METHOD DVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF CLIDINIUM BROMIDE AND CHLORDIZEPOXIDE IN BULK AND TABLET DOSAGE FORM BY A RP-HPLC

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Submitted by

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1. INTRODUCTION

HPLC introduction

The modern form of column chromatography has been called high performance, high pressure, and high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.[1]

Normal Phase Chromatography

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents.

The silica structure is saturated with silanol groups at the end. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.

- Dipole-induced dipole
- Dipole-dipole
- Hydrogen bonding
- -Complex bonding

These situations arise when the molecule has one or several atoms with lone pair electron or a double bond. The absorption strengths and hence k' values (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatics < organic halogen compounds <sulphides< ethers< esters < aldehydes and ketones < amines <sulphones< amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample molecule but also on steric factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as the aminopropyl, cyanopropyl and diol phases is useful alternatives to silica gel as stationary phase in normal phase chromatography.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phases and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in their increased homogenity of the phase surface.

Resolution with water in weak mobile phase may be most conveniently achieved by drying the solvents and then adding a constant concentration of water or some very polar modifier such as acetic acid or tri ethylamine (TEA) to the mobile phase. The addition of such polar modifiers serves to deactivate the more polar shape as well as the reproducibility of the retention times.

Reversed Phase Chromatography

In 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

A large number of chemically bonded stationary phases based on silica are available commercially. Table 1 lists some of the functional groups bonded in chemically modified silica. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbants based on polymer (styrene-divinyl benzene copolymer) are slowly gaining ground.

Simple compounds are better retained by the reversed phase surface, the less water-soluble (i.e. the more non-polar) they are. The retention decreases in the following order: aliphatics> induced dipoles (i.e. CCl_4) > permanent dipoles (e.g.CHC_{l3}) > weak lewis bases (ethers, aldehydes, ketones) > strong lewis bases (amines) > weak lewis acids (alcohols, phenols) > strong lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases.

As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimensional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecylsilane (ODS) an alkaline with 18 carbon atoms it is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

The essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures.

The systems used are often described as belonging to one of four mechanistic types, adsorption, partition, ion exchange and size-exclusion. Adsorption chromatography arises from interaction between solutes on the surface of the solid stationary phase. Partition chromatography involves a liquid stationary phase, which is immiscible with the eluent and coated on an inert support. Adsorption and partition systems can be normal phase (stationary phase more polar than eluent) or reversed phase (stationary phase less polar than eluent). Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, the large molecules enable to enter the pores eluting first.

The various components of a HPLC system are herewith described.

The HPLC system is shown in **Fig.1.0**

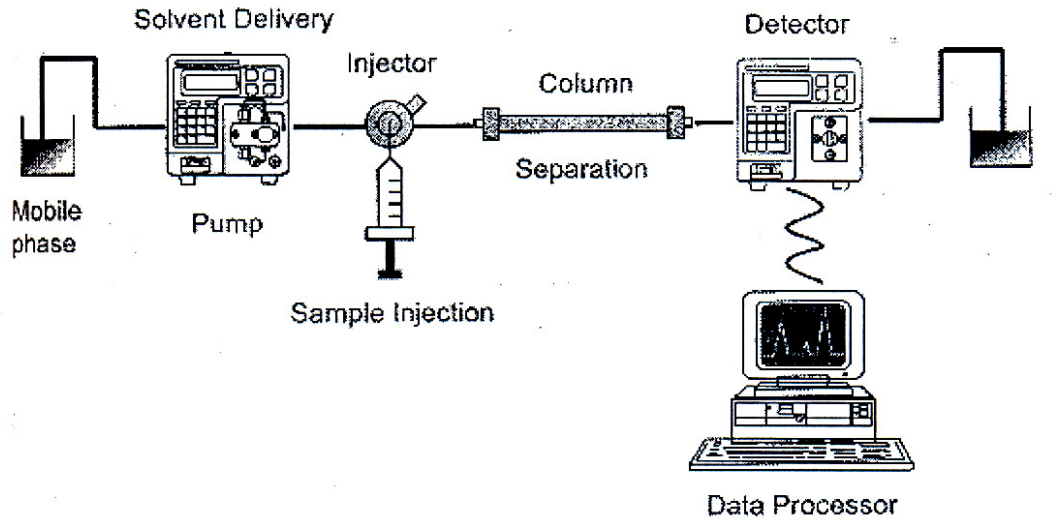

Figure no.1.0.HPLC system

Instrumentation:

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. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate, reproducibility etc.

Solvent degassing system

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filter, vacuum degassing with an air-soluble membrane, helium purging ultra sonication or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

Gradient elution devices

HPLC columns may be run isocratically, i.e., with constant eluent or they may be run in the gradient elution mode in which the mobile phase composition varies during run. Gradient elution is a means of overcoming the problem of dealing with a complex mixture of solutes.

Sample introduction systems

Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples can be loaded in to the auto injector tray. The system parameters such as flow rates, gradient, run time, volume to be injected, etc. are chosen, stored in memory and sequentially executed on consecutive injections.

Liquid chromatographic detectors

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. Generally, there are two types of HPLC detectors, bulk property detectors and solute property detectors.

Bulk property detectors

These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity and dielectric constant detectors.

Solute property detectors

Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase. These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase prior to the detection. Solute property detectors which do not require the removal of the mobile phase before detection include

spectrophotometric (UV or UV-Vis) detector, fluorescence detectors, polarographic, electro-chemical and radio activity detectors, whilst the moving wire flame ionization detector and electron capture detector both require removal of the mobile phase before detection.

UV-Vis and fluorescent detectors are suitable for gradient elution, because many solvents used in HPLC do not absorb to any significant extent.

Column and Column-packing materials

The heart of the system is the 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_2, H_2O) 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. Thus a wide range of commercial products is available with surface areas ranging from 100 to800 m^2/g. And particle sizes from 3 to 50 πm.

The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic eluents. Silica can be drastically altered by reaction with organochlorosilanes or organoalkoxysilanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon change to silica produces a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is octadecyl-silica (ODS-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. trimethylchlorosilane) to reduce further the number of silanol groups remaining on the surface (end-capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contain groups such as phenyl, nitro, amino and hydroxyl. Strong ion exchangers are also available in which sulphonic acid groups or quaternary ammonium groups are bonded to silica. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight silica may dissolve.

In HPLC, generally two types of columns are used, normal phase columns and reversed phase columns. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation. It was originally believed that separation of compounds in mixture takes place slowly by differential adsorption on a stationary silica phase.

However, it now seems that partition plays an important role, with the compounds interacting with the polar silanol groups on the silica or with bound water molecules.

While normal phase seems the passage of a relatively non-polar mobile phase over a polar stationary phase, reversed phase chromatography is carried out using a polar mobile phase such as methanol, Acetonitrile, water, buffers etc., over a non-polar stationary phase. Ranges of stationary phases (C_{18}, C_8, -NH_2, -CN, -phenyl etc.) are available and very selective separations can be achieved. The pH of the mobile phase can be adjusted to suppress the ionization of the drug and thereby increase the retention on the column. For highly ionized drugs ion-pair chromatography is used.

Derivatization

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been widely used. Ultra violet derivatization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives can be formed with reagents such as dansyl chloride, 4-bromo methyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected on to the column or by online chemical reactions between the column out let and the detector.

Gradient elution

Gradient elution or solvent programming is the change of solvent composition during a separation in which the solvent strength increases from the beginning to the end of the separation. It is well suited to the analysis of samples of unknown complexity since good resolution is automatically provided for a wide range of sample polarities. There are two types of gradient systems: Low-pressure gradient mixtures and high- pressure gradient mixtures. In the former the solvents are mixed at atmosphere pressure and then pumped to the column, where as in the later, solvents are pumped in to a mixing chamber at high pressure before going in to the column.

Performance calculations

Calculating the following values (which can be including in a custom report) used to access overall system performance.

Relative retention

Theoretical plates

Capacity factor

Resolution

Peak asymmetry

Plates per meter

The parameters used to calculate these system performance values for the separation of two chromatographic components. (Note: Where the terms W and t both appear in the same equation they must be expressed in the same units).

Relative retention (Selectivity):

$$\mu = (t_2 - t_a) / (t_1 - t_a)$$

Theoretical plates:

$$n = 16 (t / W)^2$$

Capacity factor:

$$K' = (t_2 / t_a)^{-1}$$

Resolution:

$$R = 2 (t_2 - t_1) / (W_2 + W_1)$$

Peak asymmetry:

$$T = W0.05 / 2f$$

Plates per meter:

$$N = n / L$$

HETP: L/n

Where, α = Relative retention.

t_2 = Retention time of the second peak measured from point of injection.

t_1 = Retention time of the first peak measured from point of injection.

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

n = Theoretical plates.

t = Retention time of the component.

W = Width of the base of the component peak using tangent method.

K' = Capacity factor.

R = Resolution between a peak of interest (p2) and the peak preceding it (p1)

W_2 = Width of the base of component peak 2.

W_1 = Width of the base of component peak 1.

T= Peak asymmetry, or tailing factor.

W0.05 = Distance from the leading edge to the tailing edge of the peak,

Measured at a point 5 % of the peak height from the baseline.

f = Distance from the peak maximum to the leading edge of the peak.

N = Plates per meter.

L = Column length, in meters.[2,3,4]

METHOD OPTIMIZATION

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts asymmetry, capacity, elution time, detection limits, limit of Quantitation, and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow either of two general approaches:

1. Manual
2. Computer driven

The manual approach involves varying one experimental variable at a time, while holding all others constant, and recording changes in response .The variables might include flow rates, mobile or stationary phase composition, temperature, detection wavelength, and pH this univariate approach to system optimization is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated methods development, efficiency is optimized while experimental input is minimized. Computer driven automated approaches can be applied to many applications .In addition, they are capable of significantly reducing the time, energy and cost of virtually all-instrumental methods development.

The various parameters that include to be optimized during method development

1. Mode of separation
2. Selection of stationary phase
3. Selection of mobile phase
4. Selection of detector

Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

Selection of stationary phase / column

Selection of the column is the first and the most important step in method development the appropriate choice of separation column includes three different approaches

1. Selection of separation system
2. The particle size and the nature of the column packing
3. The physical parameters of the column i.e. the length and the diameter

Some of the important parameters considered while selecting chromatographic columns are

- Length and diameter of the column.
- Packing material.
- Shape of the particles.
- Size of the particles.
- % of Carbon loading
- Pore volume.
- Surface area.
- End capping.

