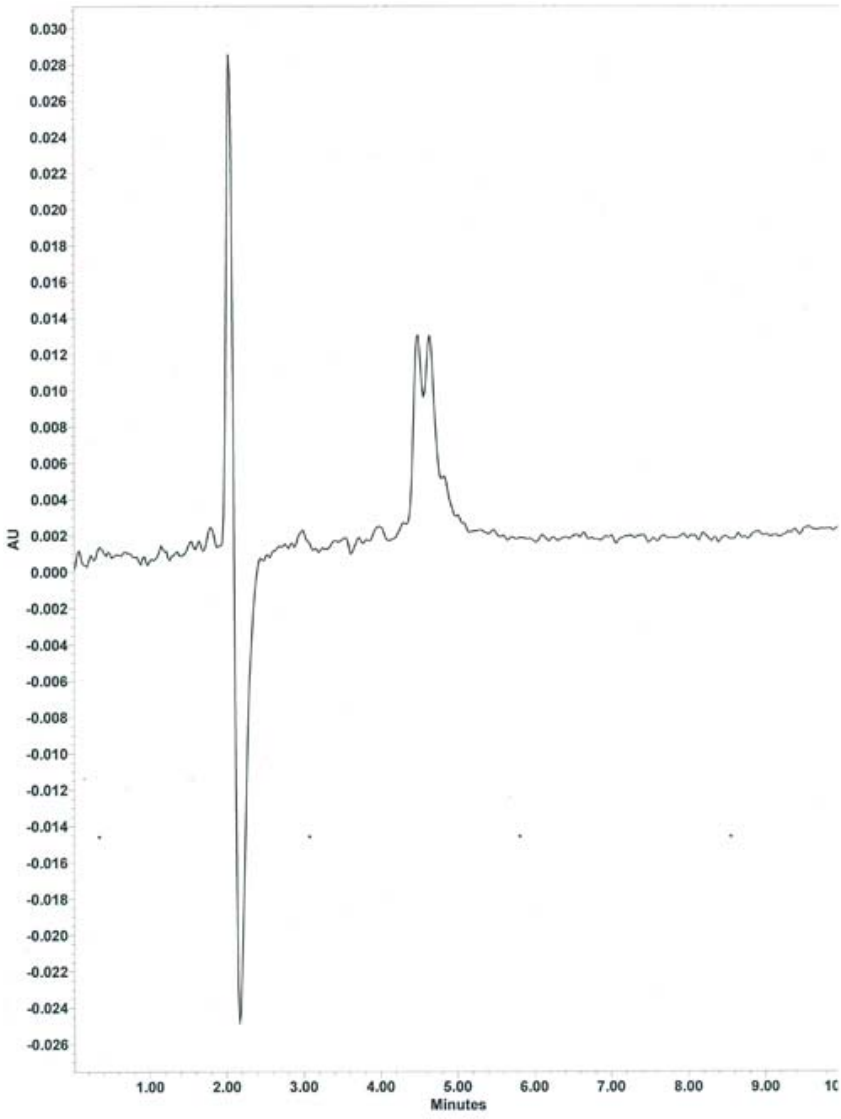
Selection of mobile phase composition

Initially experiment conducted with mobile phase having buffers with different pH an organic phase to check for the best separation between the impurities. Alternatively, solution of stressed drug substance can be used to check for separation of impurities.

After reviewing the results it was found that the mobile phase composition, Buffer: Acetonilitrile (900:100) had given best separation between the impurities, blank and placebo, so peak purity gets passed.

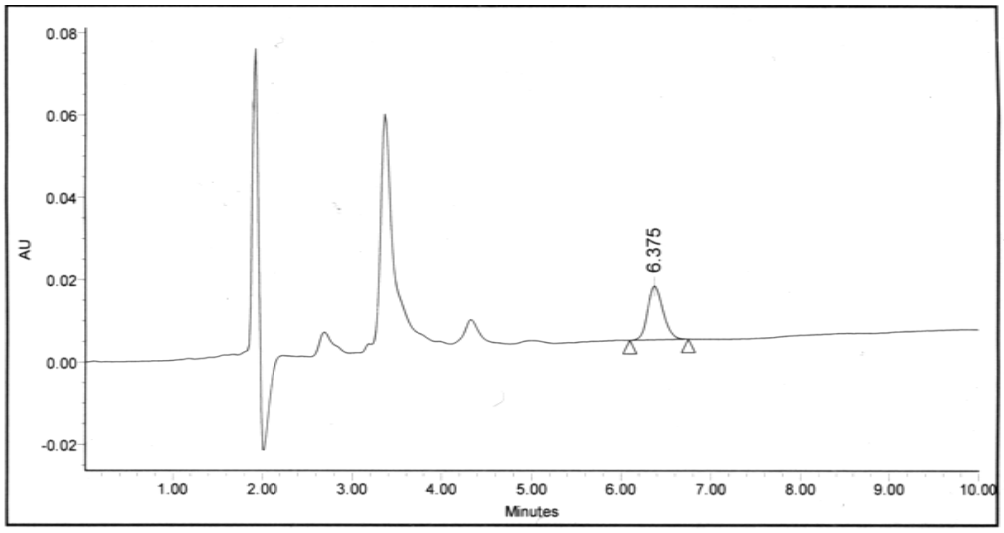
METHOD DEVELOPMENT TRIALS

Fig-2.1



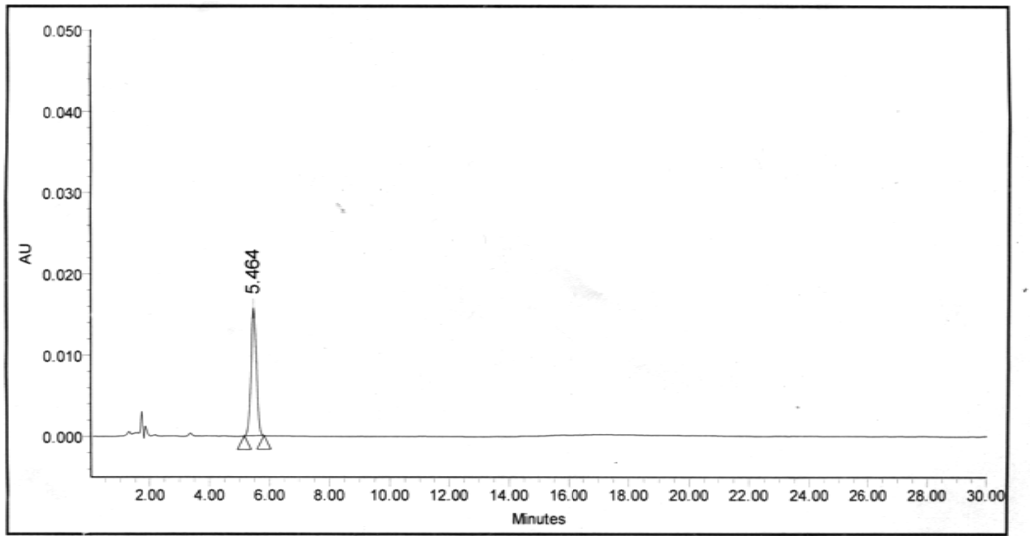
Separation using Water X-terra C-18 column

Fig-2.2



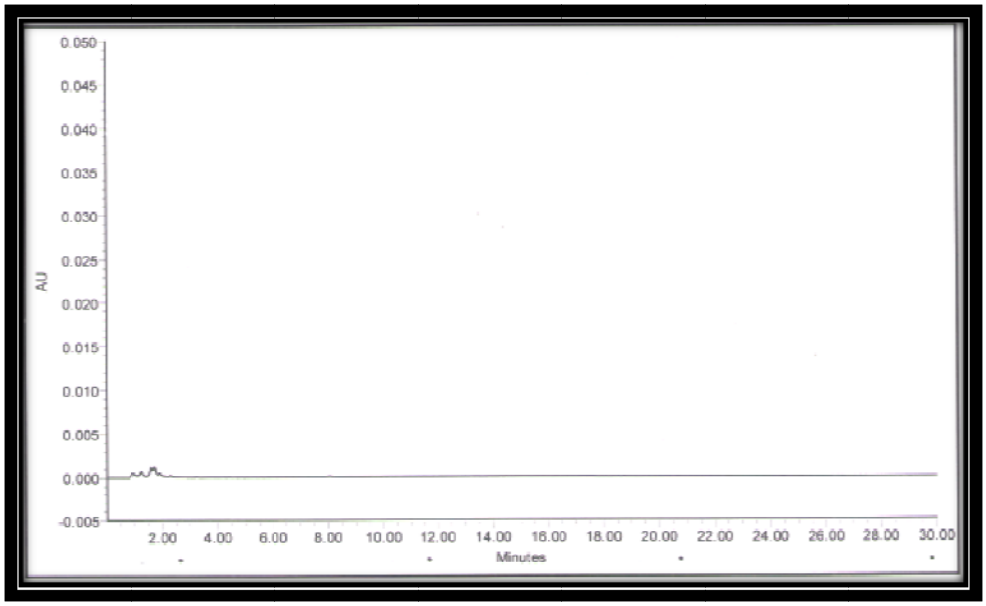
Separation using Inertsil C-18 column

Fig-2.3



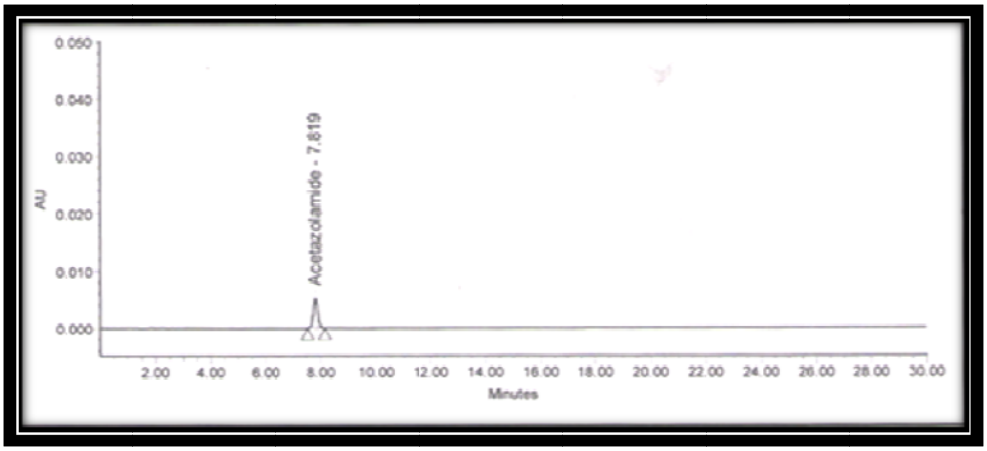
Separation using Hypersil ODS C-18 Column

Fig-2.4



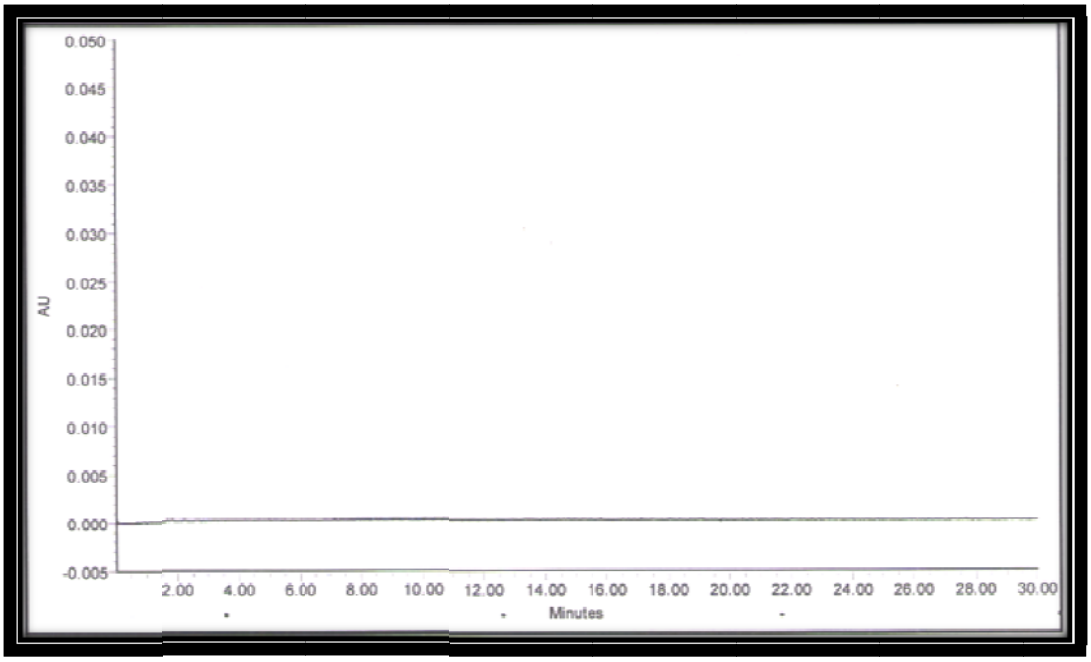
Separation using Synergic polar RP Phenomenex C-18 column (BLANK)

Fig-2.5



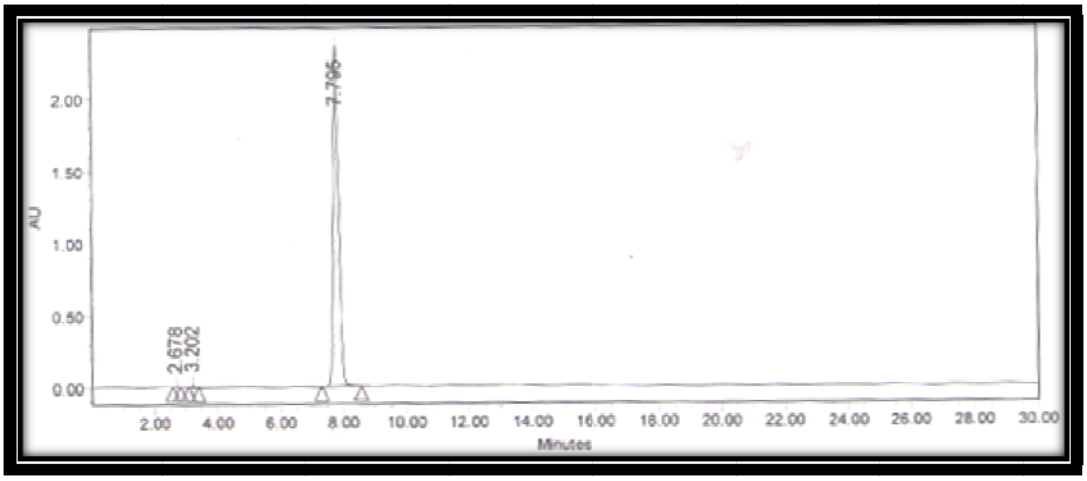
Separation using Synergic polar RP Phenomenex C-18 column (STANDARD)

Fig-2.6



Separation using Synergic polar RP Phenomenex C-18 column (PLACEBO)

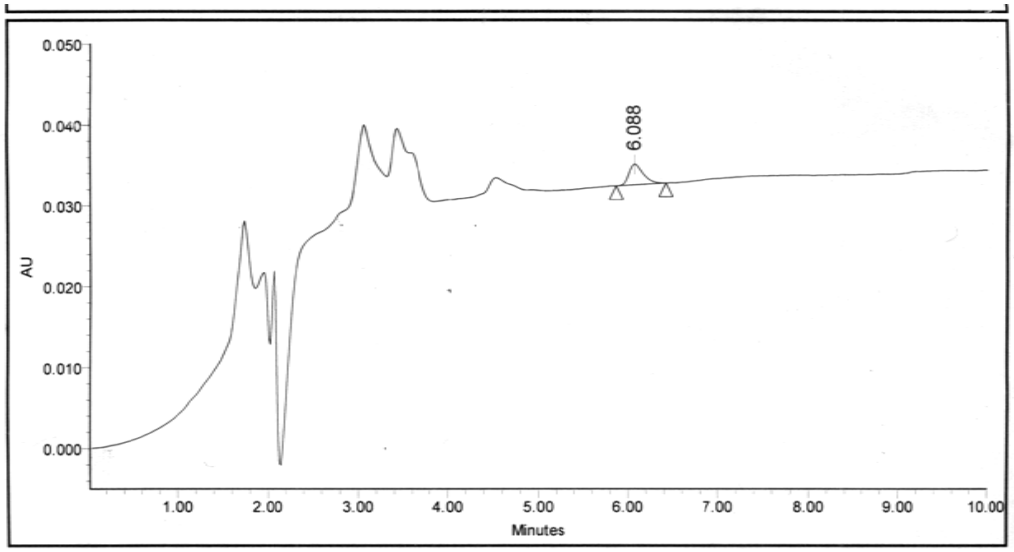
Fig-2.7



Separation using Synergic polar RP Phenomenex C-18 column (SAMPLE)