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C_2), butylsilane (C_4), octylsilane (C_8), octadecylsilane (C_{18}), base deactivated silane (C_{18}) BDS phenyl, cyanopropyl (CN), nitro, amino etc. C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column

Generally longer columns provide better separation due to higher theoretical plate numbers. As the particle size decreases the surface area available for coating increases. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability. In this case, the column selected had a particle size of 5 µm and a internal diameter of 4.6 mm

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- In accurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peak tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method

A column which gives separation of all the impurities and degradants from each other and from Analyte peak and which is rugged for variation in mobile phase shall be selected.

Selection of mobile phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, solute – mobile phase and the mobile phase – stationary phase. For a given stationary phase, the retention of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation) Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the PKa of the analytes the solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength.

The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer,
- pH of the buffer
- Mobile phase composition.

Buffer, if any and its length:

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most, commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4,NaH_2PO_4,Na_2HPO_4,etc
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers – Ammonium acetate, Sodium acetate, etc.
- Acetic acid buffers prepared using CH_3COOH.

The retention times also depend on the molar strengths of the buffer – Molar strength is increasingly proportional to retention times. The strength of the buffer can be increased, if necessary, to achieve the required separations.

The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength.

pH of the buffer:

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Experiments were conducted using buffers having different pH to obtain the required separations**.**

It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkage area cleaved below pH 2.0, while pH valued above 8.0 silica may dissolve.

Mobile phase composition:

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to that fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments were conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations between the impurities. A mobile phase which gives separation of all the impurities and degradants from each other and from analyte peak and which is rugged for variation of both aqueous and organic phase by at least ±0.2% of the selected mobile phase composition.

Selection of detector:

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction etc. characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity, facilitating trace analysis
- Negligible baseline noise. To facilitate lower detection
- Large linear dynamic range
- Low dead volume
- Non destructive to sample
- Inexpensive to purchase and operate

Pharmaceutical ingredients do not all absorb UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful.

For the greatest sensitivity λ_{max} should be used. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.[5,21]

METHOD VALIDATION

Method validation can be defined as (ICH*)* "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

Method validation is an integral part of the method development, it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Method Validation, however, is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For

chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including

(a) Recovery (b) Response function (c) Sensitivity (d) Precision (e) Accuracy

(f) Limit of detection (g) Limit of quantitation (h) Ruggedness (i) Robustness (j) stability (k) system suitability**.**

(a) Recovery

The absolute recovery of analytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample. It is best established by comparing the responses of extracted samples at low, medium and high concentrations in replicates of at least 6 with those non-extracted standards, which represent 100 % recovery.

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$$\text{Absolute recovery} = \frac{\text{response of an analyte spike into matrix (processed)}}{\text{response of analyte of pure standard (unprocessed)}} \text{ X } 100$$

If an internal standard is used, its recovery should be determined independently at the concentration levels used in the method.

(b) Response of function

In chromatographic methods of analysis, peak area or peak height may be used as response function to define the linear relationship with concentration known as the calibration model. It is essential to verify the calibration model selected to ensure that it adequately describes the relationship between response function (Y) and concentration (X).

(c) Sensitivity

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at α 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is

discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

(d) Precision

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important criteria for judging analytical procedures by their results.

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term 'set' is defined as referring to a number (n) of independent replicate measurements of some property. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$S = \sqrt{\frac{1}{n-1}\sum_{i-1}^{n}(x_i - \bar{x})^2}$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is some times multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S/x X100.

(e) Accuracy

Accuracy normally refers to the difference between the mean x, of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

Accuracy is best reported as percentage bias, which is calculated from the expression

Since for real samples the true value is not known, an approximation is obtained based on spiking drug – free matrix to a nominal concentration. The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug – free matrix sampler.

Calibration

Calibration is the most important step in bioactive compound analysis. A good precision and accuracy can only be obtained when a good calibration procedure is adopted. In the spectrophotometric methods, the concentration of a sample cannot be measured directly, but is determined using another physical measuring quantity 'y' (absorbance of a solution). An unambiguous empirical or theoretical relationship can be shown between this quantity and the concentration of an analyte. The calibration between y = g (x) is directly useful and yields by inversion of the analytical calculation function.

The calibration function can be obtained by fitting an adequate mathematical model through the experimental data. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. In practice, however, many deviations from the ideal calibration line may occur. For the majority of analytical techniques the analyst uses the calibration equation.

$$Y = a + bx.$$

In calibration, univariate regression is applied, which means that all observations are dependent upon a single variable X.

Standard deviation of slope (Sb)

The standard deviation of slope is proportional to standard error of estimate and inversely proportional to the range and square root of the number of data points.

$$Sb = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{(n-2)}} \sqrt{\frac{1}{\sum_{i=1}^{n}(x_i - \bar{x}_i)^2}}$$

Where Xi is the arithmetic mean of Xi values.

Standard deviation of intercept, (Sa)

Intercept values of least squares fits of data are often to evaluate additive errors between or among different methods.

$$Sa = \sqrt{\frac{\sum_{i=1}^{n}\left(y - \hat{y}_i\right)^2}{(n-2)}} \sqrt{\frac{1}{\sum_{i=1}^{n}(X_i - \bar{X}_i)^2}} \sqrt{\frac{\sum_{i=1}^{n} x_i^2}{n}}$$

Where Xi denote the arithmetic mean of xi, values.

Correlation Coefficient, (r)

The correlation coefficient r (x,y) is more useful to express the relationship of the chosen scales. To obtain a correlation coefficient the covariance is divided by the product of the standard deviation of x and y.

$$r = \frac{\left[\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})\right] \Big/ (n-1)}{\left[\sum_{i=1}^{n}(x_i - \bar{x})^2 (y_i - \bar{y})^2\right] \Big/ (n-1)^2}$$

Linearity and sensitivity of the method

Knowledge of the sensitivity of the color is important and the following terms are commonly employed for expressing sensitivity. According to Lambert – Beer's law, log intensity of incident radiations

$$A = \text{Log}\frac{\text{Intensity of incident light}}{\text{Intensity of transmitted light}} = \pm ct$$

The absorbance (A) is proportional to the concentration (c) of the absorbing species, if absorptivity ($\in$) and thickness of the medium (t) are constant. When c is in moles per liter, the constant is called molar absorptivity. Beer's law limits and $\in$ max values are expressed as µg ml^{-1} and $mole^{-1}$ cm^{-1}respectively.Sandell's sensitivity refers to the number of µg of the drug to be

determining, converted to the colored product, which in a column solution of cross section $1cm^2$ shows an absorbance of 0.001 (expressed as $\mu g\ cm^{-2}$).

(f) Limit of detection (LOD)

The limit of detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that reliably upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa), which may be related to LOD and the slope of the calibration curve, b, by

$$LOD = 3\ Sa / b$$

(g) Limit of quantitation (LOQ)

The LOQ is the concentration that can be quantitate reliably with a specified level of accuracy and precision. The LOQ represent the concentration of analyte that would yield a signal-to-noise ratio of 10.

$$LOQ = 10\ Sa / b$$

Where, Sa- the estimate is the standard deviation of the peak area ratio of analyte to IS (5 injections) of the drugs. b -is slope of the corresponding calibration curve.

(h) Ruggedness

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals, solvents etc. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

(i) 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 robustness of a method is the ability to remain unaffected by small changes in parameters such as pH of the mobile phase, temperature, % organic solvent strength and buffer concentration etc. to determine the robustness of the method experimental conditions were purposely altered and chromatographic characters were evaluated.

(j) Stability

To generate reproducible and reliable results, the samples, standards and reagents used for the HPLC method must be stable for a reasonable time (e.g. one day, one week, and one month depending upon need). For example, the analysis of even a single sample may require ten or more chromatographic runs to determine the system suitability, including standard concentrations to create a working analytical curve and duplicate or triplicate injections of the sample to be assayed.

(k)System suitability

System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. (or) The USP (2000) defines parameters that can be used to determine system suitability prior to analysis.[6,7,22]

2.REVIEW OF LITERATURE

- ***Lehr. G, Yuen. SM et al.,*** developed a specific liquid. The system was found to be free from any interferences from the 4 known related compounds of the 2 major components and is useful as a screening procedure for 7-chloro-1,3-dihydro-5-phenyl-2H-1, 4-benzodiazepin-2-one-4-oxide,the principal degradation product of chlordiazepoxide hydrochloride. Chromatographic method was developed for determination of clidinium bromide and clidinium bromide-chlordiazepoxide hydrochloride combinations in capsules. The procedure uses a Partisil 10 ODS-3 column and a mobile phase consisting of acetonitrile-0.3M ammonium phosphate (32 + 68) (pH = 4.3). The detection wavelength is 235 nm. Four commercial samples of the single ingredient were tested results compared favorably with the compendial method. Two commercial samples of the combination product were tested by the proposed method and results reported.[8]

- ***Savedra. M, Soto C et al.,*** performed direct and simple first derivative spectrophotometric method has been developed for the simultaneous determination of clidinium bromide and chlordiazepoxide in pharmaceutical formulations. Acetonitrile was used as solvent for extracting the drugs from the formulations and subsequently the samples were evaluated directly by derivative spectrophotometry. Simultaneous determination of the drugs can be carried out using the zero-crossing method for clidinium bromide at 220.8 nm and the graphical method for chlordiazepoxide at 283.6 nm. The calibration graphs were linear in the ranges from 0.983 to 21.62 mg/ml of clidinium bromide and from0. 740 to 12.0 mg/l of chlordiazepoxide.[9]

- ***NuhaKattan, SafwanAshour et al.,***carried out a sensitive and precise RP-HPLC method has been developed for the simultaneous estimation of clidinium bromide (CDB) and chlordiazepoxide (CDZ) in pure and pharmaceutical formulations. The separation was achieved on a Nucleodur C_8 (mm i.d., 5µm particle size) column at 25°C. CH_3CN: MeOH: NH_4OAc0.1M (30:40:30, v/v/v) was used as the mobile phase at a flow rate of 1.0mLmin^{-1} and detector wavelength at 218nm. Almotriptan (ALT) was used as internal standard. The method showed good linearity in the ranges of 2.5–300.0 and 3.0–500.0µgmL^{-1} for CDB and CDZ, respectively. The percentage recovery obtained for CDB

and CDZ was 100.40–103.38 and 99.98–105.59%, respectively. LOD and LOQ were 0.088 and 0.294µgmL^{-1} for CDB and 0.121 and 0.403µgmL^{-1} for CDZ, respectively.[10]