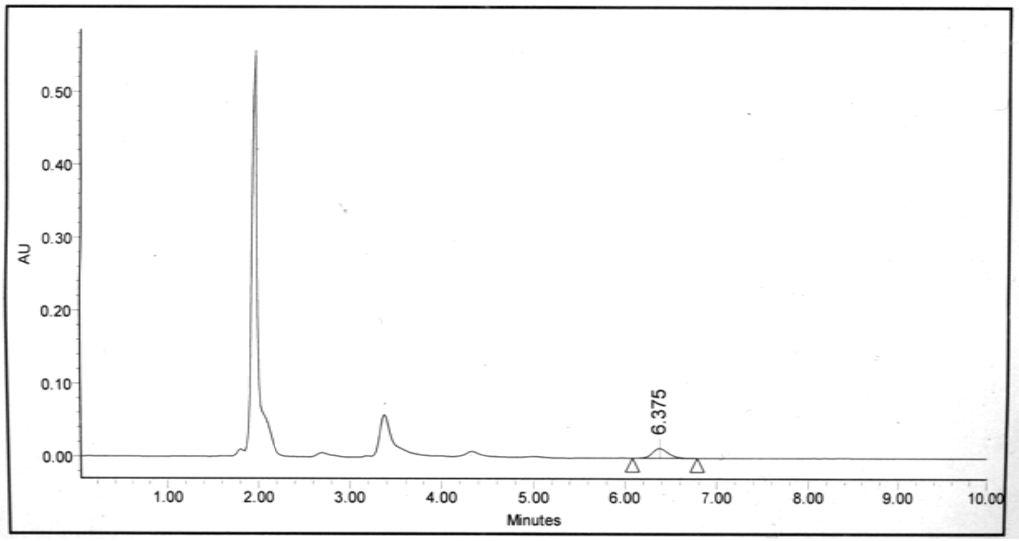
TRIALS WITH CHANGE IN COMPOSITION OF MOBILE PHASE

Fig-3.1



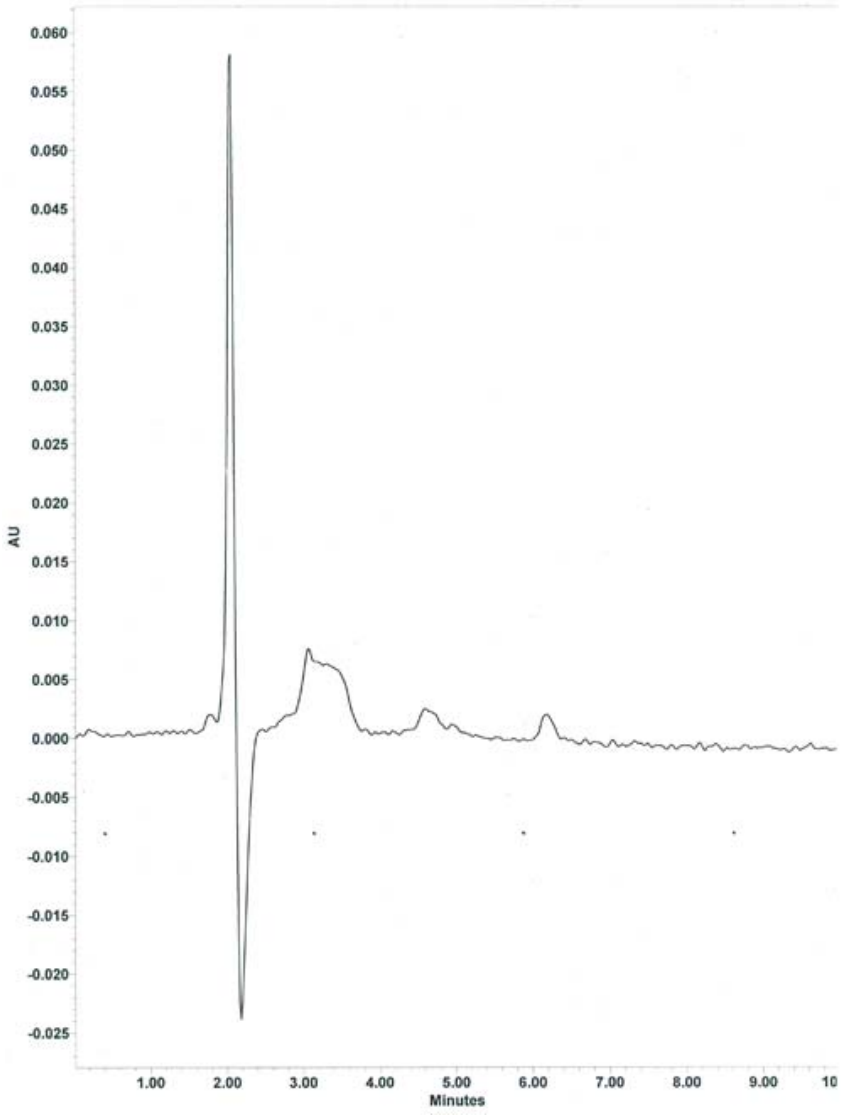
Separation using Acetonitrile : Water

Fig-3.2



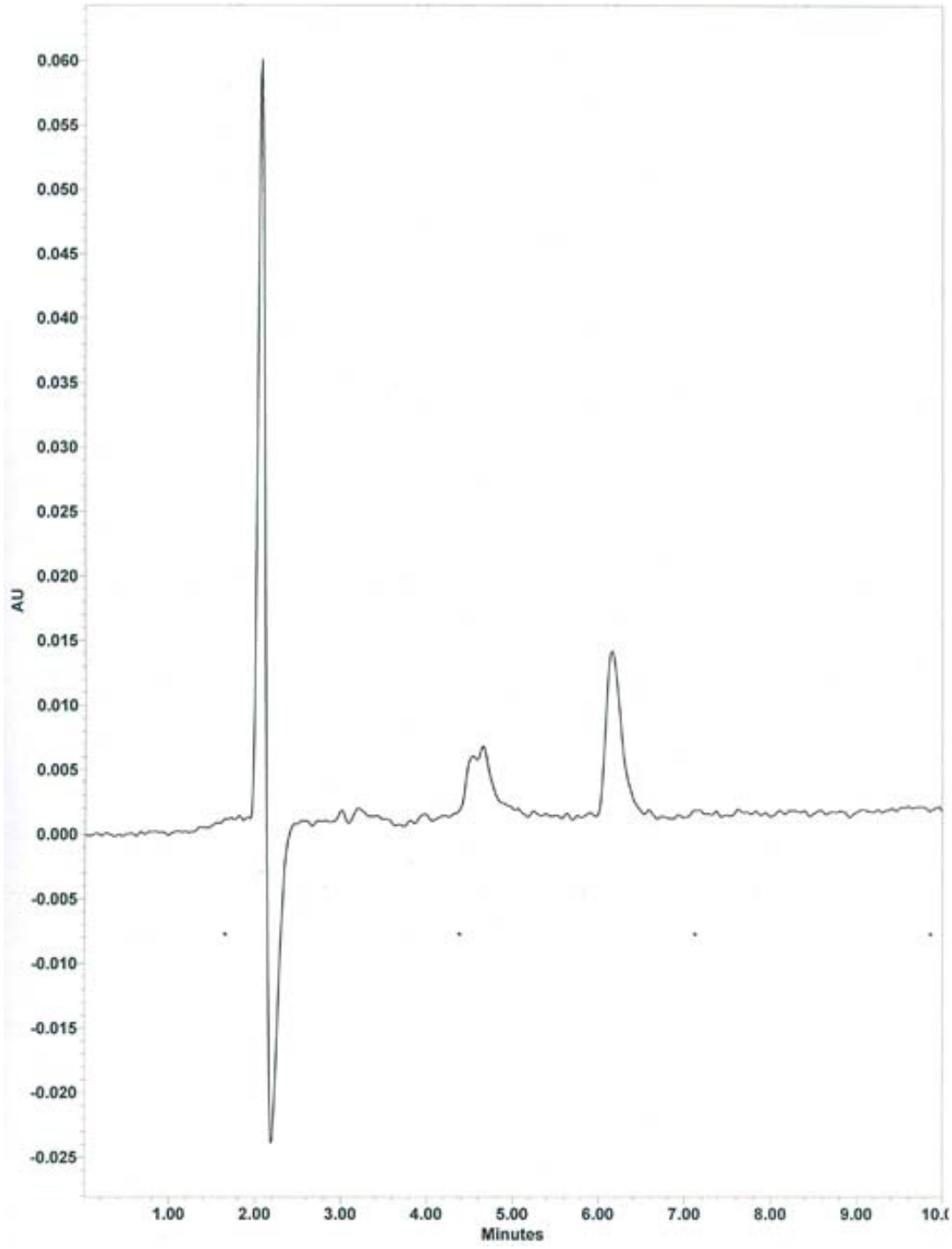
Separation using Methanol : Water

Fig-3.3



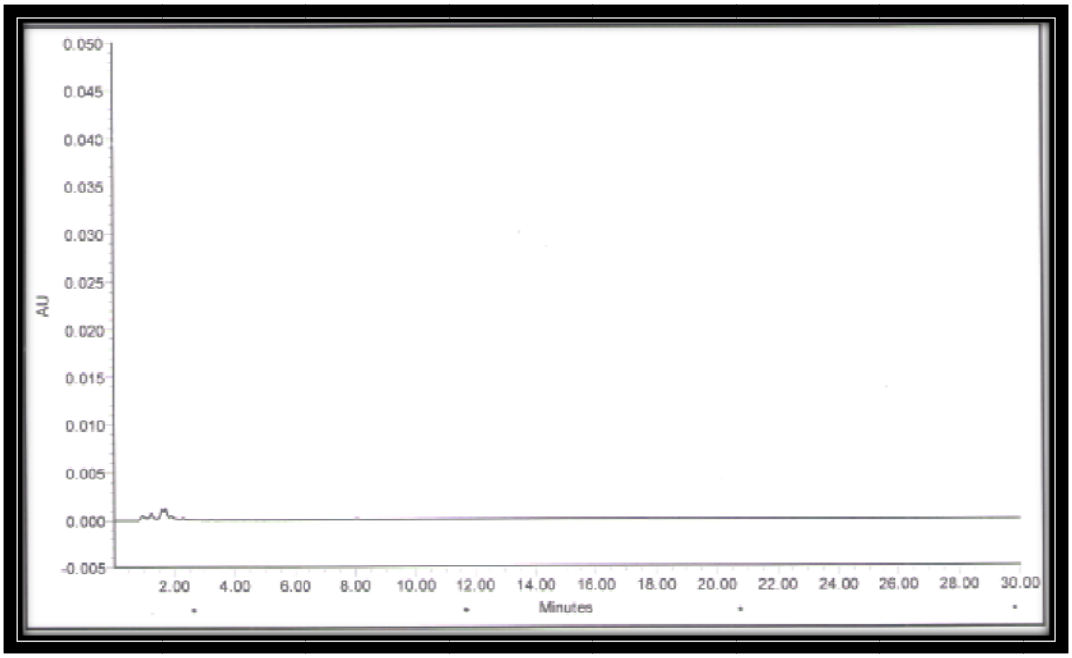
Separation using Methanol : Buffer

Fig-3.4



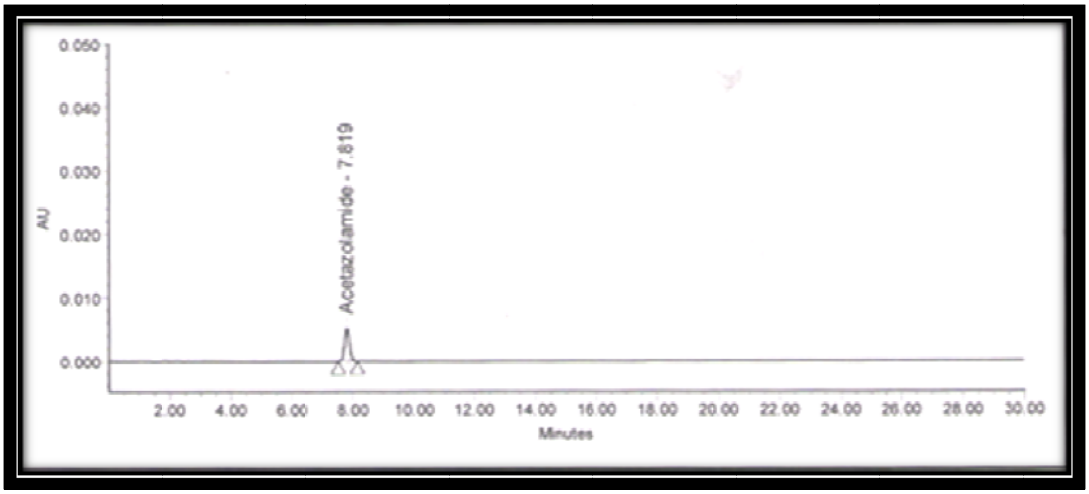
Separation using Acetonitrile:Buffer (50:50)

Fig-3.5



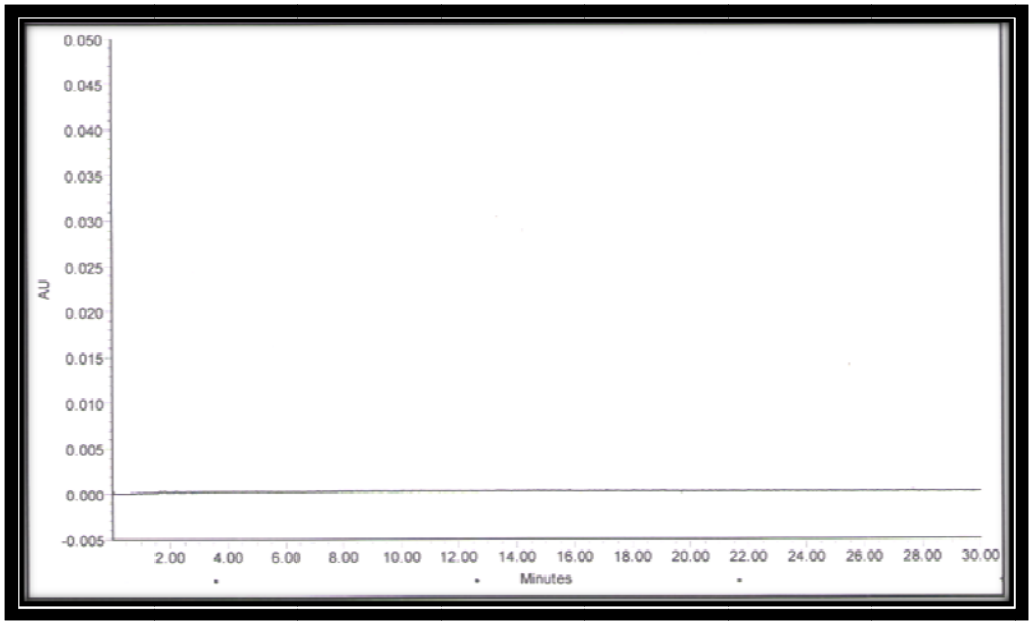
Separation using Acetonitrile : Buffer (900:100) BLANK

Fig-3.6



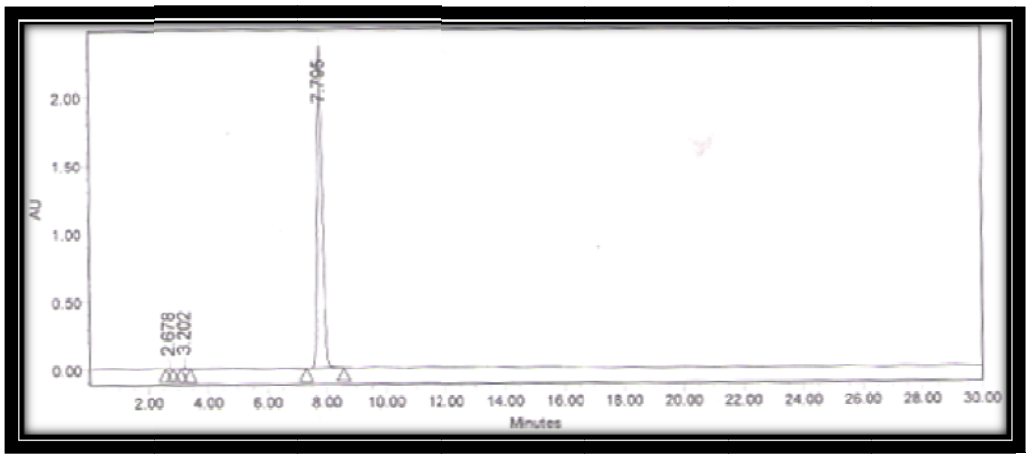
Separation using Acetonitrile : Buffer (900:100) STANDARD

Fig-3.7



Separation using Acetonitrile : Buffer (900:100) PLACEBO

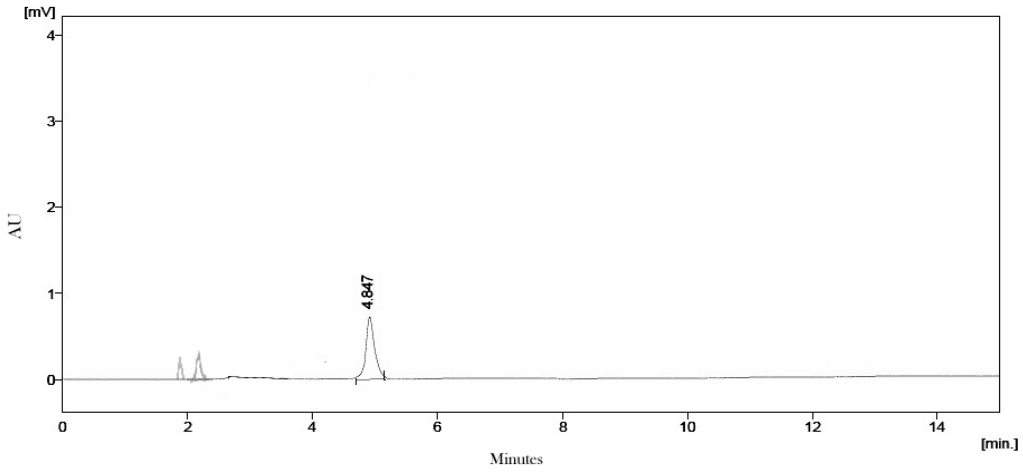
Fig-3.8



Separation using Acetonitrile : Buffer (900:100) SAMPLE

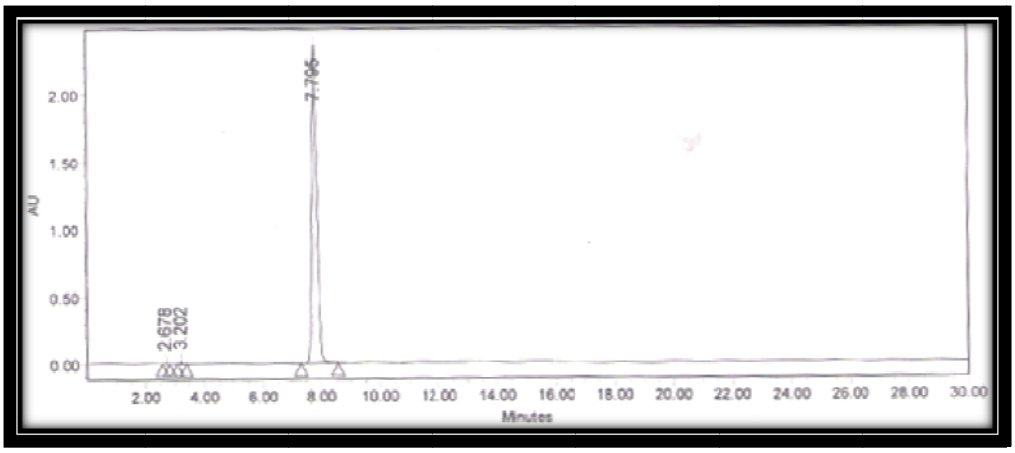
TRIALS WITH CHANGE IN FLOW RATE

Fig-4.1



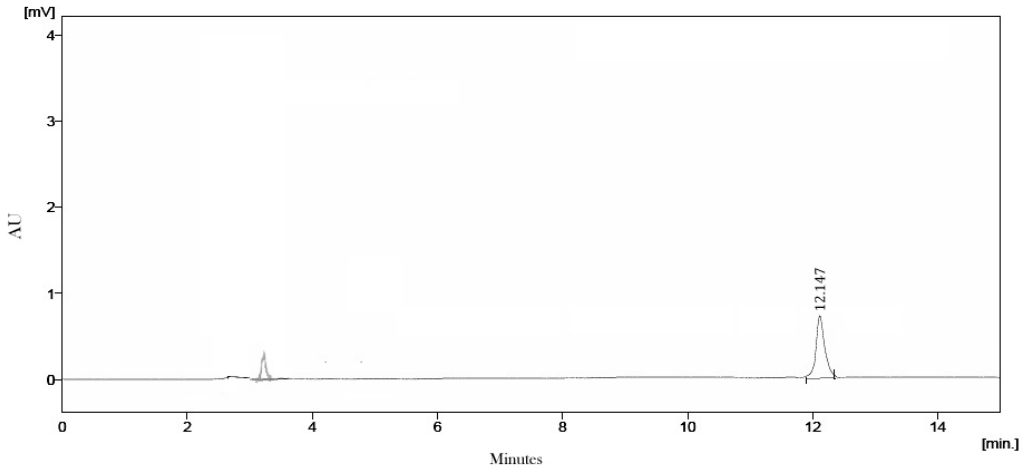
Separation using Acetonitrile : Buffer (900:100) at flow rate 0.8ml/min

Fig-4.2



Separation using Acetonitrile : Buffer (900:100) at flow rate 1ml/min

Fig-4.3



Separation using Acetonitrile : Buffer (900:100) at flow rate 1.2ml/min

METHOD VALIDATION

PRECISION

System Precision

Six replicate injections of standard solution was injected as per method. The mean and percentage RSD for the peak areas of Acetazolamide were calculated. The result are tabulated in the table below

Acceptance Criteria

The percentage RSD for peak area of Acetazolamide is not be more than 5.0

System Precision Data

TABLE-8

S. No	Peak Area
1	59938
2	60167
3	60010
4	60301
5	60691
6	61097
Mean	60367
% RSD	0.7

Conclusion

The percentage RSD value indicate an acceptable level of precision of the analytical system for the estimation of Acetazolamide.

METHOD PRECISION

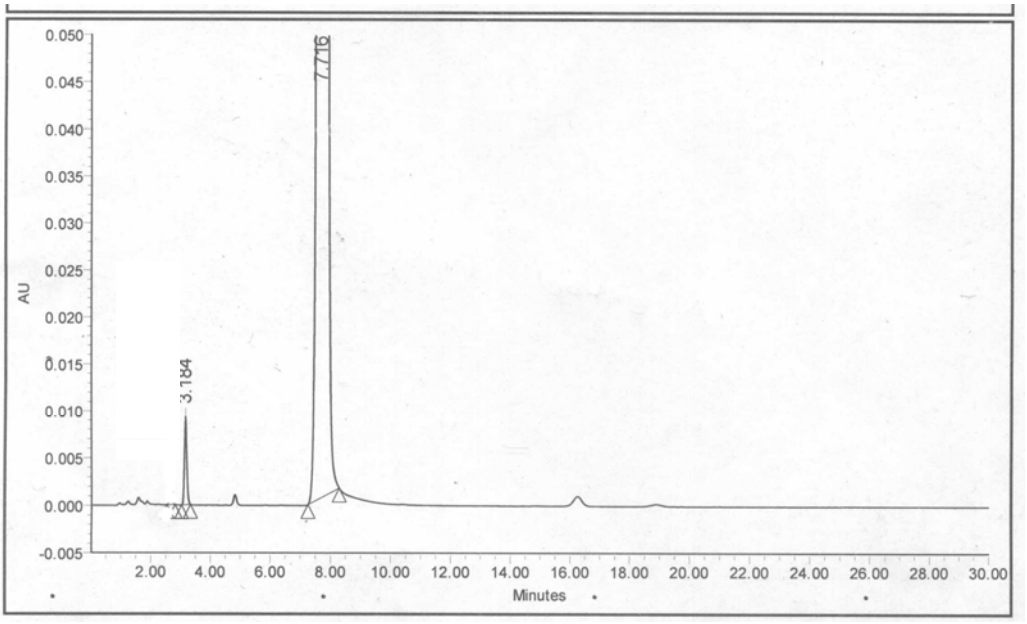
Six samples were analysed as per test method with the spiking of known impurities in the specification level. Percentage of known impurities and total impurities in six sample and percentage RSD for percentage of known impurities and total impurities were calculated.

Acceptance Criteria

The percentage RSD for known impurities and total impurities of six sample is not more than 10.0

Method precision Chromatogram

fig-5.0



Method Precision Data

Impurity-D

TABLE-9

S.No	Peak Area	% w/w
1	48942	0.25
2	47425	0.24
3	49223	0.25
4	49945	0.25
5	49735	0.25
6	47964	0.24
Average	48872	0.25
% RSD	-	2.1

Conclusion

The percentage RSD value indicates an acceptable level of precision of the analytical method for estimation of impurities

INTERMEDIATE PRECISION

Six samples were analyzed as per test method with the spiking of known impurity in the specifications level by different analyst, different instrument and different column and different day. Percentage of known impurity and total impurities in six samples were calculated. Over all percentage RSD for method and intermediate precision result calculated for known and total impurity calculated

Acceptance Criteria

The percentage RSD for known and total impurities of six samples is not more than 10.0

$$\text{Percentage RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

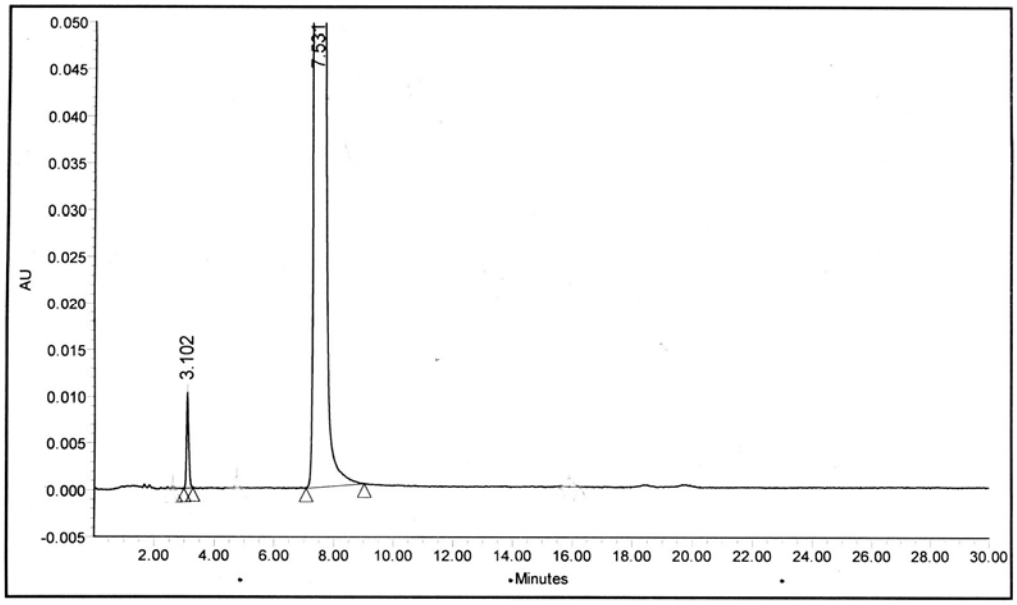
The overall percentage RSD for method precision and intermediate precision result is not more than 10.0 for known and total impurities

Intermediate Precision

Analyst	Analyst-I	Analyst-II
Name	DIBIN.E	M.Venkatesan
Instrument No.	QHPL4012	QHPL4013
Column Id No.	SFAD/LC/05/134	SFAD/LC/02/002
Date	5/11/2011	5/11/2011

Intermediate precision chromatogram (Analyst-1)

Fig-6.1



Intermediate Precision Data (Analyst-I)

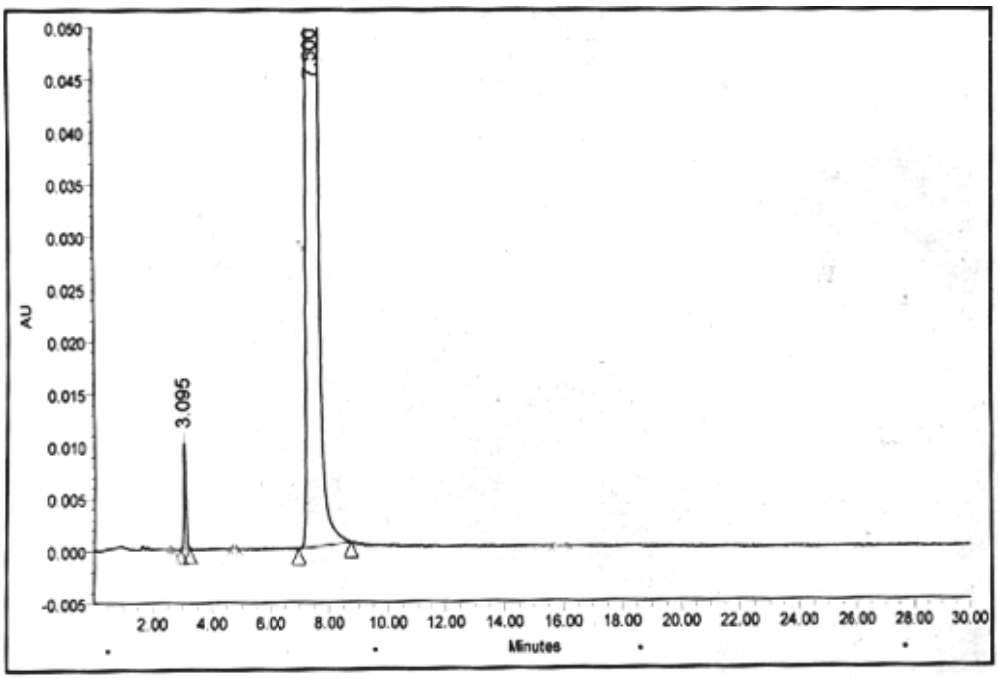
Impurity-D

TABLE-10

Sample No	Peak Area	% w/w
1	53071	0.25
2	52383	0.24
3	52630	0.25
4	52193	0.25
5	52936	0.25
6	52578	0.24
Average	52632	0.25
% RSD	-	2.1

Intermediate precision chromatogram (Analyst-2)

Fig-6.2



Intermediate Precision Data (Analyst-II)

Impurity-D

TABLE-11

Sample No	Peak Area	% w/w
1	53071	0.29
2	52384	0.29
3	52640	0.29
4	52193	0.29
5	52936	0.29
6	52678	0.29
Average	52650	0.29
% RSD	-	0.00

The overall % RSD = 8.5

Conclusion

The percentage RSD values indicates and acceptable level of precision of the analytical method for the related substance of Acetazolamide in Acetazolamide capsules.

SPECIFICITY

Blank, placebo, sample solution, sample solution spiked with known impurity at specification level, individual impurity at specification level and standard solution were injected in to the HPLC system. There was no interference of the blank and placebo at the retention time of Acetazolamide and known impurity peak in blank and placebo solution.