- ***Pallavi R, AshutoshPathak et al.,***has donea validation of a stability indicating reverse-phase high-performance liquid chromatography method for the simultaneous estimation of clidinium bromide and chlordiazepoxidefrom their combination drug product. Chromatographic separations are performed at ambient temperature on a Phenomenex Luna C_{18} (250 mm × 4.6 mm, i.d., 5 µm) column using a mobile phase consistingof potassium dihydrogen phosphate buffer (0.05 M, pH 4.0 adjusted with 0.5% orthophosphoric acid): methanol: acetonitrile (40:40:20, v/v/v). The flow rate is 1.0 mL/min, and the detection wavelength is 220 nm.[11]

- ***Sajel.kpatel, N. J. Patel, et al.,*** performed a binary mixture of imipramine HCl and chlordiazepoxide was determined by three different spectrophotometric methods. The first method involved determination of imipramine HCl and chlordiazepoxide using the simultaneous equations and the second method involved absorbance ratio method. Imipramine has absorbance maxima at 251 nm, chlordiazepoxide has absorbance maxima at 264.5 nm and isoabsorptive point is at 220 nm in methanol. Linearity was obtained in the concentration ranges of 1-25 and 1-10 µg/ml for Imipramine HCL and Chlordiazepoxide, respectively. The third method involved determination of these two drugs using the first-derivative spectrophotometric technique at 219 and 231.5 nm over the concentration ranges of 1-20 and 2-24 µg/ml with mean accuracies 99.46±0.78 and 101.43±1.20%, respectively.[12]

- ***Hoffmann-La, Nutleyet al.,***developed capillary electrophoresis (CE) method utilizing indirect ultraviolet (UV) detection was developed for the determination of a non-UV absorbing degradation product, Ro 5–5172, in clidinium bromide drug substance. The electrophoresis buffer consisted of sodium phosphate and benzyltrimethylammonium bromide. Rinsing the capillary with sodium hydroxide followed by water then fresh capillary electrophoresis buffer was found to significantly improve the reproducibility of the migration times of the analytes. To further improve run-to-run reproducibility, an internal marker was used to account for differences in injection volumes and migration times between runs. The precision of the method was found to be less than 1% relative standard deviation for the migration time ratio and peak area ratio of Ro 5–5172 to the internal standard. The method

was found to be linear for 0.05–1% Ro 5–5172 with respect to a 10 mg ml^{-1} sample preparation. The limit of detection was found to be less than 0.01% Ro 5–5172. Results obtained for the analysis of a clidinium bromide drug substance lot using this CE method and a thin layer chromatography method were compared and found to be in agreement.[13]

- ***Aravind.Doki, Kamarapu.SK et al.,***the study describes method development and subsequent validation of RP-HPLC method for simultaneous estimation of Clidinium bromide (CDB), Chlordiazepoxide (CDZ) and Dicyclomine hydrochloride (DICY) in bulk and combined tablet dosage forms. Chromatographic separation was achieved on a Kromasil C18 (250 mm × 4.6 mm id, 5µm) column using a mobile phase ratio consisting of (40:30:30) Methanol: Acetonitrile: Potassium di hydrogen phosphate buffer (0.05M, PH 4.0 adjusting with 0.5% Ortho phosphoric acid) at flow rate 1.0 ml/min. The detection wavelength is 270 nm. The retention times of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride were found to be 7.457 min, 4.400 min and 3.397 min respectively.[14]

- ***Butterfield AG, Matsui FFet al.,***carried out a rapid, precise, forward-phase (adsorption) high-performance liquid chromatographic procedure is presented for the determination of chlordiazepoxideand two common impurities, 7- chloro-1,3-dihydro-5 – phenyl-2H-1,4-benzodiazepine – 2 – one 4- oxidant 2amino – 5-chlorobenzophenone, in commercial formulations and for the determination of the benzophenone in the chlordiazepoxide drug substance. The method involves simultaneous quantitation of chlordiazepoxide and the 1,3-dihhydro impurity, followed by quantitation of the benzophenone from a separate sample extract using a second mobile phase. A single microparticulate silica gel column is used throughout. Nitrazepam and o-dinitrobenzene are the internal standards; Quantitation is by peak area using a computing integrator, except that the peak due to the benzophenone is quantitated by peak height. The described procedure is of equivalent precision, but superior accuracy, to the BP 1973 spectrophotometric procedure for the analysis of chlordiazepoxide in chlordiazepoxide formulations. Quantitation of the 1,3-dihydro and the benzophenone impurities at levels as low as 6.3 and 0.9 mg, respectively, is demonstrated.[15]

- ***Roberts SE, Delaney MFet al.,*** performed a quantitative high-performance liquid chromatographic method using an octadecylsilane column and a methanol-water mobile

phase was employed for the determination of chlordiazepoxide, chlordiazepoxideand related impurities in capsule and tablet preparations. Each component is well separated and directly detected by 254 nm absorption. For chlordiazepoxide and chlordiazepoxidethe coefficient of variation for replicated injections was below 1%. Recovery of authentic samples ranged from 98.4 to 101.6% for both capsule and tablet formulations.[16]

3. AIM OF WORK

The literature survey revealed that few analytical methods are reported for the estimation of Clidinium bromide and Chlordiazepoxide in the pharmaceutical combined tablets dosage forms.

Now adays HPTLC, HPLC are most valuable analytical techniques for pharmaceutical they will remain a useful tool in the future despite further advances in analytical chemistry, because of several overwhelming advantages which include speed, specificity and sensitivity availability of multiple methods for the estimation of drugs their formulation is advantageous and a suitable method may be adopted by an analyst as per purpose of analysis and infrastructural facilities available. In the project it was planned to optimize the assay method for estimation of Clidinium bromide and Chlordiazepoxide based on the literature survey made. So the present work is aimed at development of Isocratic Reverse Phase HPLC method for the Clidinium bromide and Chlordiazepoxide in combined tablet dosage form and its validation as per ICH guide lines.

4. PLAN OF WORK

The plan of the proposed work includes the following steps.

Step 1 - To undertake solubility studies and analytical studies of Clidinium bromide and chlordiazepoxide and to develop initial HPLC chromatographic conditions.

Step 2 - Development of suitable mobile phase.

Step 3 - Selection of suitable detection wavelength.

Step 4 - Setting up of initial HPLC chromatographic conditions for the assay of Clidiniumbromide and chlordiazepoxide.

Step 5 - Optimization of initial chromatographic conditions.

Step 6 - Analytical method validation of the developed RP-HPLC methods as per I.C.H guidelines.

Step 7 - Evaluation of analytical method validation report generated for the developed method.

5. DRUG PROFILE

Clidiniumbromide:-[17, 18]

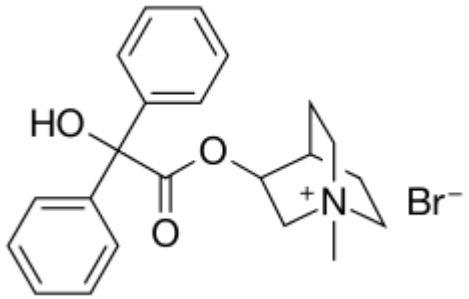

Fig no: 2 Structure of clidinium bromide

Molecular formula	**:**	$C_{22}H_{26}NO_3$
Molecular weight	**:**	352.447 $g.mol^{-1}$
Chemical name	**:**	3-[(2-hydroxy-2,2-diphenylacetyl)oxy]-1-Methyl-Azabicyclo[2.2.2]octan-1-ium bromide
Category	**:**	Anti cholinergic/Antiplasmodic
CAS number	**:**	7020-55-5
Description	**:**	White crystalline
Solubility	**:**	Clidinium bromide is soluble in water and very Soluble in DMSO and methanol
PK_avalue	**:**	11.6
Storage	**:**	Stored at 4^0C
Melting point	**:**	240-241^{0}C
Therapeutic Uses	**:**	Peptic ulcer disease,GI motility disturbances, Acute enter colitis

Pharmacology:

Indication For the treatment of peptic ulcer disease and also to help relieve abdominal or stomach spasms or cramps due to colicky abdominal pain, diverticulitis, and irritable bowel syndrome.

Pharmacodynamics:

Clidinium is a synthetic anticholinergic agent which has been shown in experimental and clinical studies to have a pronounced antispasmodic and antisecretory effect on the gastrointestinal tract.

Mechanism of Action:

Clidinium – AntiCholinergics inhibit the muscarinic actions of acetylcholine on structures innervated by postganglionic cholinergic nerves as well as on smooth muscles that respond to acetylcholine but lack cholinergic innervation. These postganglionic receptor sites are present in the autonomic effecter cells of the smooth muscle, cardiac muscle, sinoatrial and atrioventricular nodes, and exocrine glands. Depending on the dose, anticholinergics may reduce the motility and secretary activity of the gastrointestinalsystem.

Inhibits muscarinic actions of acetylcholine at postganglionic parasympathetic neuroeffector sites primarily inhibiting the M1 muscarinic receptors

PHARMOCOKINETICS:

Absorption:

It is a good adsorbent.

Bioavailability:

Incompletely absorbed from the GI tract (apparently from the intestines) because completely ionized.

Onset of action:

Following oral administration, Anti-Secretary activity occurs within <1 hour.

Duration:

Following oral administration, anti-secretary activity persists for ≤hours.

Distribution:

Does not readily penetrate the CNS or the eye.

Not known whether clidinium bromide crosses the placenta or is distributed into milk.

Metabolism:

Principally in the liver to its 3-hydroxyalcohol.

Elimination Route:

Limited data in 2 adults, approximately 36% of dose excreted in urine within 7 days of oral administration, with 90% of urinary excretion occurring within the first day; 20–46% eliminated in feces.

Half-life:

Biphasic; in 2 adults, initial half-life was 2.4 hours and terminal half-life was 20 hours

Contraindications:

- Glaucoma (to avoid mydriasis).
- Prostatic hypertrophy or benign bladder neck obstruction

Adverse Effects:

Xerostomia,Blurred vision,Constipation, Urinary hesitancy.

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DRUG PROFILE

Chlordiazepoxide :[19,20]

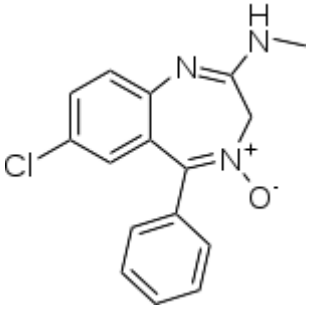

Fig no: 3 Structure of chlordiazepoxide

Molecular formula : $C_{22}H_{26}CLNO_3$

Molecular weight : 299.75 g-mol^{-1}

Chemical name : 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide

Category : Antianxiety, hypnotics and sedatives

CAS number : 58-25-3

PK_a value : 4.8

Description : White, light yellow crystal or crystalline powder

Solubility : Chlordiazepoxide is slightly soluble in water and soluble in Ethanol,glacial acetic acid

Storage : Stored at room temperature

Melting point : 236.2^0

Mechanism of Action:

Chlordiazepoxidebindstostereo specific benzodiazepine binding sites on GABA(A) receptor complexes at several sites within the central nervous system, including the limbic system and reticular formation. This results in an increased binding of the inhibitory neurotransmitter GABA to the GABA (A) receptor. BZDs, therefore enhance GABA-mediated chloride influx through GABA receptor channels, causing membrane hyper polarization. The neuro-inhibitory effects result in the observed sedative, hypnotic,anxiolytic,and muscle relaxant properties.

Pharmacokinetics:

Chlordiazepoxide is a long acting benzodiazepine drug. The half-life of Chlordiazepoxide is 5 – 30 hours but has an active benzodiazepine metabolite (desmethyldiazepam) which has a half-life of 36 – 200 hours. The half-life of chlordiazepoxide increases significantly in the elderly which may result in prolonged action as well as accumulation of the drug during repeated administration. Delayed body clearance of the long half-life active metabolite also occurs in those over 60 years of age which further prolongs the effects of the drugs with additional accumulation after repeated dosing.

Interactions:

Some of the major interactions involving Chlordiazepoxide are listed below.

- ACE inhibitors, Adrenergic neuroneblockers, Angiotensin II receptor antagonists, Betablockers, Calciumchannelblockers,
- Clonidine, Diazoxide,Diuretics,Hydralazine, Methyldopa, Minoxidil,
- SodiumNitroprussideenhanced hypotensive effect
- Alcohol, barbiturates, opiates, antihistamines, antipsychotics - increased sedative effect in combination with Benzodiazapines.
- Cimetidine - metabolism of benzodiazepines inhibited by cimetidine (increased plasma concentration)

- Disulfiram - metabolism of benzodiazepines inhibited by disulfiram (increased sedative effects)

Trade name **:** Librax

Strength **:** (Clidinium bromide 2.5mg,Chlordiazepoxide 5.0mg)

Side effects **:** Confusion,Constipation,Drowsiness,Fainting,

Altered sex drive,Liver problems,Nausea,

Lack of muscle coordination,Yellow eyes and skin

Minor menstrual irregularities, Skin rash or eruptions

Swelling due to fluid retention

Contraindications:

Use of chlordiazepoxide should be avoided in individuals with the following conditions:

- Myasthenia gravis
- Acute intoxication with alcohol, narcotics, or other psychoactive substances
- Ataxia
- Severe hypoventilation
- Acute narrow-angle glaucoma

6. EXPERIMENTAL WORK

6.1 MATERIALS AND METHODS:-

Table No.1

List of Reagents and chemicals used:-

S. No	Name of chemical	Grade	Manufactured By
1.	Methanol	HPLC	Merck Specialty Pvt. Ltd Mumbai
2.	Ortho phosphoric acid	Analytical	Ranbaxy fine chemical Industry Mumbai
3.	Tri-ethylamine	HPLC	Merck Specialty Pvt. Ltd Mumbai
4.	Potassium di-hydrogen phosphate	HPLC	Merck Specialty Pvt. Ltd Mumbai
5.	Water	HPLC	MILLI-Q

Table.No.2

List of Instruments used:-

S. No	Name of Instrument	Manufactured by
1.	Control dynamics pH meter	Lab India
2.	Balance	Sartorius
3.	LC-2010 CHT HPLC	Waters
4.	Sonicator	Spectra lab
5.	UV-Visible Spectroscopy	PERKINELMER with LAMDA25

TableNo.3

Active pharmaceutical Ingredient (pure drug)used:-

S. No	Name	% Purity	Supplier	Specification
1.	Clidinium bromide	99.8%	Lara labs	Reference Standard
2.	Chlordiazepoxide	99.8%	Lara labs	Reference Standard

***TableNo.*4**

Marketed Formulationused:-

S.No	Brand Name	Manufacturer
1.	Librax (7.5mg)	Torrent Pharmaceuticals

Solubility:

Solubility of drugs were observed by dissolving it in different solvents and it was found that drug having good solubility in water. Based on solubility we have selected the phosphate buffer for this work. Solubility of drug was observed by dissolving it in different solvents and it was found that drug having good solubility in followings.

SOLVENT	SOLUBILITY Clinidiumbromide and Chlordizepoxide
Water	+
Phosphate Buffer	+
Acetonitrile	+
Methanol	+

6.2 METHOD DEVELOPMENT

Determination of ISOBESTIC point of Clidinium bromide and Chlordizepoxide by UV-Visible spectrophotometry

Sample Preparation:-

Accurately Weighed and transferred Clidinium bromide and Chlordiazepoxide equivalent to 25 mg and 25 mg into 50 mL clean dry volumetric flask, 50 mL of methanol was added, sonicated for 5 minutes, and filtered through 0.45 µm nylon membrane filter. Pipette out both 5 mL of each solutions transferred into 50 mL volumetric flask, and diluted to 50 mL with methanol in 50 mL volumetric flask, and scanned between 200 to 400 by UV spectroscopy. Shown in ***Figure 1***

Initialization of the instrument

Initially, Column was placed on the instrument and the instrument was switched on and washed with Methanol:Water (20:80 V/V) for 30min. Then the mobile phase ran into 30min to obtain column saturation.

Selection of mobile phase:

The method development and validation of Clidinium bromide and Chlordiazepoxide requires greater resolution. Hence different solvent systems were tried.

The trails are using LC-2010 CHT HPLC waters equipped with UV detector and isocratic pump. The system controlled by WATERS EMPOWER2 software.

Selection of flow rate:

The flow rate of Clidinium bromide and Chlordiazepoxide were tried from 1.0 mL to 1.5 mL

TRIAL-1

Preparation of Buffer:

The buffer was prepared by dissolving 5.4 g of potassium di-hydrogen orthophosphate in 2000ml of water and adjusted pH to 4.0 with orthophosphoric acid. Filter through 0.45µm membrane filter.

Preparation of Mobile phase:

Degassed mixture of Buffer: Methanol (60:40V/V) in the ratio of respectively.Filter through 0.45 µ membrane filter and degas.

Standard preparation:

The quantity containing 5mg of Clidinium bromide and 10mg of Chlordiazepoxide was weighed and transferred in to a 25ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45µm membrane filter. From this 5ml was pipetted out in to a 25ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure-2.***

Chromatographic Condition:

Equipment	:	High performance liquid chromatography equipped with Auto Sampler and UV detector
Column	:	OC C_8 (250 x 4.5mm, 5.0µm) or equivalent
Mode of operation	:	Isocratic
Flow rate	:	1.0ml/min
Wave length	:	240nm
Injection volume	:	10µl
Column oven	:	Ambient

Run time : 30min

Temperature : 25^0C

TRIAL-2

Preparation of Buffer:

The buffer was prepared by dissolving 5.4g of potassium di-hydrogen orthophosphate in 2000 ml of water and adjusted pH to 4.0 with orthophosphoric acid. Filter through 0.45 µm membrane filter.

Preparation of Mobile phase:

Degassed mixture of Buffer: Methanol (V/V) in the ratio of 70:30%respectively Filter through 0.45 µ membrane filter and degas.

Standard preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 3.***

Chromatographic Condition:

Equipment : High performance liquid chromatography equipped with Auto Sampler and UV detector

Column : OC C_8 (250 x 4.5mm, 5.0µm) or equivalent

Mode of operation : Isocratic

Flow rate : 1.2ml/min

Wave length : 240nm

Injection volume : 10µl

Column oven : Ambient

Run time : 30min

Temperature : 25^0C

TRIAL-3

Preparation of Buffer:

The buffer was prepared by dissolving 5.4g of potassium di-hydrogen orthophosphate in 2000ml of water. To 2000ml of phosphate buffer and adjust pH to 4.0 with phosphoric acid. Filter through 0.45µmmembrane filter.

Preparation of Mobile phase:

Degassed mixture of Buffer: Methanol (V/V) in the ratio of 70:30% respectively Filter through 0.45 µ membrane filter and degassed.

Standard preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 4.***

Chromatographic Condition:

Equipment : High performance liquid chromatography equipped with Auto Sampler and PDA detector

Column : OC C_8 (250 x 4.5mm, 5.0µm) or equivalent

Flow rate : 1.5ml/min

Mode of operation : Isocratic

Wave length : 240nm

Injection volume : 10µl

Column oven : Ambient

Run time : 30min

Temperature : 25^0C

TRIAL-4

Preparation of Buffer:

The buffer was prepared by dissolving 5.4g of potassium dihydrogen orthophosphate in 2000ml of water. To 2000ml of phosphate buffer and adjust pH to 4.0 with phosphoric acid. Filter through 0.45µ membrane filter.

Preparation of Mobile phase:

Filtered and degassed mixture of Buffer (pH 4.0): Methanol (80:20) v/v in the ratio of 80:20. And filter through 0.45µ membrane filter.

Standard preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 5.***

Chromatographic Condition:

Equipment : High performance liquid chromatography equipped with Auto Sampler and UV detector

Column : OC C_8 (250 x 4.5mm, 5.0µm) or equivalent

Flow rate : 1.5ml / min

Mode of operation : Isocratic

Wave length : 240nm

Injection volume : 10µl

Column oven : Ambient

Run time : 30min

Temperature : 25^0C

OPTIMIZED METHOD

Preparation of Buffer:

The buffer was prepared by dissolving 5.4 g of potassium di-hydrogen orthophosphate in 2000ml of water and adjust pH to 4.5 with phosphoric acid. Filter through a 0.45µ nylon membrane filter.