Acceptance Criteria

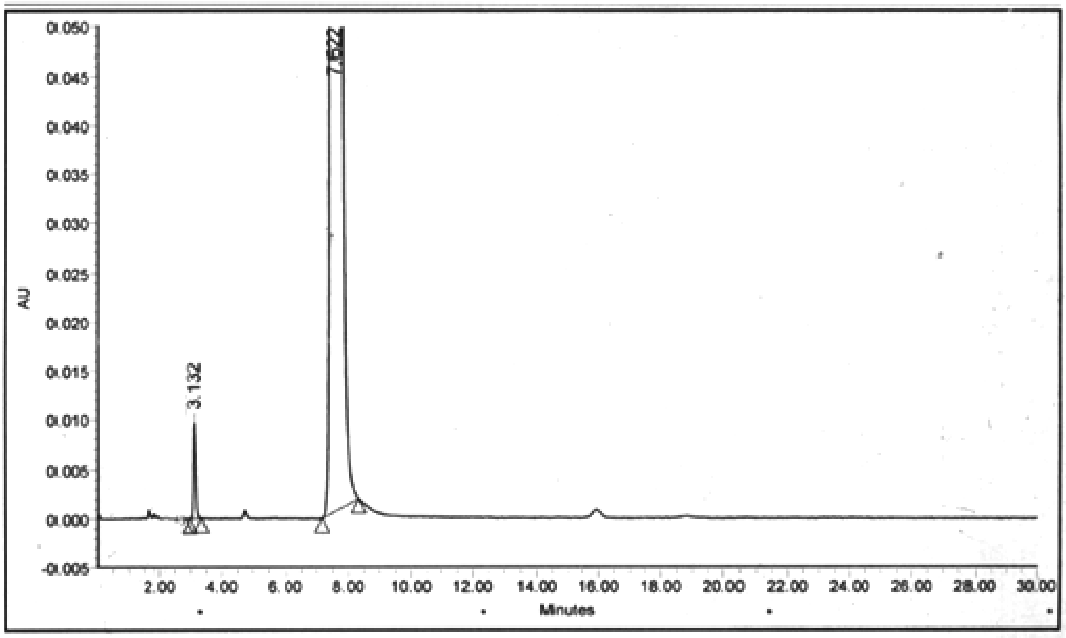
No peak elutes at the retention time of Acetazolamide and known impurity in blank and placebo.

Peak purity for Acetazolamide and known impurity passes.

Resolution is not less than 2.0.

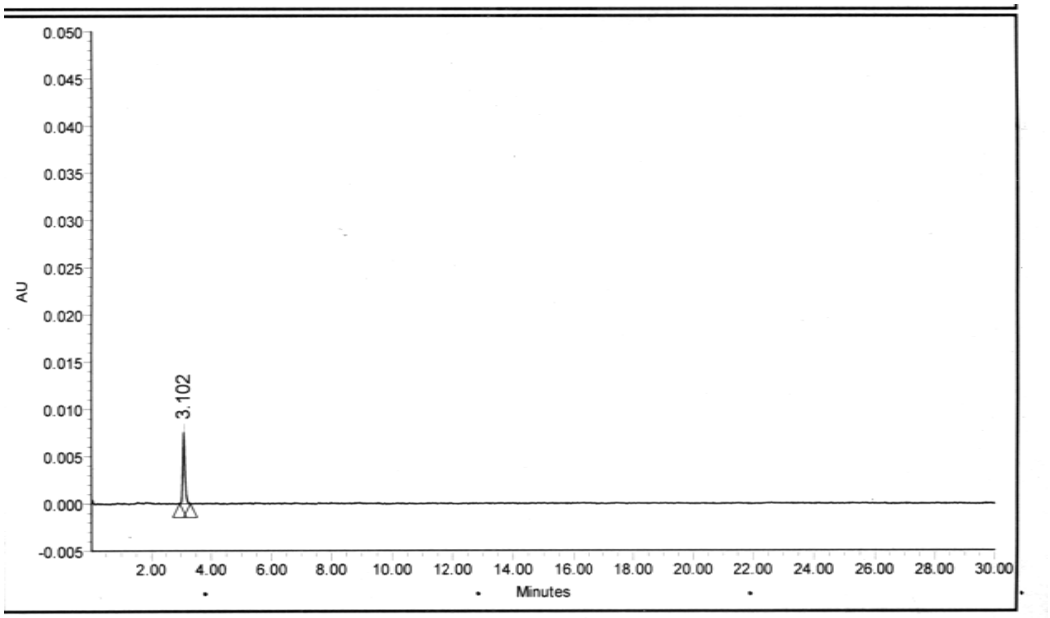
Specificity Chromatogram

Fig-7.1



Impurity - D identification (chromatogram)

Fig-7.2



Specificity Data

TABLE-12

Peak Name	Retention time(mins)	Relative retention time(RRT)
Acetazolamide	7.510	1.00
Impurity-D	3.104	0.41

Specificity Data

TABLE-13

Sample Name	Peak Name	Purity Angle	Purity Threshold
Standard	Acetazolamide	2.633	2.794
Sample	Acetazolamide	1.247	1.749
	Impurity-D	4.816	5.350
Impurity spiked sample	Acetazolamide	1.298	1.633
	Impurity-D	1.316	1.706

Conclusion

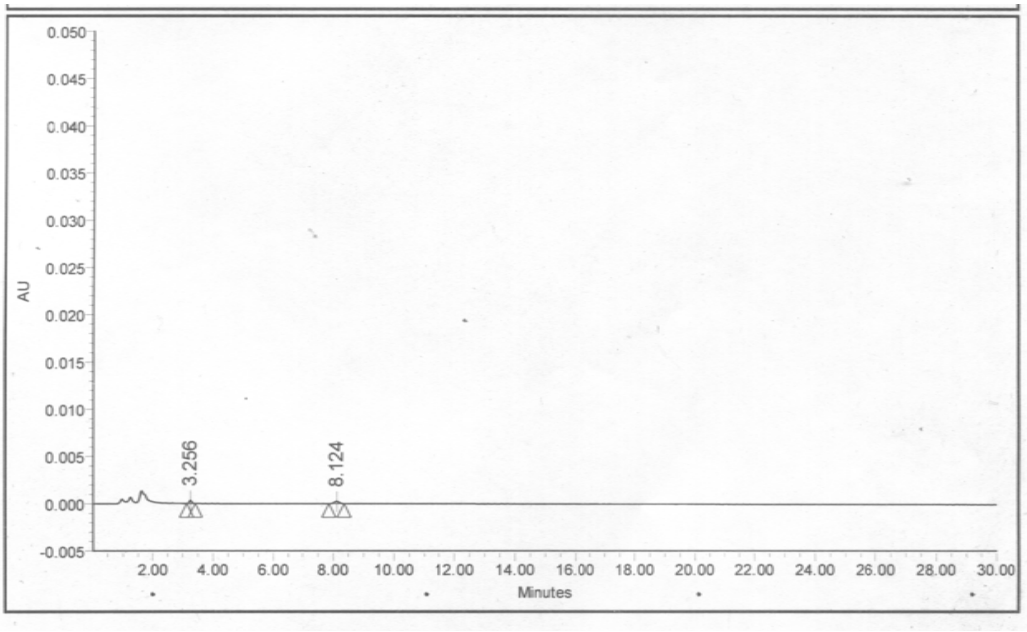
No peak was eluted at the retention time of Acetazolamide and unknown impurity in blank and placebo.

LINEARITY

The linearity of Acetazolamide and impurity –D was performed using standard solution in the range of about LOQ to 150% of specification limit. A graph was plotted with concentration (in µg/ml) on x-axis and peak areas on y- axis . slope , y-intercept, Correlation co –efficient (r-value) and residual sum of squares (RSS) were determined.

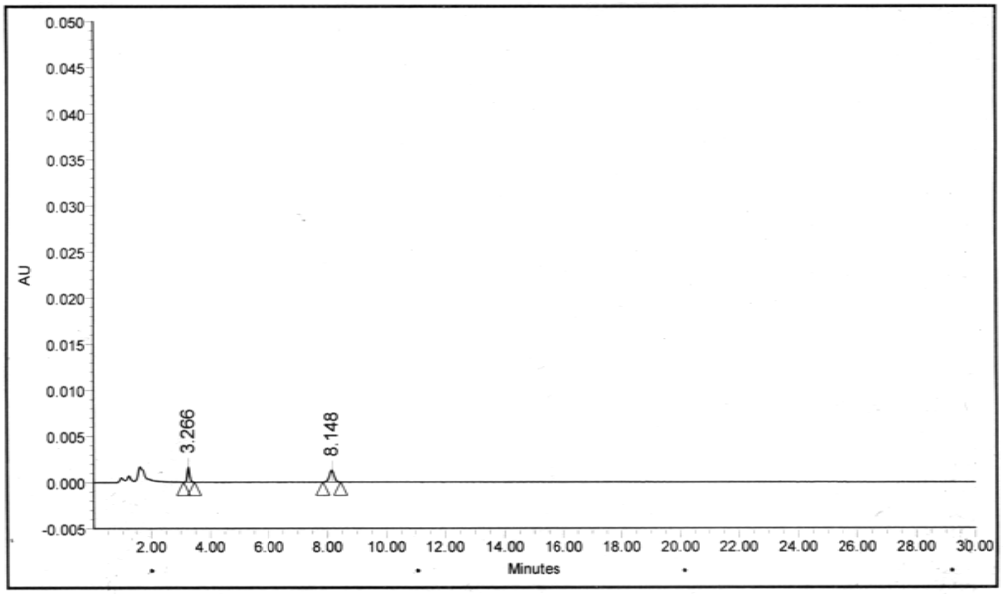
Linearity Solution-1

Fig-8.1



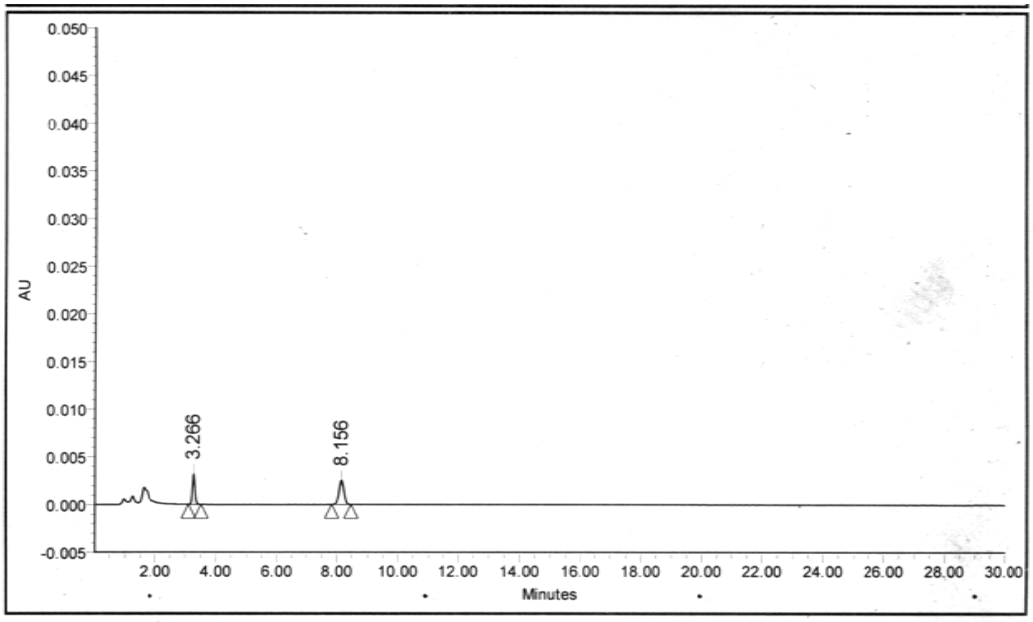
Linearity Solution-2

Fig-8.2



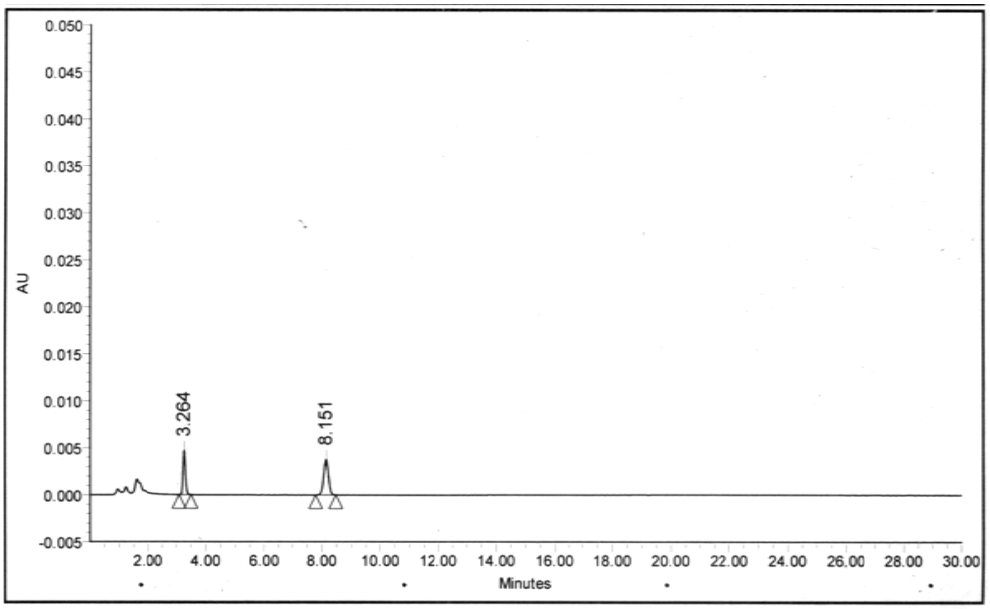
Linearity Solution-3

Fig-8.3



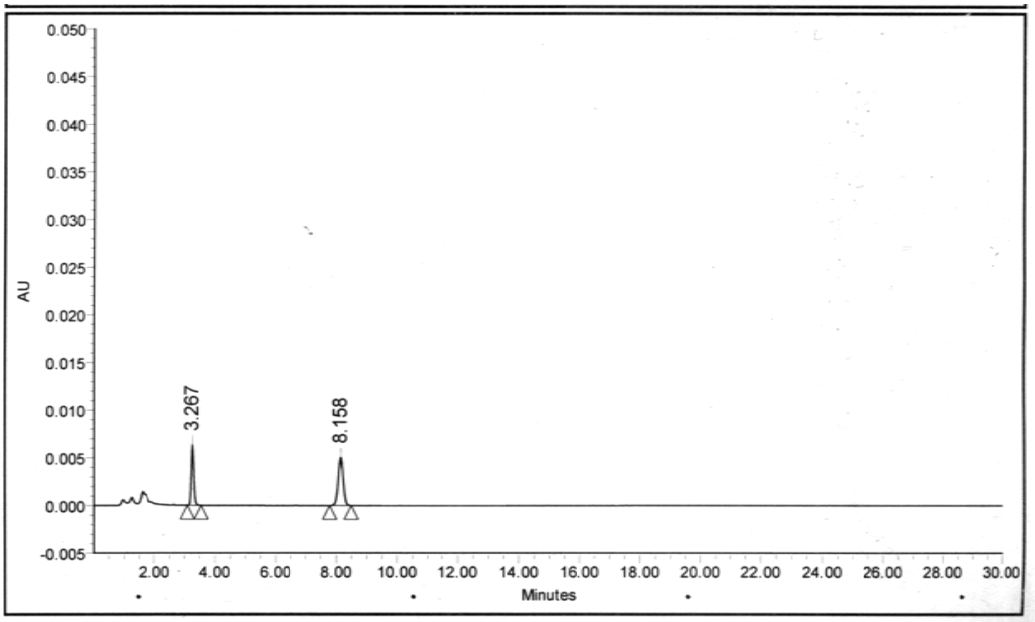
Linearity Solution-4

Fig-8.4



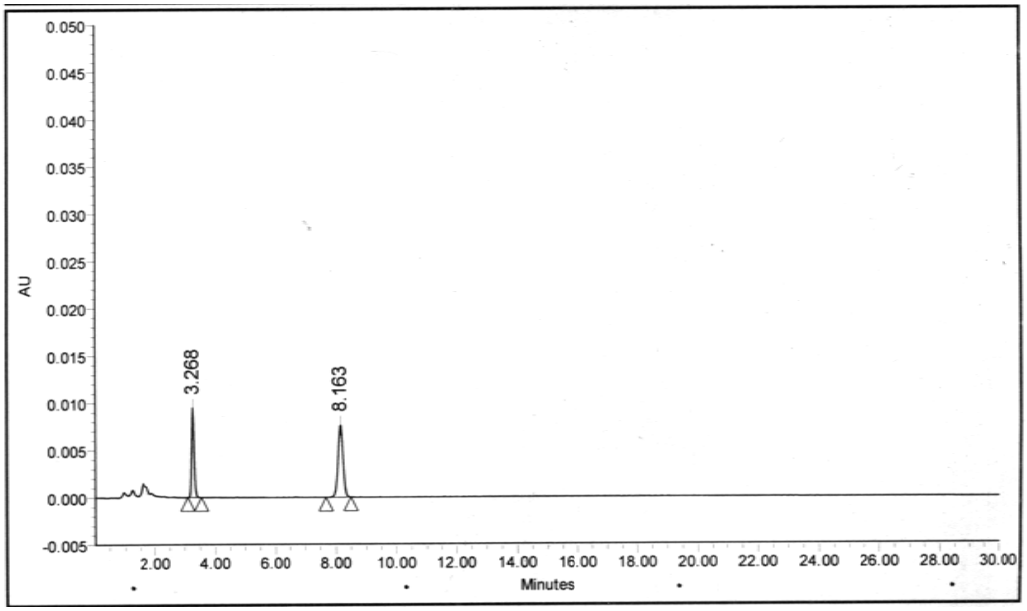
Linearity Solution-5

Fig-8.5



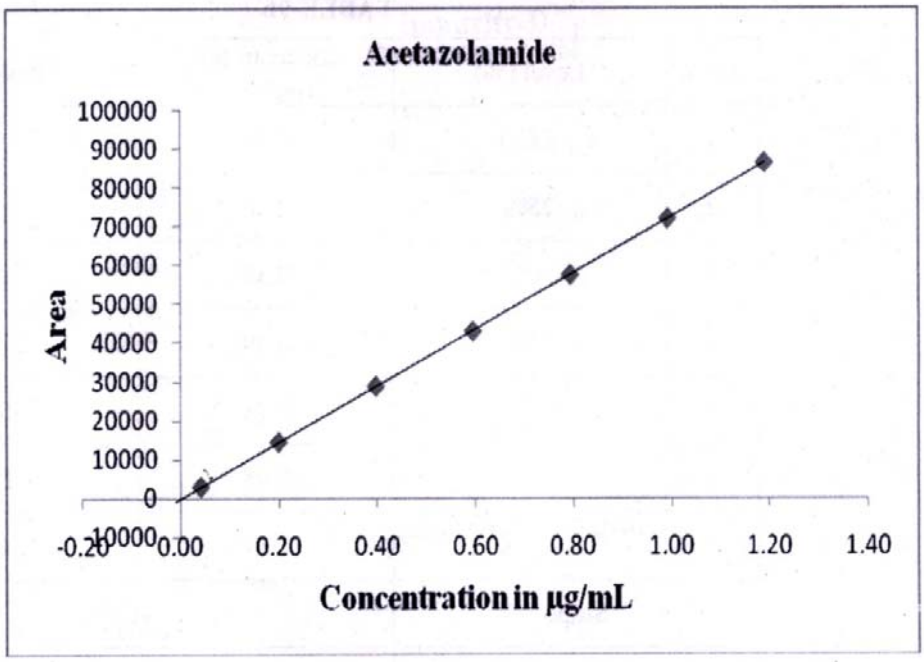
Linearity Solution-6

Fig-8.6



Linearity plot for Acetazolamide

Fig-8.7



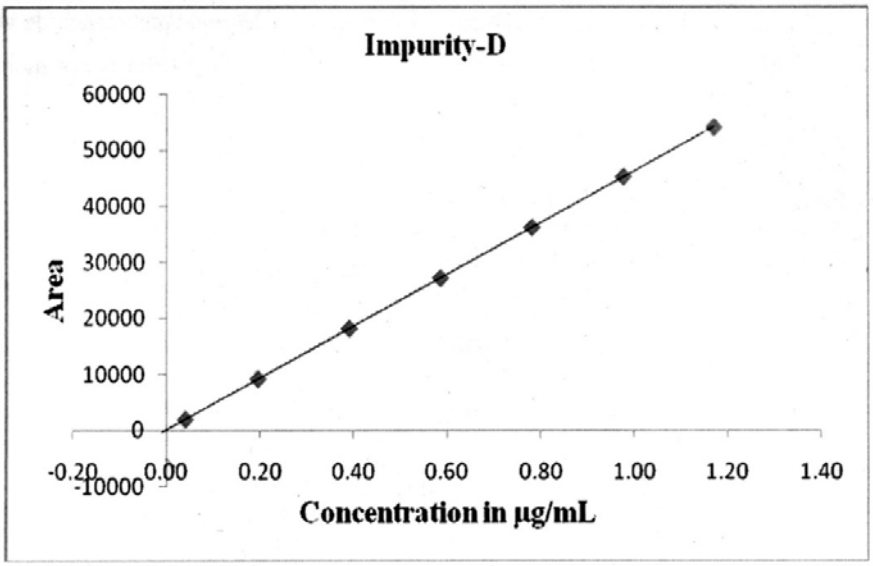
Linearity Data of Acetazolamide

TABLE-14

S.No	Level (%)	Concentration (µg/mL)	Peak Area
1	LOQ	0.04	2803
2	25 %	0.20	14393
3	50 %	0.40	28711
4	75 %	0.60	42882
5	100 %	0.79	57439
6	125 %	0.99	71923
7	150 %	1.19	86314
Slope		72463	
y-intercept		-90.34	
r-value		0.999	
RSS		57389	

Linearity plot for impurity – D

Fig-8.8



Linearity Data of Impurity-D

TABLE-15

S.No	Level (%)	Concentration (µg/mL)	Peak Area
1	LOQ	0.04	1932
2	25 %	0.20	9156
3	50 %	0.39	18110
4	75 %	0.59	27120
5	100 %	0.78	36177
6	125 %	0.98	45253
7	150 %	1.17	54079
Slope		46134	
y-intercept		115.36	
r-value		1.000	
RSS		20238	

Conclusion

The detector response of impurity – D and Acetazolamide is directly proportional to concentration ranging from LOQ to 150 % (with respect to specification limit)

LOQ AND LOD

The LOD and LOQ of Acetazolamide related compounds and Acetazolamide has been determined by signal to noise ratio.

Acceptance Criteria

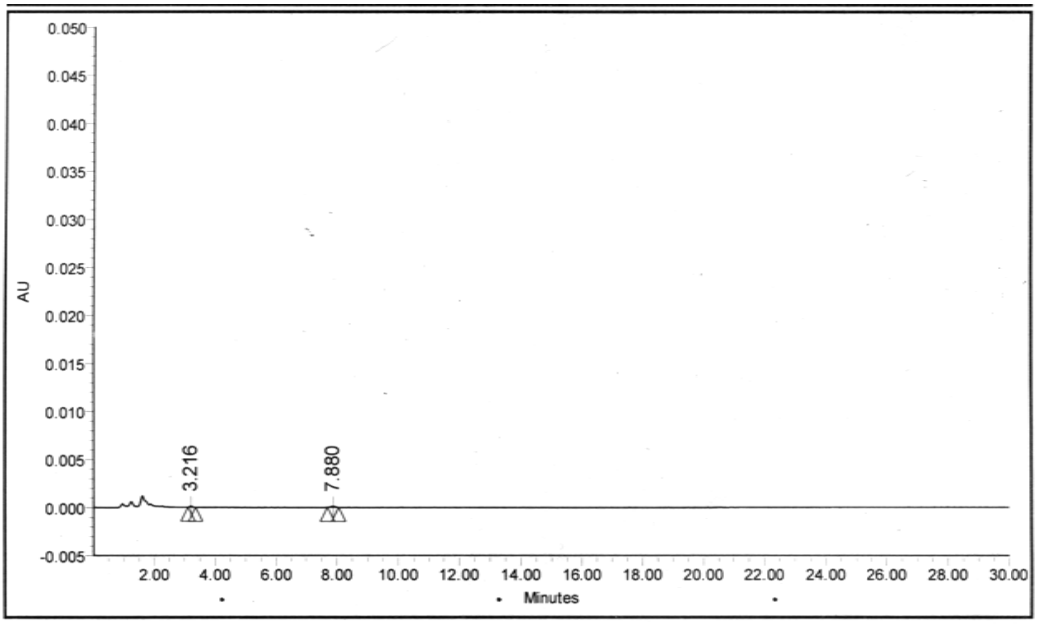
Signal to noise ratio 10:1 at the level of LOQ and 2 or 3 :1at the level of LOD

.

The percentage RSD for the peak area at LOQ level is not more than 10.0

LOD and LOQ Chromatogram

Fig-9.0



LOQ AND LOD DATA

Name of the compound	Limit of detection	Limit of quantitation
	Con. µg/ mL	Con. µg/ MI
Impurity – D	0.01	0.04
Acetazolamide	0.01	0.04

SOLUTION STABILITY

Stability of analytical solution was verified by analyzing the standard solution and filtered impurity spiked sample solution at initially and also different time intervals by storing in sample compartment of HPLC instrument at room temperature.

Calculated the cumulative percentage RSD for peak areas of Acetazolamide in standard solution. Calculated cumulative percentage RSD for peak areas of impurity – D and percentage content of total impurities in spiked sample.

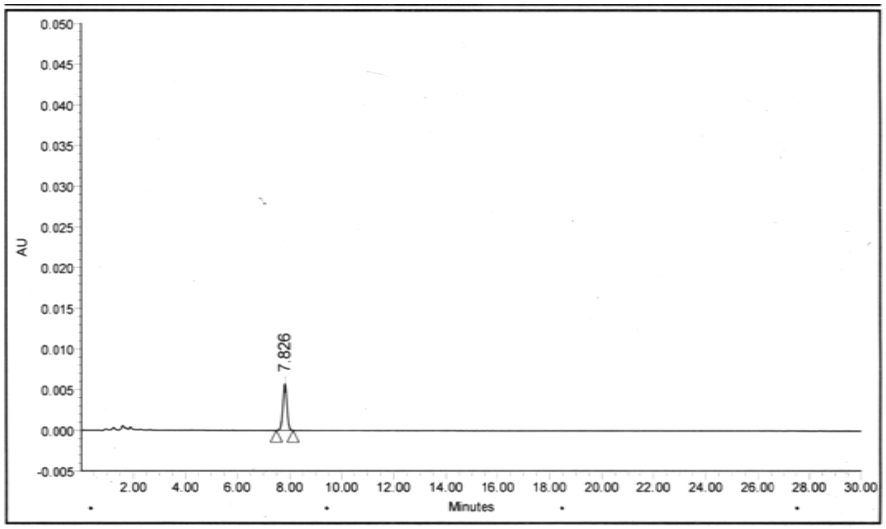
Acceptance Criteria

Cumulative percentage RSD is not more than 5.0 for peak area of Acetazolamide in standard.

Cumulative percentage RSD is not more than 10.0 peak area of known impurity and percentage of total impurity in spiked sample solution.

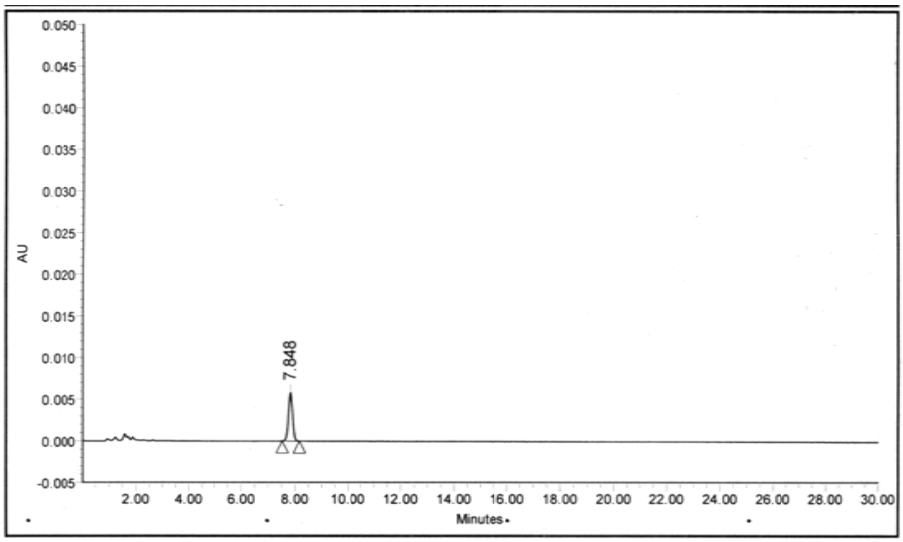
Standard Solution (2hrs)

Fig-10.1



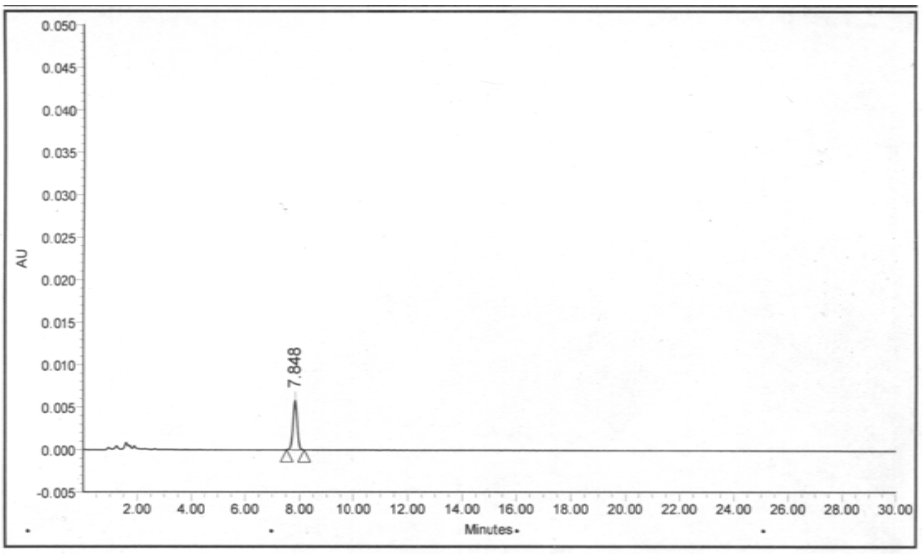
Standard Solution (9hrs)

Fig-10.2



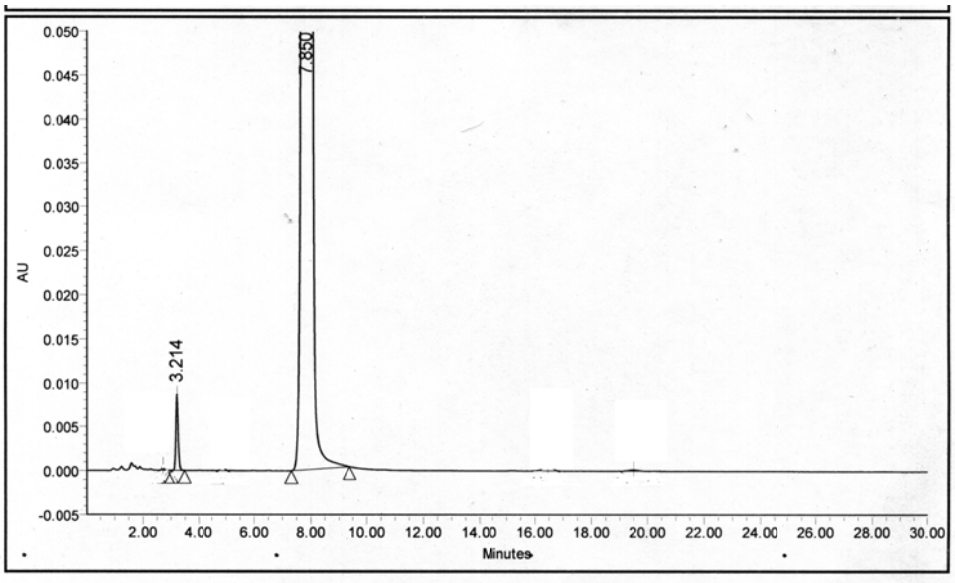
Standard Solution (48hrs)