Preparation of Mobile phase:

Filtered and degassed mixture of Buffer (pH 4.5): Methanol (80:20) v/v in the ratio of 80:20. And filter through 0.45µ membrane filter.

Standard Preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 6.***

Chromatographic Conditions:

Equipment : High performance liquid chromatography equipped with Auto Sampler and PDA detector

Column	:	OC C_8 (250 x 4.5mm, 5.0µm) or equivalent
Flow rate	:	1.5ml/min
Mode of operation	:	Isocratic
Wave length	:	240nm
Injection volume	:	10µl
Column oven	:	Ambient
Run time	:	30min
Temperature	:	25^0C

6.2.1 ASSAY OF CLIDINIUM BROMIDE AND CHLORDIAZEPOXIDE:

Standard Preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively.The results was tabulated in ***Table 6*** & The chromatogram was shown in ***Figure 7.***

Sample preparation:

Weigh and powder 20 tablets and weigh powder equivalent to135mg of the sample was taken and transferred in to a 25ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45µm membrane filter. From this 5ml was pipetted out in to a 25ml volumetric flask and made up to the volume with mobile phase.Furthur diluted to obtain final concentration of 80 µg/ mLof Clidinium bromide and Chlordiazepoxide respectivelyWith optimized Chromatographic condition a steady base line was recorded with mobile phase 10 µl quantity of sample solution was injected and the Chromatogram was recorded. The results was tabulated in ***Table 7*** & the chromatogram is shown in ***Figure 8.***

Procedure:

Inject 10 l of the standard, sample into the chromatographic system and measure the areas for theClidinium bromide and Chlordiazepoxide.

Amount of drug in tablet was calculated using following formula

Amount present:

$$\frac{\text{Sample area X standard weight}}{\text{Standard area X sample weight}} \text{ X dilution factor X average weight}$$

Percentage purity:

Amount present / label claim X 100

6.3 METHOD VALIDATION

The chromatographic conditions were validated by evaluating linearity, accuracy, method precision, limit of detection (LOD), limit of quantitation (LOQ), ruggedness and robustness in accordance with ICH guidelines.

6.3.1 SPECIFICITY:

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. It is a measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to only to a single component; that is, that no co-elution exists. Specificity is measured and documented in a separation by the resolution, plate count, and tailing factor.

Preparation of solutions:

a) Placebo interference:

Sample was prepared by taking the placebo equivalent to about the weight in portion of test preparation as per the test method and injected into the HPLC system. The chromatogram was shown in ***Figure 9.***

Acceptance criteria:

Chromatogram of placebo should not show any peak at the retention time of analyte peak.

b) Blank Interference

Mobile phase was prepared as per the test method and injected and into the HPLC system. The chromatogram was shown in ***Figure 10.***

Acceptance criteria:

Chromatogram of blank should not show any peak at the retention time of analyte peak.

6.3.2 SYSTEM SUITABILITY

System Suitability was the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factor, resolution, and reproducibility are determined and compared against the specification set for the method. These parameters are measured during the analysis of a system suitability sample that is a mixture of main components and expected by-product. The results was tabulated in ***Table-8*** & the chromatogram was shown in ***Fig no 13***.

6.3.3 LINEARITY:

Preparation of stock solution:

The quantity containing 5mg of Clidinium bromide and 10mg of Chlordiazepoxide was weighed and transferred in to a 25ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45µm membrane from this 5ml was pipetted out in to a 25ml volumetric flask and made up to the volume with the mobile phase. The

solution contains 200 µg/ mL and 400 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively.

Preparation of linearity solution (50%):

2.5 mL of stock solution was taken in 25 mL of volumetric flask dilute up to the mark with diluent. The solution contains 20 µg/ mL and 40 µg/ mL concentration ofClidinium bromide and Chlordiazepoxiderespectively.

Preparation of linearity solution (75%):

3.75 mL of stock solution was taken in 25 mL of volumetric flask dilute up to the mark with diluent. The solution contains 30 µg/ mL and 60 µg/ mL concentration ofClidinium bromide and Chlordiazepoxiderespectively.

Preparation of linearity solution (100%):

5.0 mL of stock solution was taken in 25 mL of volumetric flask dilute up to the mark with diluent. The solution contains 40 µg/ mL and 80 µg/ mL concentration ofClidinium bromide and Chlordiazepoxiderespectively.

Preparation of linearity solution (125%):

6.25 mL of stock solution was taken in 25 mL of volumetric flask dilute up to the mark with diluent. The solution contains 50 µg/ mL and 100 µg/ mL concentration ofClidinium bromide and Chlordiazepoxiderespectively.

Preparation of linearity solution (150%):

7.5 mL of stock solution was taken in 25 mL of volumetric flask dilute up to the mark with diluent. The solution contains 60 µg/ mL and 120 µg/ mL concentration ofClidinium bromide and Chlordiazepoxiderespectively.

Procedure:

Each level of the above solutions was injected into the chromatographic system for five replicate and the peak area was measured. A graph was plotted (peak area versus concentration)

and the correlation coefficient (r^2) was calculated. The results were tabulated in ***Table 9***& the chromatograms were shown in ***Figure 15-21.***

6.3.4 PRECISION:

Precision was the measure of the degree of repeatability of an analytical method under normal operation and it was normally expressed as the relative standard deviation for a statistically number of samples. Precision should be performed at three different levels: repeatability, intermediate precision and reproducibility.

Standard Preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 4.***

Sample Preparation:

Weigh and powder 20 tablets and weigh powder equivalent to 444.8 mg of the sample was taken and transferred in to a 25ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5ml was pipetted out in to a 25ml volumetric flask and made up to the volume with mobile phase. About 10µl of the sample was injected separately through auto injector the results and the chromatogram shown in ***Figure no 22-27.*** And ***Table no 10***.

Procedure:

Six replicate Sample solutions were prepared as per the test method and injected as per the test procedure

6.3.5 ACCURACY:

The accuracy of the test method was carried out by preparing the samples at a level of 50%, 100% and 150% of target concentration. The samples were prepared in six times for lower and higher concentration levels and triplicate the other level and perform the assay as per test

Preparation of Stock Solution:

The quantity containing 5mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane from this 5ml was pipetted out in to a 25ml volumetric flask and made up to the volume with the mobile phase.

50% Sample preparation:

From the above stock solution1.25 mL containing of Clidinium bromide and Chlordiazepoxidewas taken into a 25 mL volumetric flask and diluted up to the mark with diluent. The solution was sonicated for 15 min and filtered through 0.45 µm nylon membrane filter. The above solution were inject into the HPLC column same procedure was repeated for three replicate. The results are tabulated in***Table11-12*** & the chromatograms were shown in ***Figure 28-36***.

100% Sample preparation:

From the above stock solution2.5 mL containing of Clidinium bromide and Chlordiazepoxidewas taken into a 25 mL volumetric flask and diluted up to the mark with diluent. The solution was sonicated for 15 min and filtered through 0.45 µm nylon membrane filter. The above solution were inject into the HPLC column same procedure was repeated for three replicate. The results are tabulated in ***Table 11-12*** & the chromatograms were shown in ***Figure 28-36***.

150% Sample preparation:

From the above stock solution3.75 mL containing of Clidinium bromide and Chlordiazepoxidewas taken into a 25 mL volumetric flask and diluted up to the mark with diluent. The solution was sonicated for 15 min and filtered through 0.45 µm nylon membrane filter. The above solution were inject into the HPLC column same procedure was repeated for

three replicate. The results are tabulated in ***Table 11-12***& the chromatograms were shown in ***Figure 28-36***.

Procedure:

The standard solution was injected in triplicate for Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Clidinium bromide and Chlordizepoxideand calculate the individual recovery and mean recovery values.

$$\%\ Recovery = \frac{\text{Sample peak area x weight of standard}}{\text{Standard peak area x weight of sample}} X\ 100$$

6.3.6 RUGGEDNESS (INTERMEDIATE PRECISION):

Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. Theincludesdifferent analysts, laboratories, columns, and instruments, source of reagents, chemicals, and solvents.In the present study, ruggedness was performed with different analysts.

Standard Preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 4.***

Sample Preparation:

Weigh and powder 20 tablets and weigh powder equivalent to 444.8 mg of the sample was taken and transferred in to a 25ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5ml was pipetted out in to a 25ml volumetric flask and made up to the volume with mobile

phase. About 10µl of the sample was injected separately through auto injector the results and the chromatogram shown in ***Figure no 13-14.*** And ***Table no 37-48***.

6.3.7 ROBUSTNESS:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by a small, but deliberate variation in the method parameters and provides an indication of its reliability during normal usage. Robustness of the method was investigated under a variety of conditions including deliberate changes in the mobile phase ratio and change in flow rate. The degree of reproducibility of the results obtained as a result of small variations in the method parameters has proven that the method is robust.

Standard Preparation:

The quantity containing 5 mg of Clidinium bromide and 10 mg of Chlordiazepoxide was weighed and transferred in to a 25 ml volumetric flask and made up to the volume with mobile phase and sonicated for about 15 min and filtered through 0.45 µm membrane filter. From this 5 ml was pipetted out in to a 25 ml volumetric flask and made up to the volume with mobile phase. The solution contains 40 µg/ mL and 80 µg/ mL concentration of Clidinium bromide and Chlordiazepoxide respectively. The chromatogram was shown in ***Figure 49-52 &table 15-16***

6.3.8 LIMIT OF DETECTION:

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantities under the stated experimental conditions. The detection is usually expressed as the concentration of the analyte in sample, for example, percentage, parts per million (ppm), or parts per billion (ppb).

The LOD value determined during method validation isaffected by the separation conditions like columns, reagents and special instrumentation and data Systems. Instrumental changes, particularly pumping systems and detectors or the use of contaminated reagents can result in large changes in result.

The standarddeviation of the Y-intercept and the slope of the calibration curves were used to calculate the LOD. The chromatogram was shown in ***fig 62.***

$$LOD = 3.3 * \sigma / slope$$

6.3.9 LIMIT OF QUANTIFICATION:

The Limit of Quantification (LOQ) is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and Degradation products in finished pharmaceuticals. The detection is usually expressed as the concentration of the analyte in the sample, for example, percentage, parts per million (ppm), or parts per billion (ppb).