Fig-10.3



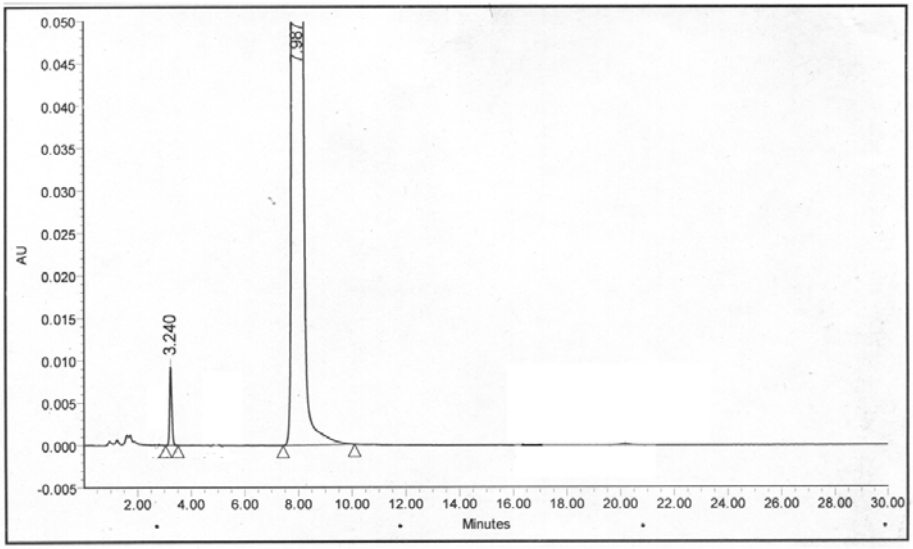
Sample Solution (7hrs)

Fig-10.4



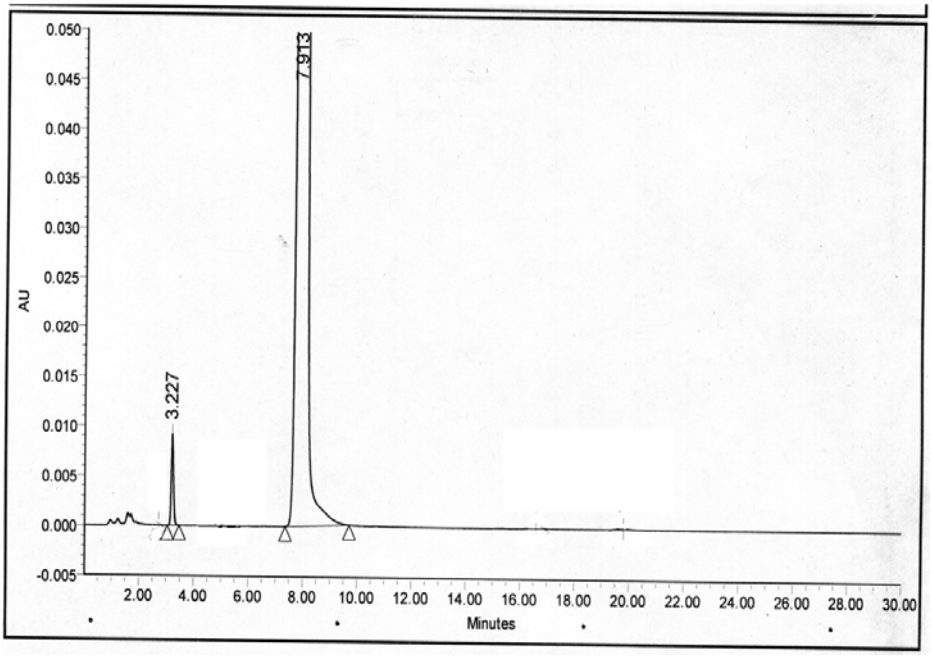
Sample Solution (28hrs)

Fig-10.5



Sample Solution (38hrs)

Fig-10.6



Solution Stability for Sample

TABLE-16

Sample solution		
Time in hours	Ambient condition	
	Peak area	Cumulative % RSD
Initial	48942	-
7	48715	0.3
15	49836	1.2
28	52384	3.4
38	52201	3.5
48	52311	3.5

Solution Stability For Standard

TABLE-17

Standard Solution		
Time in hours	Ambient Condition	
	Peak Area	Cumulative % RSD
Initial	61482	-
2	61483	0.0
9	62318	0.8
17	62441	0.9
30	67257	3.9
40	67574	4.1
48	66640	4.1

Conclusion

Cumulative percentage RSD is not more than 5.0 for peak area of Acetazolamide in standard.

ACURACY

Known amount of impurity – D spiked to the sample solution at about LOQ , 100% , and 150% . The percentage recovery was calculated from the amount recovered and actual amount added.

The percentage recovery was calculated by using the following formula

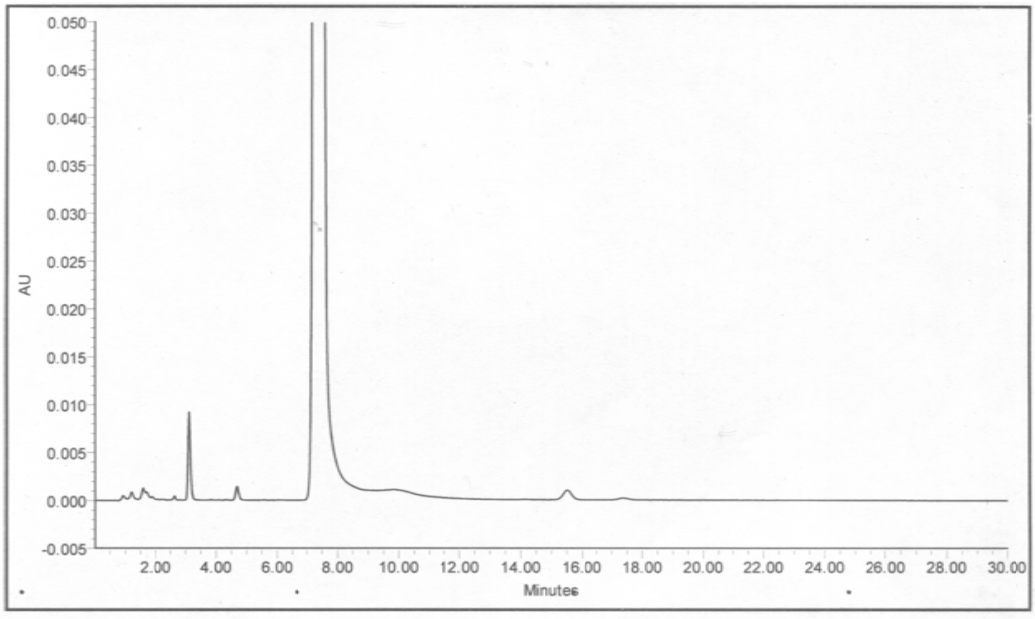
$$\% \text{ recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

Acceptance criteria

The percentage recovery of known impurity and Acetazolamide at each spiking level is between 90 .0 and 110.0 and percentage RSD is not more than 10.0 at each level.

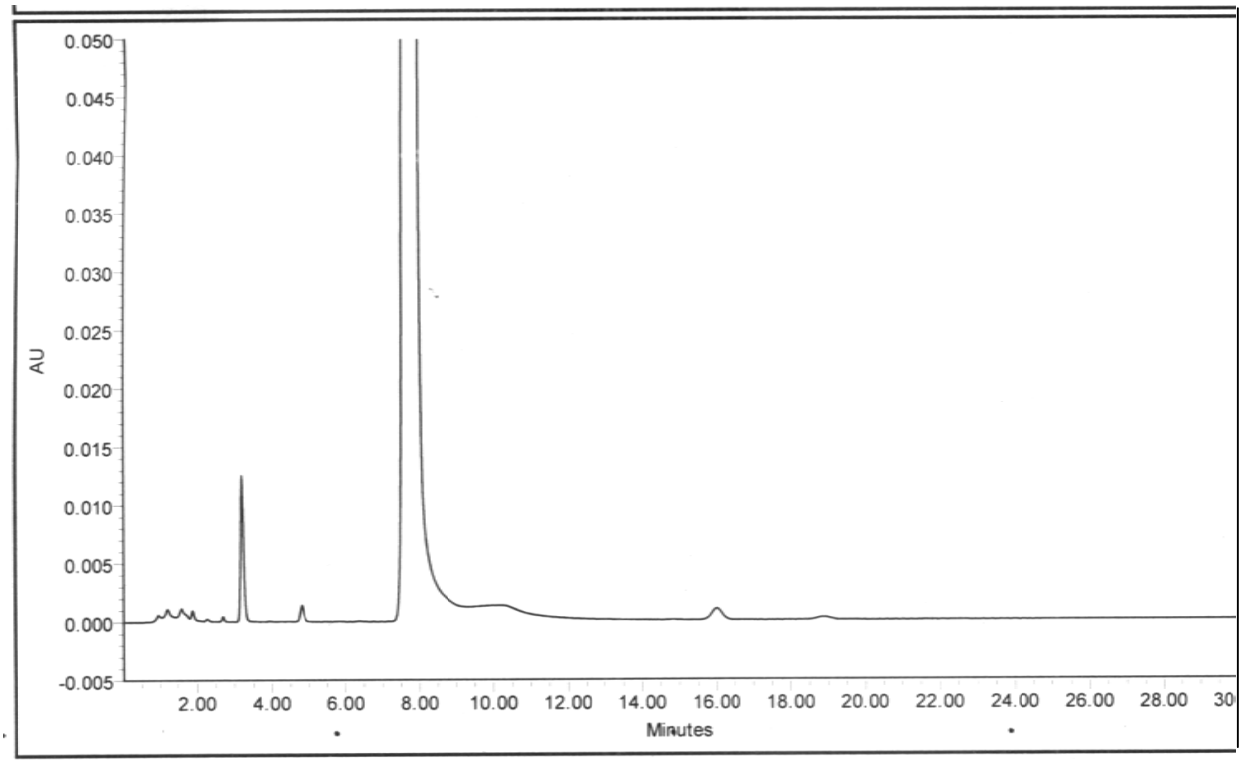
Accuracy at 100% level Chromatogram

Fig-11.1



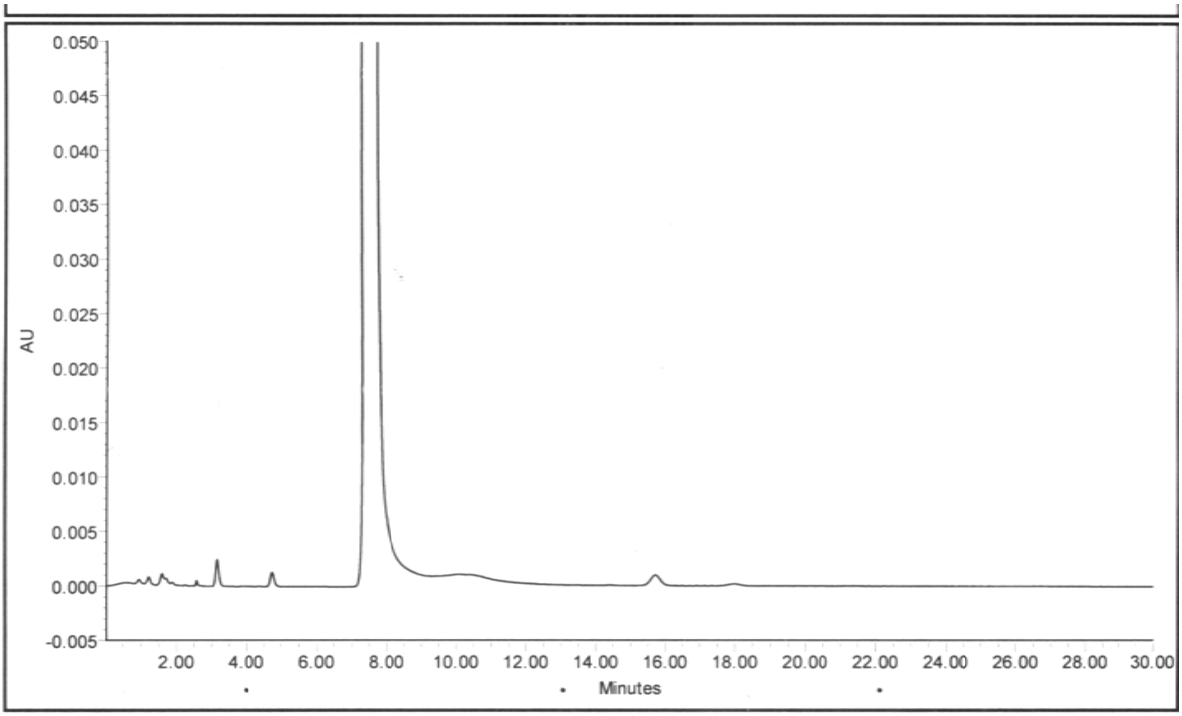
Accuracy at 150% level Chromatogram

Fig-11.2



Accuracy at LOQ level Chromatogram

Fig-11.3



Accuracy of Acetazolamide

TABLE-18

Level	Sample No	Amount added in µg	Amount recovered in µg	% recovery	Mean	% RSD
LOQ	1	0.040	0.041	102.5	102.5	0.0
	2	0.040	0.041	102.5		
	3	0.040	0.041	102.5		
100%	1	0.798	0.780	97.7	98.1	0.4
	2	0.798	0.782	98.0		
	3	0.798	0.786	98.5		
150%	1	1.196	1.159	96.9	97.5	1.4
	2	1.196	1.155	96.6		
	3	1.196	1.185	99.1		

Accuracy of Impurity-D

TABLE-19

Level	Sample No	Amount added in µg	Amount recovered in µg	% recovery	Mean	% RSD
LOQ	1	0.041	0.041	100.0	99.2	1.4
	2	0.041	0.041	100.0		
	3	0.041	0.040	97.6		
100%	1	0.828	0.826	99.8	102.2	0.5
	2	0.828	0.834	100.7		
	3	0.828	0.835	100.8		
150%	1	1.242	1.245	100.2	100.2	0.1
	2	1.242	1.243	100.1		
	3	1.242	1.246	100.3		

Conclusion

The analytical method meets the pre established acceptance criteria for accuracy study as per protocol.

FORCED DEGRADATION

The method was found to be specified as demonstrated by forced degradation studies in that all degradation impurity are resolved from the analyte peak.

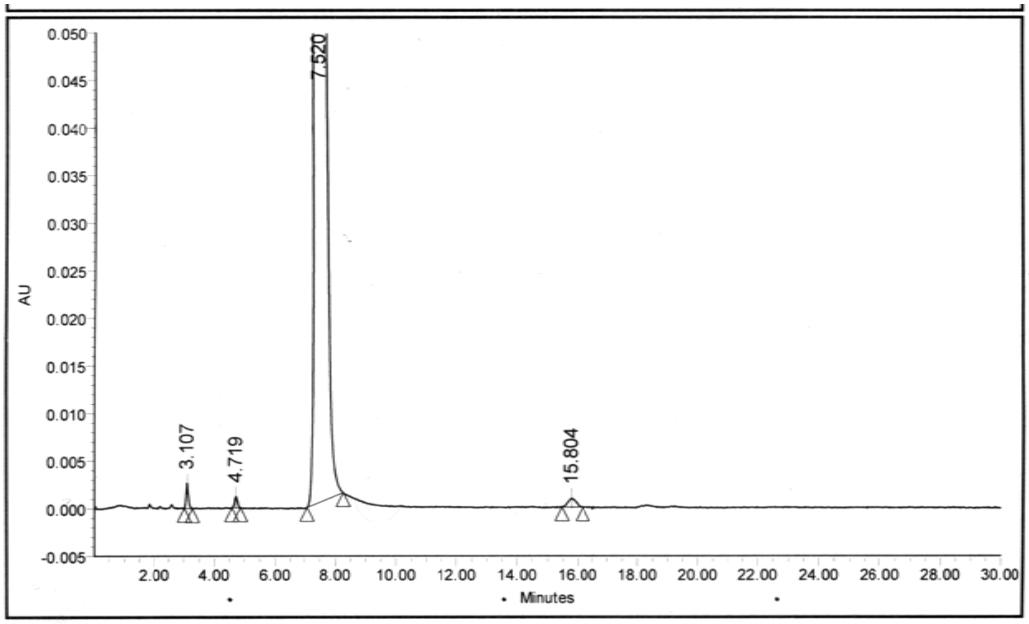
In addition, photo diode array detector (PDA) was use to demonstrate the peak spectral homogeneity with the read of peak purity results.

Acceptance Criteria

No peak elutes at the retention time of Acetazolamide and known impurity in blank and placebo.

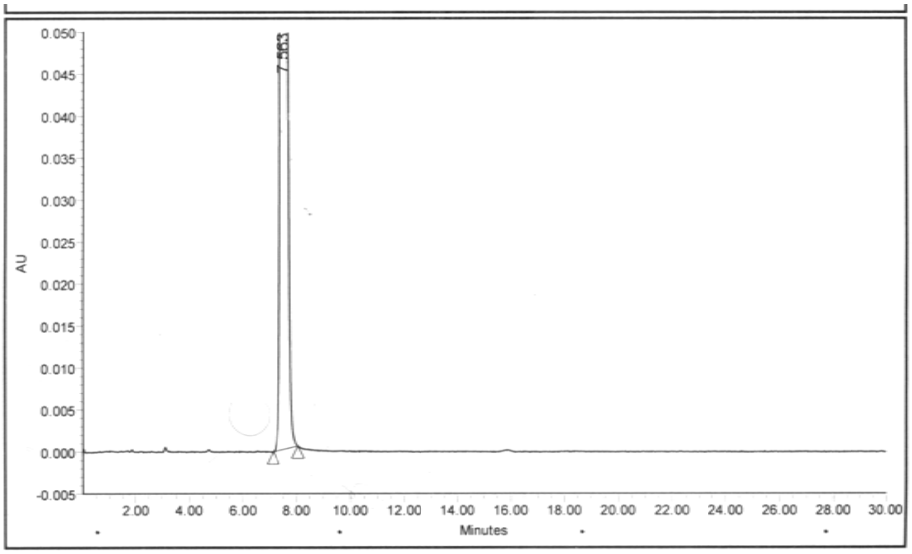
Photos stability uncontrolled sample (chromatogram)

Fig-12.1



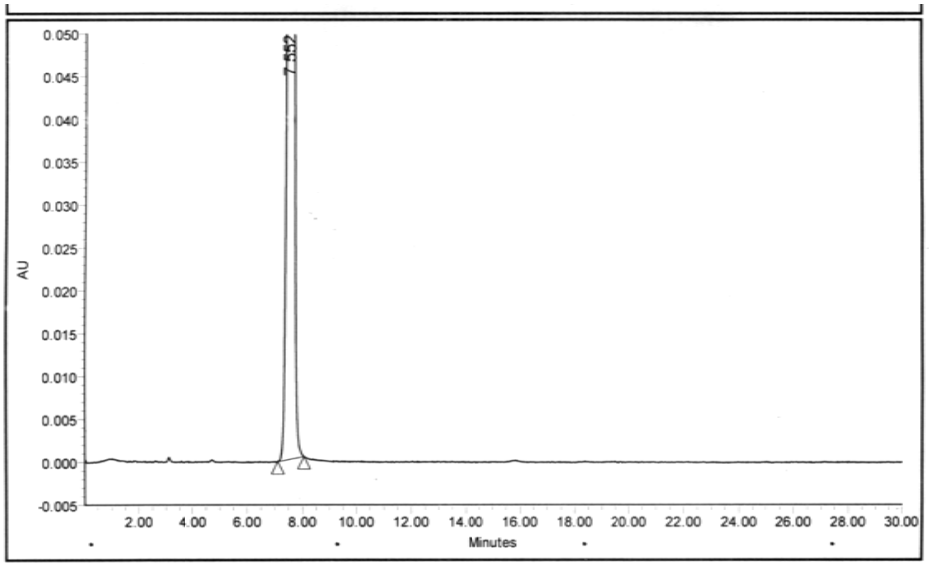
Photos stability controlled sample (chromatogram)

fig-12.2



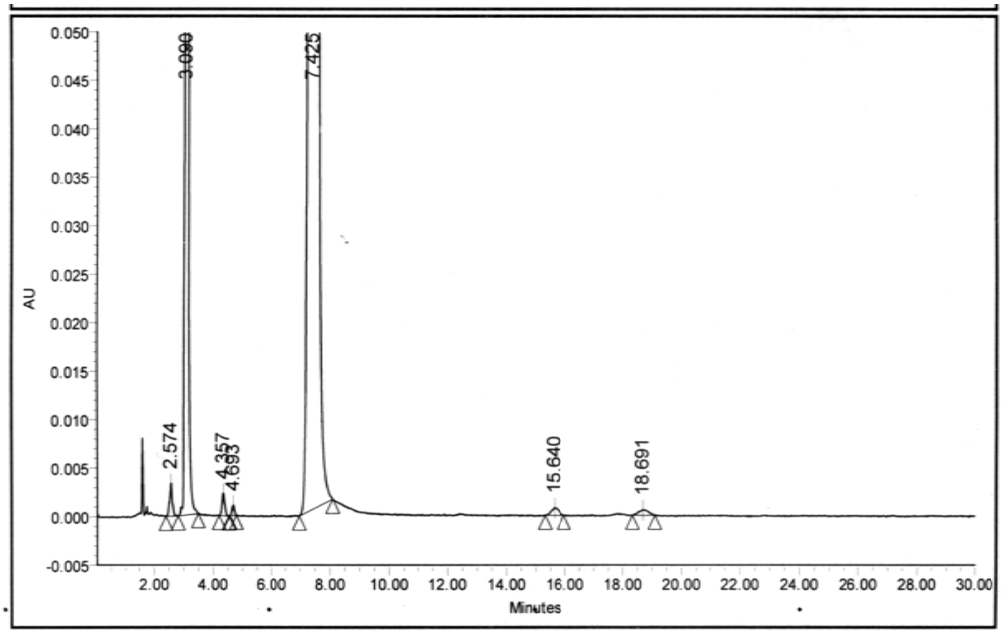
Thermal stressed sample (chromatogram)

Fig-12.3



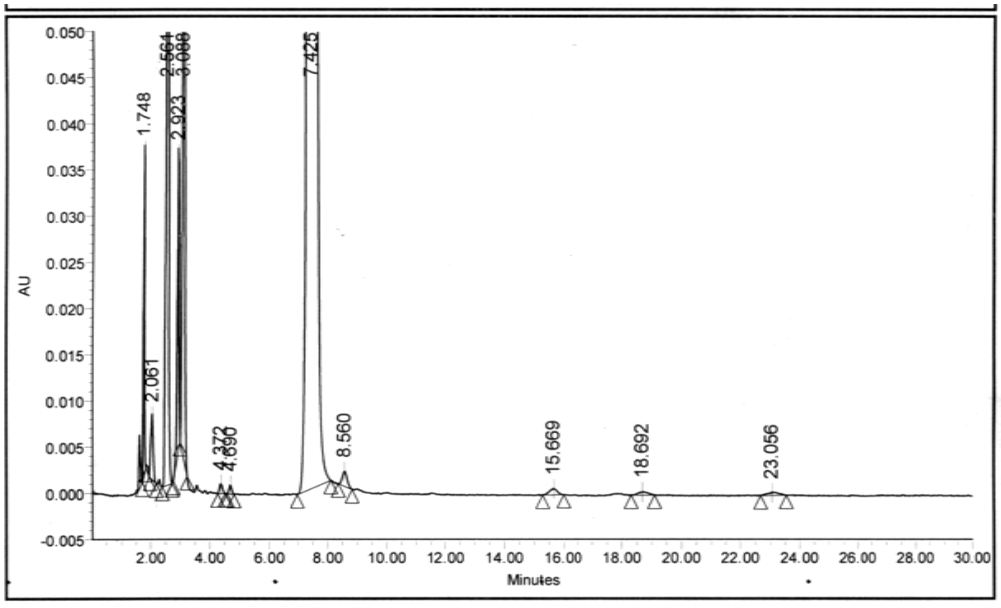
Acid stressed sample (chromatogram)

Fig-12.4



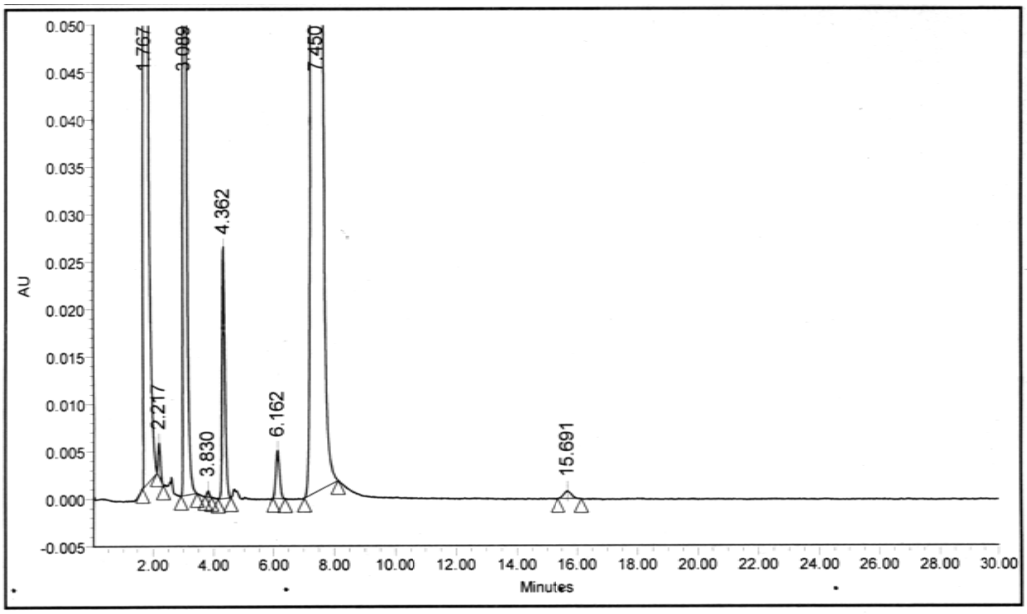
Base stressed sample (chromatogram)

Fig-12.5



Peroxide stressed sample (chromatogram)

Fig-12.6



Acetazolamide

TABLE-20

Sample No	Purity Angle	Purity Threshold
Unstressed	0.042	0.266
Acid Stressed	0.024	0.250
Base Stressed	0.031	0.244
Peroxide Stressed	0.040	0.254
Thermal Stressed	0.046	0.270
PhotoStability Controlled	0.043	0.268
PhotoStability UnControlled	0.043	0.268

Impurity-D

TABLE-21

Sample No	Purity Angle	Purity Threshold
Unstressed	4.816	5.350
Acid Stressed	0.073	0.264
Base Stressed	0.239	0.278
Peroxide Stressed	0.082	0.263
Thermal Stressed	2.373	2.856
PhotoStability Controlled	4.667	5.770
PhotoStability UnControlled	5.021	5.239

ROBUSTNESS

Robustness of the method was verified by deliberately changing the following method conditions.

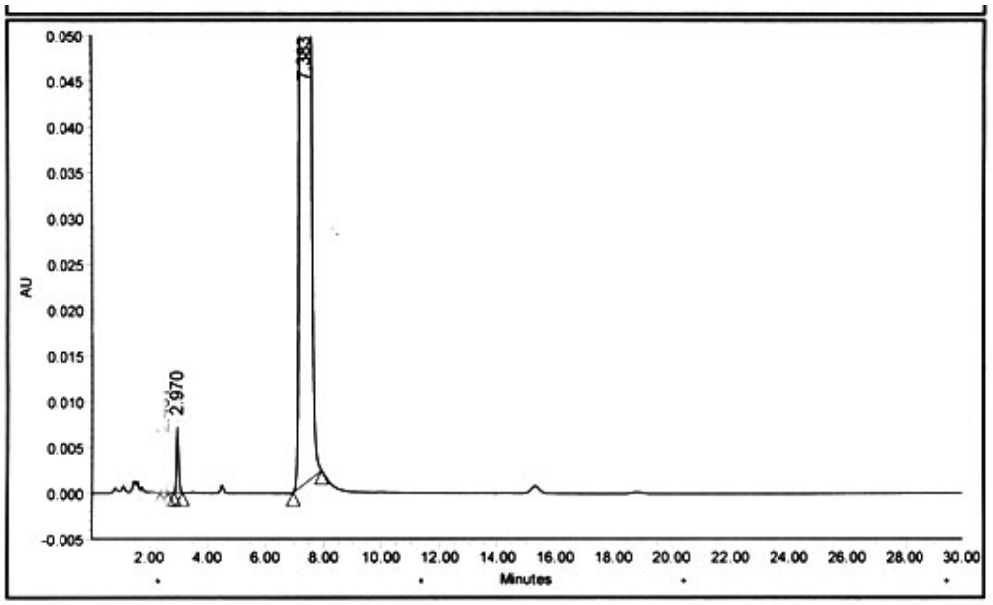
1. Flow rate (± 10%)
2. Column oven temperature (± 5 ^{o}C)
3. Wave length (± 2nm)
4. Organic content in mobile phase (± 2% absolute)
5. PH of the mobile phase buffer (± 0.2 units)

   System suitability was evaluated in each condition and impurity spiked sample was analyzed.

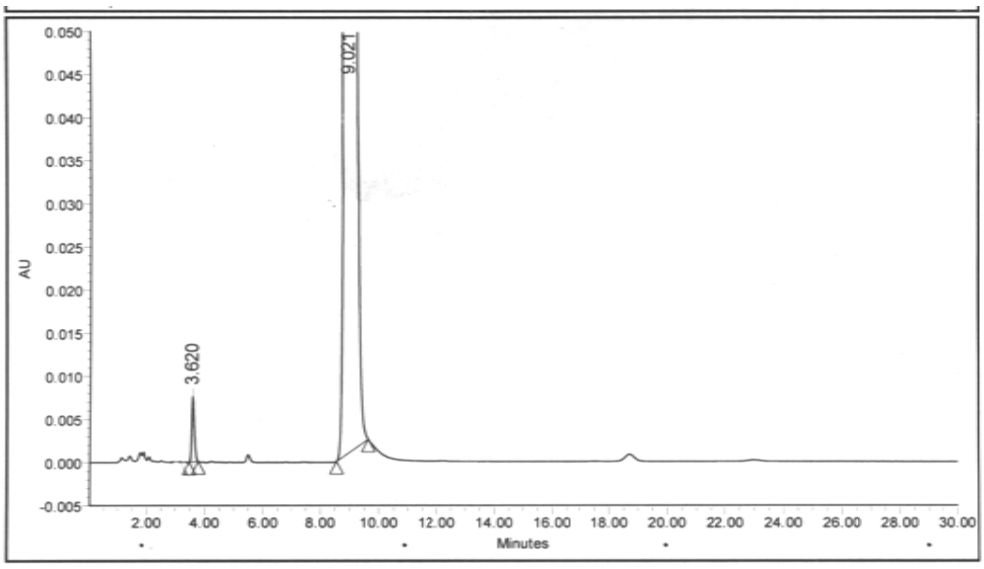
Acceptance Criteria

RRT`s of known impurities shall be comparable

Robustness high flow rate (chromatogram)
Fig-13.1

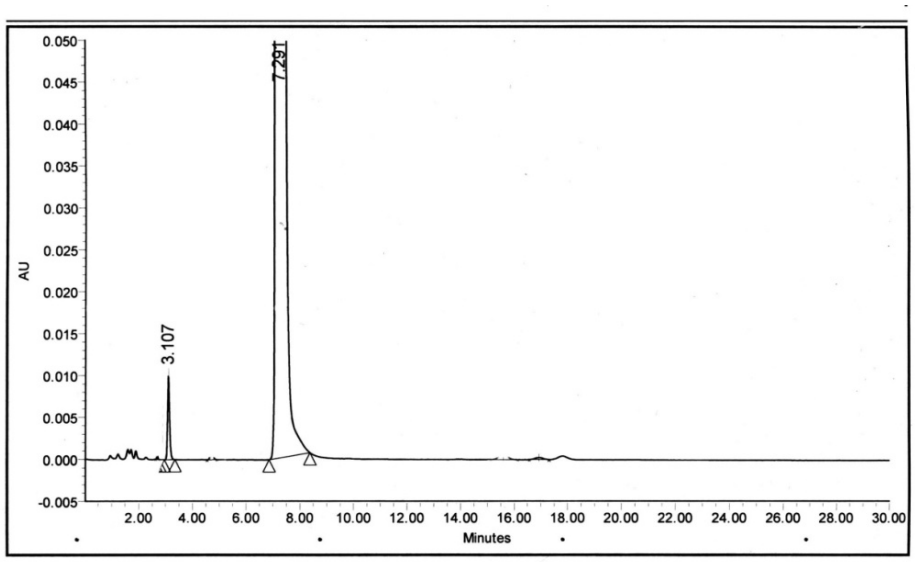


Robustness low flow rate (chromatogram)
Fig-13.2



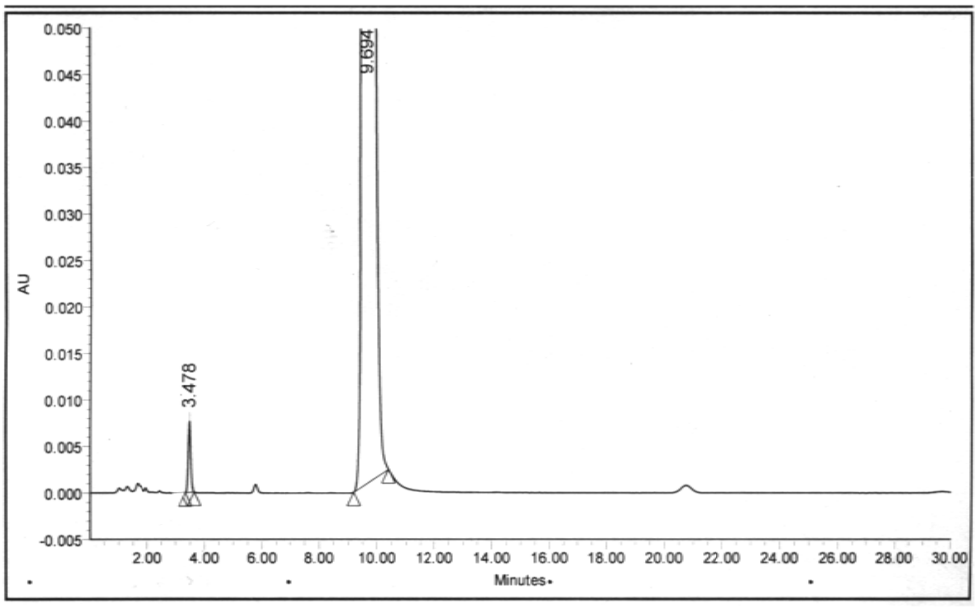
Robustness - temperature (chromatogram)