The LOQ value determined during method validation is affected by the separation conditions: columns, reagents and special instrumentation and data Systems. Instrumental changes, particularly pumping systems and detectors or the use of contaminated reagents can result in large changes in result.

The standarddeviation of the Y-intercept and the slope of the calibration curves were used to calculate the LOD by using the equations 10*std.dev/slope for LOQ. The chromatogram was shown in ***fig 63.***

$$LOD = 10 * \sigma / slope$$

7. RESULTS AND DISCUSSION

7.1 METHOD DEVELOPMENT:

7.1.1 Isobestic point of Clidinium bromide and Chlordiazepoxide by UV-Visible spectrophotometry

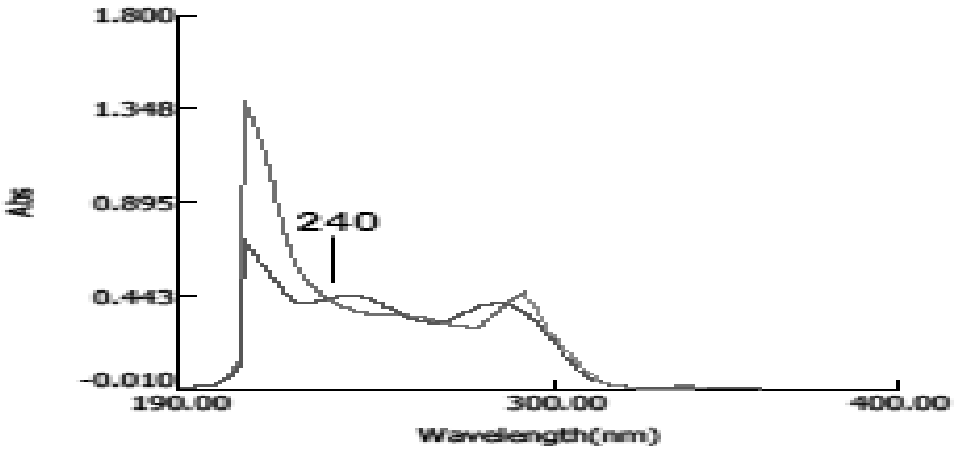

Fig. 1: Isobestic pointClidinium bromideandChlordiazepoxide 240 nm

Observation:

The overlaid spectrum of Clidinium bromide of andChlordiazepoxide shows maximum absorbance at 240 nmand was used for method development.

7.1.2 DEVELOPMENT TRAILS:

Selection of flow rate:

The flow rate was changed from 1.0-1.5 mL min^{-1} and the chromatogram was developed. The drugs eluted with small differences in fronting and tailing except at 1.5 mL min^{-1} and it was selected for analysis.

Trial-1

Table 1: Chromatographic condition

Parameters	**Description**
Column	OC C_8 (250 mm x 4.5 mm, 5 µm)
Mobile Phase	Buffer pH 4.0 : Methanol(60:40)
Diluent	Water : Methanol(60:40)
Flow rate	1.0 mL min^{-1}
Wavelength	240 nm
Injection mode	Auto injector (vial)
Injection volume	10 µL

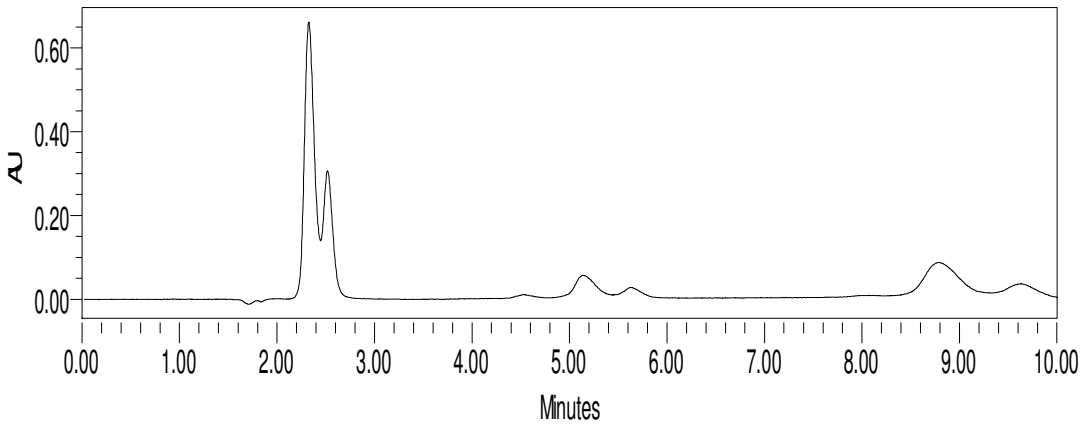

Fig. 2: Chromatogram of Clidinium bromideand Chlordiazepoxide

Observation

Two peaks were merged with the same retension time.Clidinium bromide and Chlordiazepoxidewere not eluted properly. So the mobile phase has been changed for next trial.

Trail 2

Table 2: Chromatographic condition

Parameters	**Description**
Column	OC C_8 (250 mm x 4.5 mm, 5 µm)
Mobile Phase	Buffer pH 4.0 : Methanol(70:30)
Diluent	Water : Methanol (70:30)
Flow rate	1.2 mL min^{-1}
Wavelength	240 nm
Injection mode	Auto injector (vial)
Injection volume	10 µL

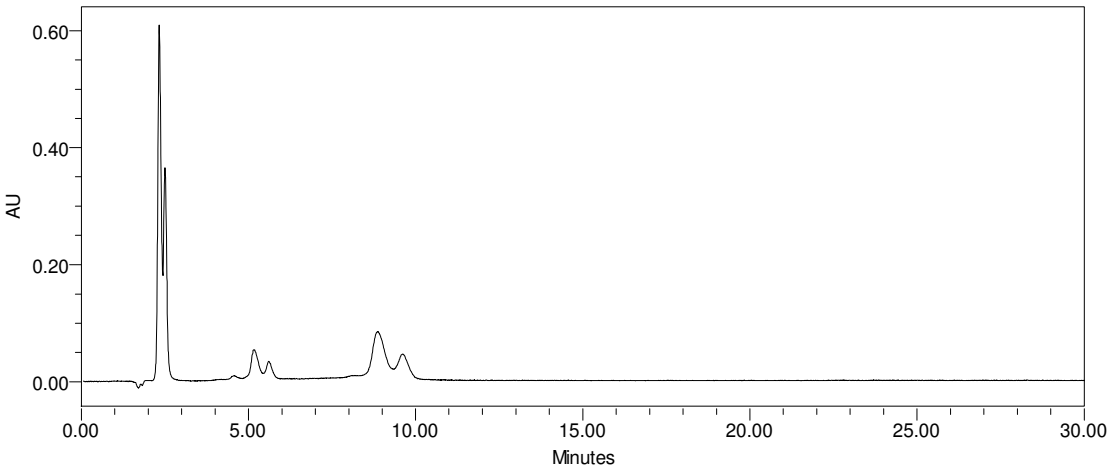

Fig. 3:

Observation

Cleft peak was obtained both Clidinium bromide and Chlordiazepoxidewere not eluted properly. So flow rate has been changed for next trial.

Trail 3

Table 3: Chromatographic condition

Parameters	**Description**
Column	OC C_8 (250 mm x 4.5 mm, 5 µm)
Mobile Phase	Buffer pH 4.0 : Methanol(70:30)
Diluent	Water : Methanol (70:30)
Flow rate	1.5 mL min^{-1}
Wavelength	240 nm
Injection mode	Auto injector (vial)
Injection volume	10 µL

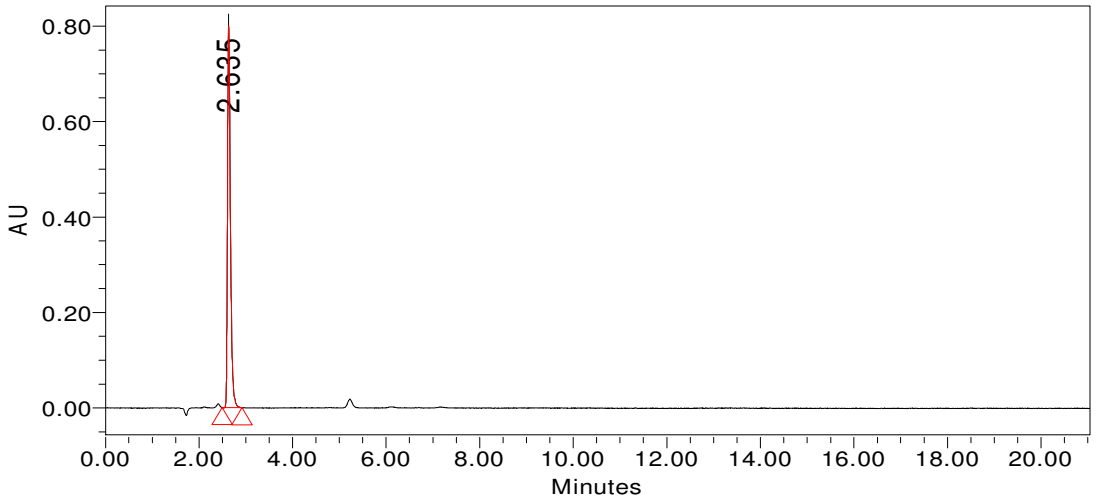

Fig. 4: Chromatogram of Clidinium bromideand Chlordiazepoxide

Observation

Single peak was obtained for both components. Henceanother trail was carried out by changing the mobile phase ratio.

Trail 4

Table 4: Chromatographic condition

Parameters	**Description**
Column	OC C_8 (250 mm x 4.5 mm, 5 µm)
Mobile Phase	Buffer pH 4.0 : Methanol(80:20)
Diluent	Water : Methanol (80:20)
Flow rate	1.5 mL min^{-1}
Wavelength	240 nm
Injection mode	Auto injector (vial)
Injection volume	10 µL

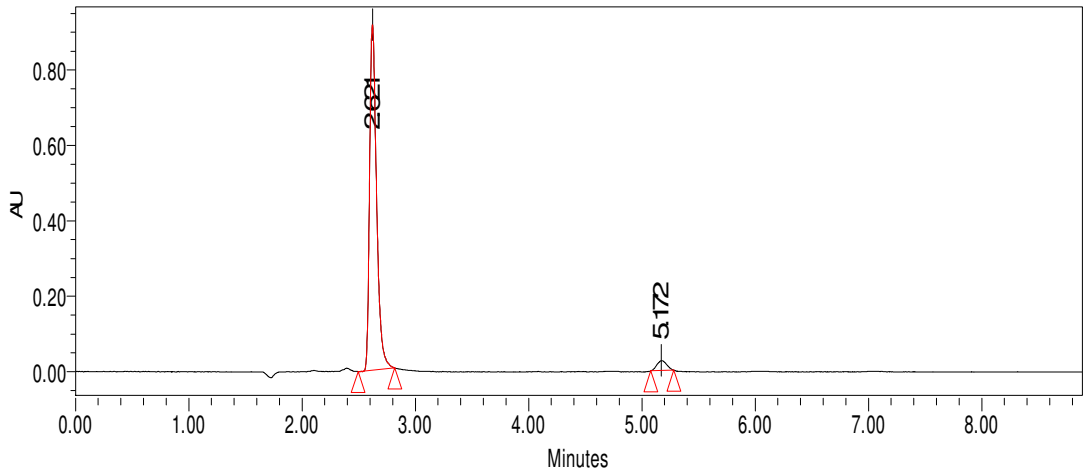

Fig. 5: Chromatogram of Clidinium bromideand Chlordiazepoxide

Observation

Clidinium bromide and Chlordiazepoxidepeaks were obtained but Retention time is very high and second peak was not obtained properly next performed optimization method by changing the flow rate and pH.

OPTIMIZED METHOD

Table 5: Chromatographic condition

Parameters	**Description**
Column	OC C_8 (250 mm x 4.5 mm, 5 µm)
Mobile Phase	Buffer pH 4.5 : Methanol(80:20)
Diluent	Water : Methanol (80:20)
Flow rate	1.5 mL min^{-1}
Wavelength	240 nm
Injection mode	Auto injector (vial)
Injection volume	10 µL

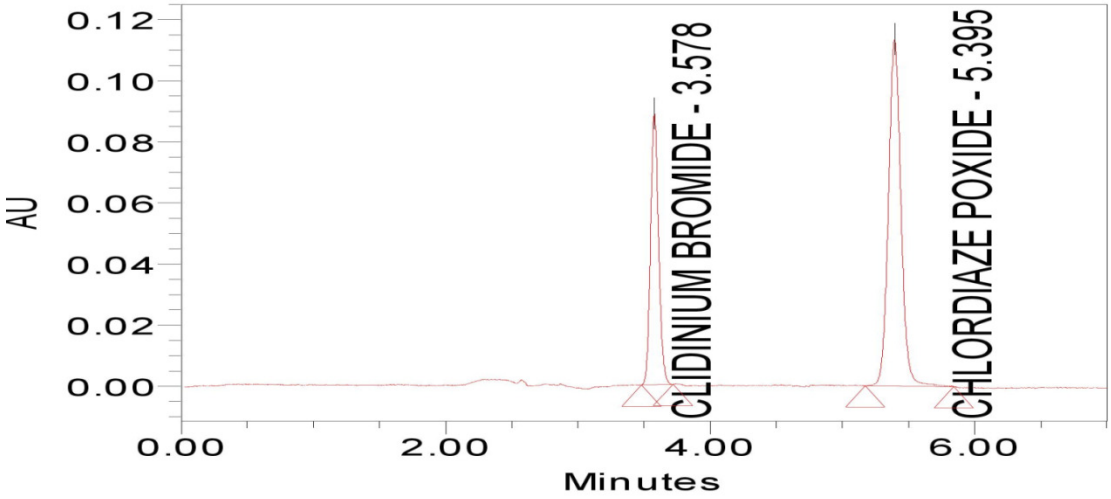

Fig. 6: Chromatogram of Clidinium bromideand Chlordiazepoxide

Observation:

Resolution, theoretical plates and symmetric factor were found to be within the limit. So this method is finalized as the optimized method.

ASSAY:

Table 6: **Assay data for standard Clidinium bromide and Chlordiazepoxide**

S.No	Standard	
	Clidinium bromide	**Chlordiazepoxide**
1	374688	753761
2	375210	753617
3	374921	753711
Mean	374939	753696

Table 7: **Assay data for tablets of Clidinium bromide and Chlordiazepoxide**

S.No	Standard	
	Clidinium bromide	**Chlordiazepoxide**
1	387781	751750
2	387688	751617
3	387921	751711
Avg	387796	751692

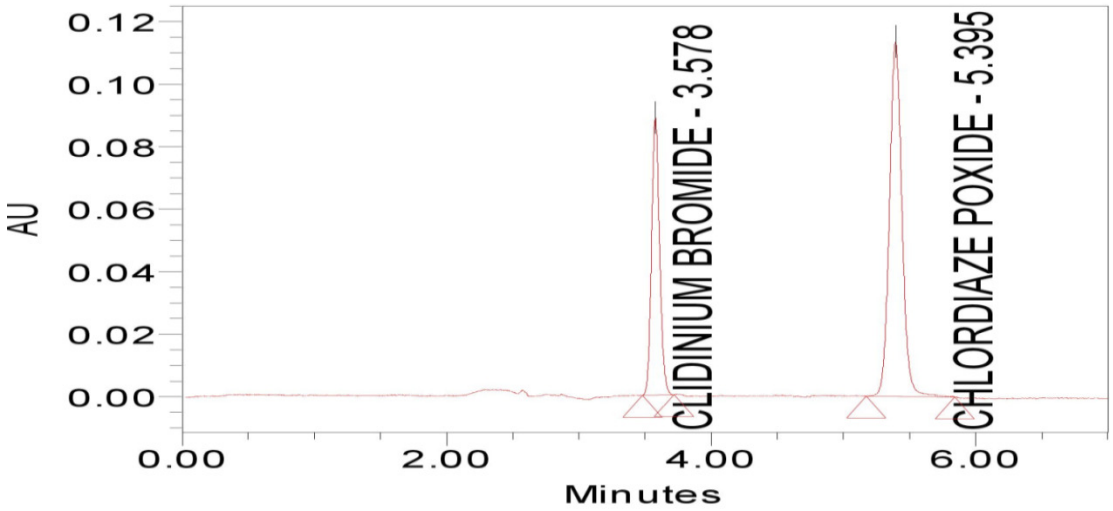

Fig. 7:Standard chromatogram of Clidinium bromide and Chlordiazepoxide

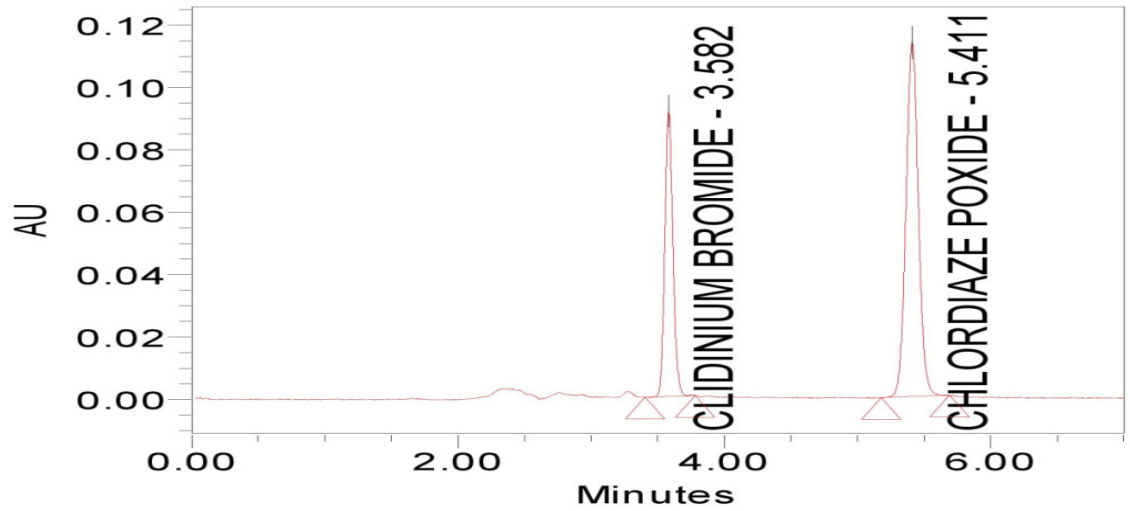

Fig.no.8

Fig. 8:Chromatogram of marketed formulation ofClidinium bromide and Chlordiazepoxide

Formula:

Amount present: Sample area X standard weight

———————————— X dilution factor X average weight

Standard area X sample weight

Percentage purity: Amount present/label claim X 100

For Clidinium bromide:

$$= \frac{387796\times5\times5\times25\times25\times99.8\times222.4\times100}{374939\times25\times25\times444.8\times5\times100\times2.5}$$

$= 1.0026\times99.8$

$= 100.05\%$

ForChlordiazepoxide:

$$=\frac{751692\times10\times5\times25\times25\times99.8\times222.4\times100}{753696\times25\times25\times444.8\times5\times100\times5}$$

$= 1.0039\times99.8$

$= 100.1\%$

7.2 METHOD VALIDATION

7.2.1 SPECIFICITY:

Specificity is the ability to assess unequivalently the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, matrix, degradants etc. It is evaluated by injecting the blank and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of Clidinium bromide and Chlordiazepoxide.

The specificity of the HPLC method was complete separation of Atazanavirsulphate and Ritonavir was noticed in presence of tablet excipients. In addition there was no interference at the retention time of Clidinium bromide and Chlordiazepoxidein the chromatogram of placebo solution.