Fig-13.3

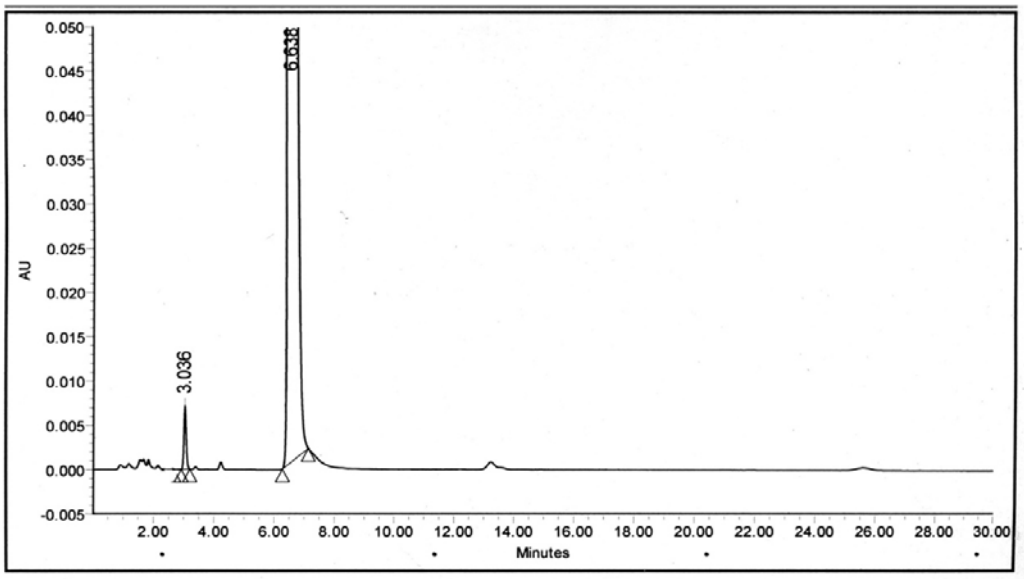


Robustness – lower organic (chromatogram)

Fig-13.4



Robustness – higher organic (chromatogram)
Fig-13.5



ROBUSTNESS

TABLE-22

Sr. No	I		II		III	
	Impurity -D	Total impurity	Impurity -D	Total impurity	Impurity -D	Total impurity
1	0.25	0.36	0.26	0.38	0.30	0.40
2	0.24	0.33	0.26	0.37	0.30	0.39
3	0.25	0.37	0.26	0.37	0.29	0.39
4	0.25	0.37	-	-	-	-
5	0.25	0.36	-	-	-	-
6	0.24	0.35	-	-	-	-
Over all mean	-	-	0.25	0.36	0.26	0.37
Overall % RSD	-	-	3.1	4.1	9.7	6.0

ROBUSTINESS

TABLE-23

Sr. No	IV		V		VI	
	Impurity -D	Total impurity	Impurity -D	Total impurity	Impurity -D	Total impurity
1	0.27	0.39	0.28	0.39	0.29	0.41
2	0.27	0.39	0.28	0.42	0.29	0.41
3	0.29	0.42	0.30	0.46	0.31	0.45
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
Over all mean	0.26	0.37	0.26	0.38	0.26	0.38
Overall % RSD	6.5	7.1	7.8	9.5	9.9	9.8

ROBUSTINESS

TABLE-24

Sr. No	IV		V	
	Impurity -D	Total impurity	Impurity -D	Total impurity
1	0.24	0.34	0.29	0.40
2	0.24	0.34	0.28	0.39
3	0.24	0.34	0.29	0.39
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
Over all mean	0.24	0.35	0.26	0.37
Overall % RSD	2.2	4.1	7.9	6.0

TABLE-25

Note	Experiment (Actual value)
I	Method precision data
II	Wavelength (263 nm)
III	Wavelength (267 nm)
IV	Column oven temperature (30^0C)
V	Flow rate (0.9 m L/ min)
VI	Flow rate (1.1 mL/ min)
VII	Organic content (8%)
VIII	Organic content (12%)

Conclusion

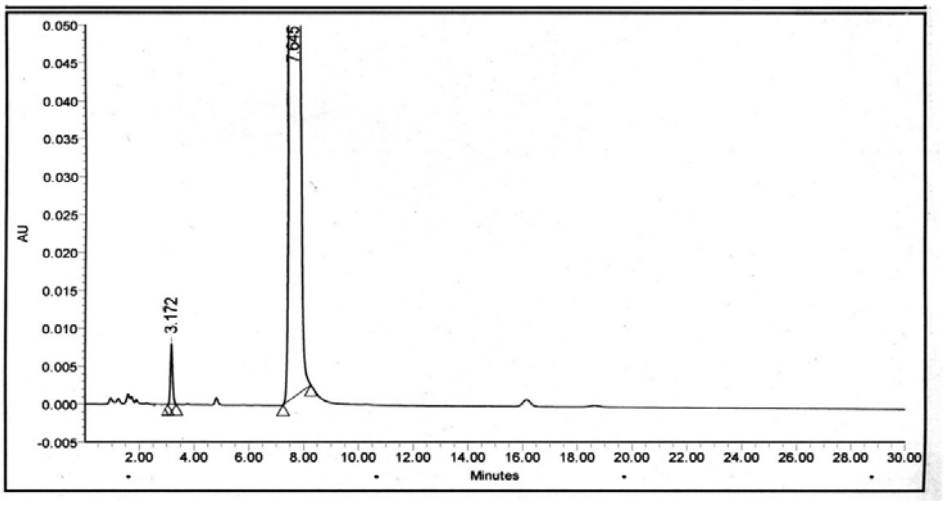
RRT`s of known impurity was within the limit. System suitability gets passed.

SYSTEM SUITABILITY

System suitability was evaluated by injecting resolution and standard solution during various days of validation. Resolution, symmetry factor, theoretical plate count and % RSD for the peak areas of Acetazolamide were verified.

System Suitability Chromatogram

Fig-14.0



SUMMARY OF SYSTEM SUITABILITY

TABLE-26

S.No	Name of experiment	Resolution	Tailing factor	Theoretical plate	% RSD
1	LOD and LOQ	3.9	1.1	11941	0.7
2	Method precision	3.8	1.0	12156	0.2
3	Intermediate precision	4.2	1.0	12242	1.6
4	Robustness	3.9	1.0	12176	1.0
5	Accuracy	4.0	1.0	11972	0.3
6	Robustness	3.7	1.1	12027	0.4
7	Robustness	4.1	1.1	12114	0.1
8	Robustness	4.0	1.0	12206	1.3
9	Specificity	4.3	1.0	12531	1.6
10	Specificity	4.4	1.0	12330	1.1

FILTER INTERFERENCE STUDY

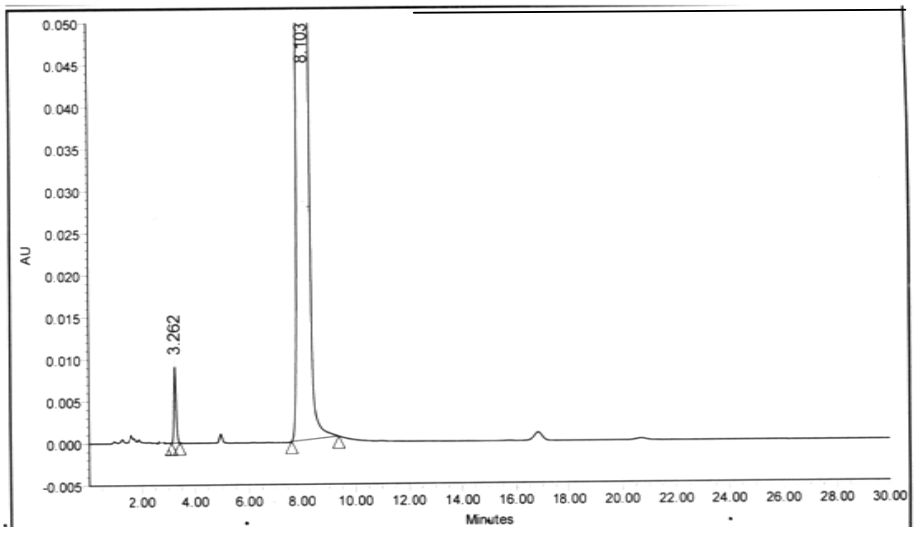
Injected the centrifuged impurity spiked sample solution and impurity spiked sample solution filtered through 0.45µm nylon filter.

Acceptance Criteria

The percentage difference between the centrifuged and filtered sample with respect to centrifuged sample is not more than ±10.0 for known and total impurities.

FILTER INTERFERENCE CHROMATOGRAM

Fig-15.0



FILTER INTERFERENCE DATA

TABLE-27

Filter name	Pore size	L.No	Manufacturer
Nylon filter	0.45µm	SN3190	Advanced micro devices pvt.ltd

TABLE-28

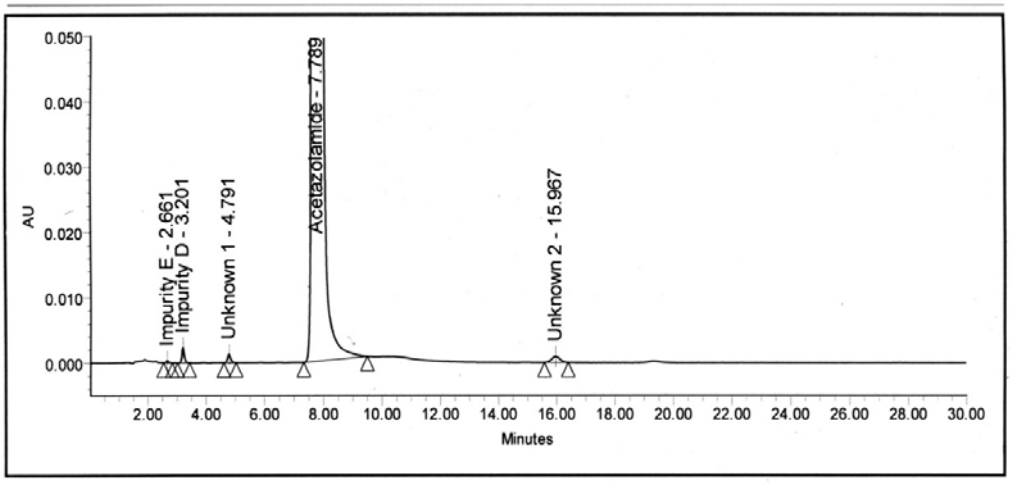
Name	Result in % w/w		% difference
	Filtered sample	Centrifuged sample	
Impurity-D	0.24	0.25	4.0

Conclusion

0.45µm nylon filter suitable for filtering the sample solution.

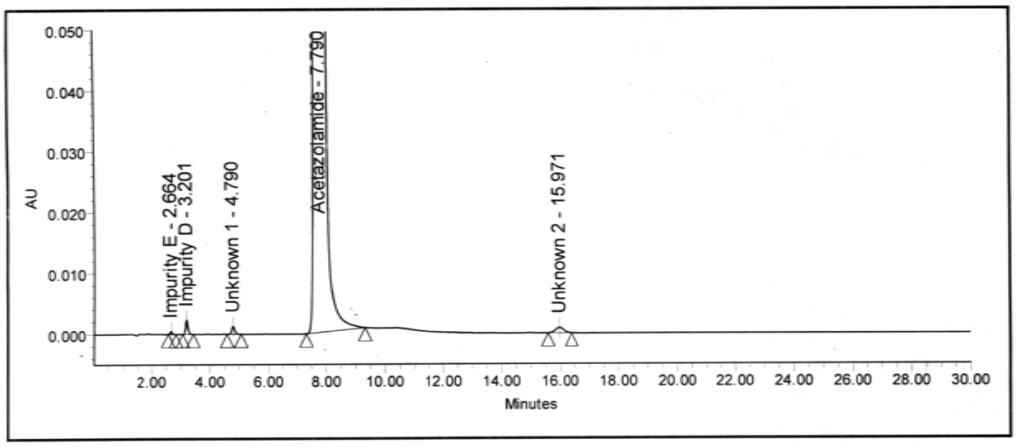
STABILITY INDICATING METHODS (25^0C/ 90 %RH)

Fig-16.1



STABILITY INDICATING METHODS (40^0C/ 90 %RH)

Fig-16.2



Conclusion

Impurity - E is arised

8. CONCLUSION

A simple, economic, selective, precise and stability-indicating HPLC method has been developed and validated for analysis of Acetazolamide SR Capsules and its related substances. Reversed phase chromatography was performed on a C-18 column with Acetonitrile : Buffer as mobile phase at a flow rate of 1.0mL/min. Detection was performed at 265nm and a sharp peak was obtained for Acetazolamide at a retention time of 7.5 min. The %RSD for the peak area of LOQ level is not more than 2. The method was validated for accuracy, precision, reproducibility, specificity and robustness, in accordance with ICH guidelines. Statistical analysis proved the method was precise, reproducible, selective, specific, and accurate for the analysis of Acetazolamide SR capsules. The wide linearity range, sensitivity, accuracy, short retention time and simple mobile phase imply the method is suitable for routine quantification of Acetazolamide with high precision and accuracy.

The proposed method can be used as alternative method to the reported ones for the routine determination of selected drugs under the study in bulk and pharmaceutical dosage forms. In RS method all the peaks were well separated, peak purity was passing for the peaks and finally method was a stability-indicating method.

Thus the purpose of the present investigation was successfully achieved. Pharmaceutical analysis simply means analysis of pharmaceuticals. Today pharmaceutical analysis entails much more than the analysis of active pharmaceutical ingredients or the formulated product. The pharmaceutical analyst plays in a major rule in assuring identity, safety, efficacy, purity, and quality of a drug product. New methods are now being developed with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.

The result of the validation parameters meet the ICH guidelines.

S.No	Parameters	Limit	Pass/Fail
1	System suitability data	RSD NMT 2% Tailing NMT 2% Plate count NLT 10000	Passes
2	Specificity	No interference with the retention time of analyte peak.	Passes
3	Precision	RSD NMT 2%	Passes
4	Linearity	Correlation coefficient NLT 0.999	Passes
5	Accuracy	% recovery in the range of 90-110%	Passes
6	Robustness	RSD NMT 2%	Passes

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