Name of the solution	Retention time in min
Blank	No peak
Placebo	No peak
Standard	3.57 and 5.39
Sample	3.57 and 5.40

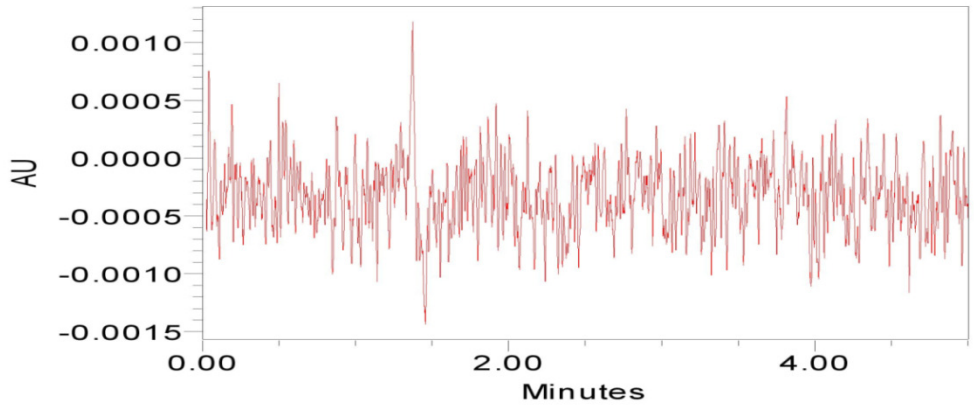

Fig. 9: Chromatogram of placebo solution

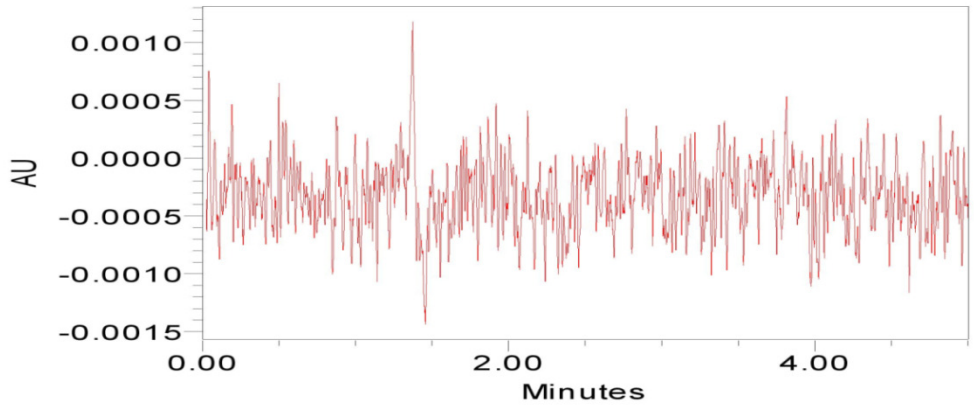

Fig. 10: Chromatogram of blank solution

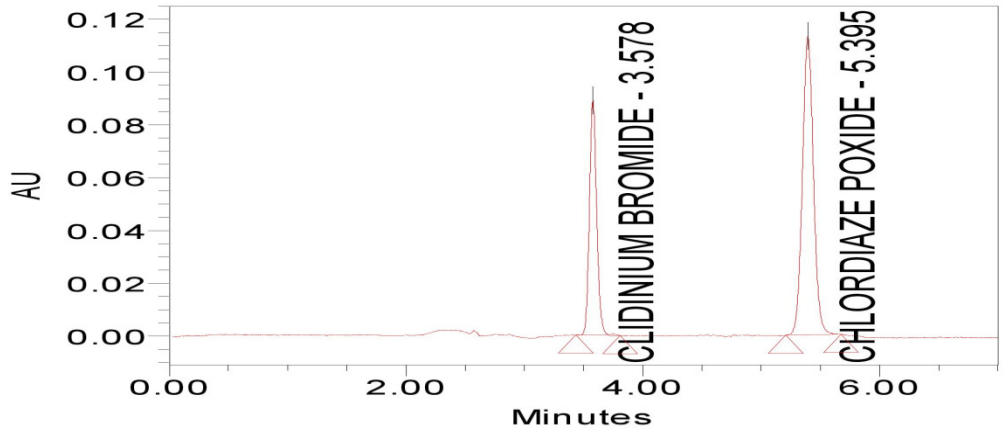

Fig. 11: Chromatogram of standard formulation of Clidinium bromide and Chlordiazepoxide

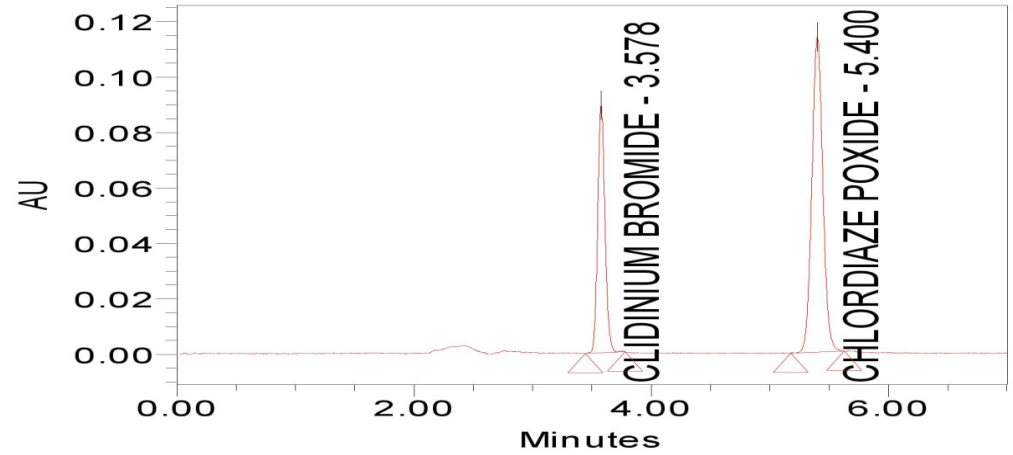

Fig. 12: Chromatogram of marketed formulation of Clidinium bromide and Chlordiazepoxide

Result:

The method was found to be specific because it did not show any extra peaks and there is no interference from excipients.

7.2.2 SYSTEM SUITABILITY

Suitability is a measure of the precision under the same operating conditions over a short interval of time, that is, under normal operating conditions of the analytical method with the same equipment. The chromatograms obtained for suitability were shown in ***fig no 13***. and the parameters were shown in ***table no 8*.**

Table 8: System Suitability Parameters

S. No	Parameters	Clidiniumbromide	Chlordiazepoxide
1	Theoretical plates	4688	5761
2	RT	3.578	5.39
3	Resolution	12.36	12.68
4	Tailing factor	1.129	1.072

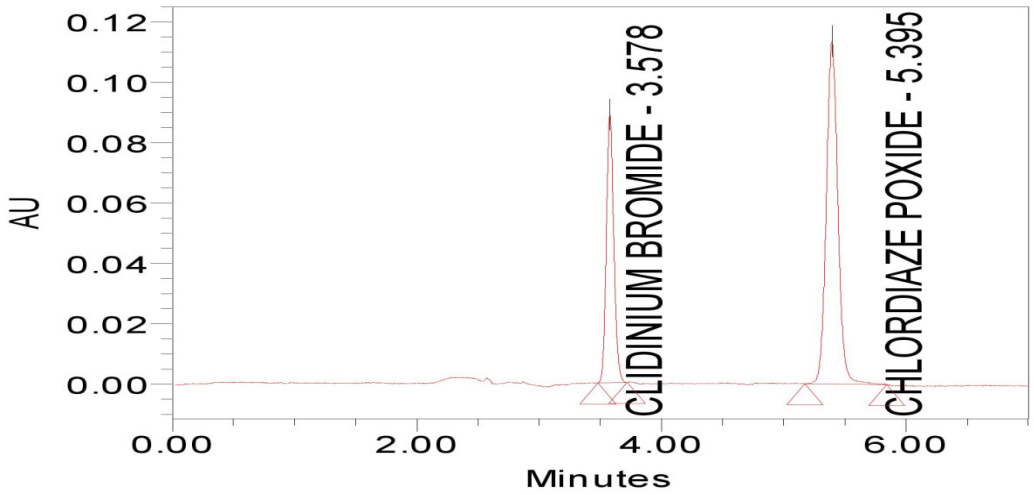

Fig. 13: Chromatogram of standard formulation of Clidinium bromide and Chlordiazepoxide

Acceptance criteria:

Theoretical plate's ≥ 2000, symmetric factor ≤ 2, tailing factor ≤ 2.

Result:

From the above table it was concluded that result obtained was within the limit only.

7.2.3 LINEARITY:

The linearity of calibration curves (Absorbance V_S concentration) in pure solution was checkedover the concentration ranges of about 60-180µg/ml for Clidinium bromide and chlordiazepoxide. The results were tabulated in ***table no 9***and ***figure no 15-21***.

Table 9: Linearity of Clidinium bromide and Chlordiazepoxide

S.No.	Clidinium bromide		Chlordiazepoxide	
	Conc. (µg mL^{-1})	Peak Area	Conc. (µg mL^{-1})	Peak Area
1	20	190127	40	366951
2	30	285491	60	550758
3	40	380605	80	733006
4	50	476653	100	917184
5	60	571015	120	1105711
Slope		3174.6		6132.9
Y-Intercept		141.9		1020.1
Correlation coefficient		0.999		0.999

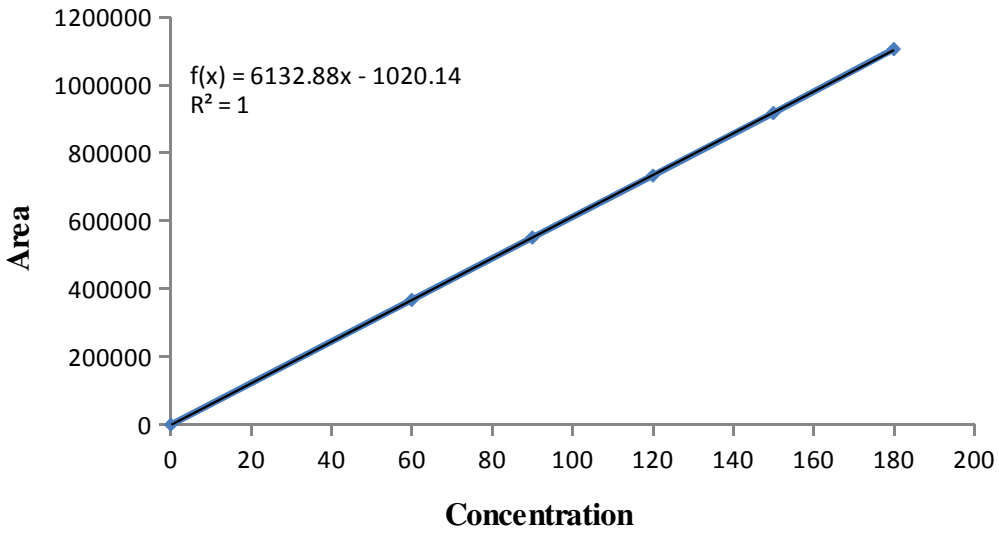

Fig. 15: Calibration plot of Clidinium bromide

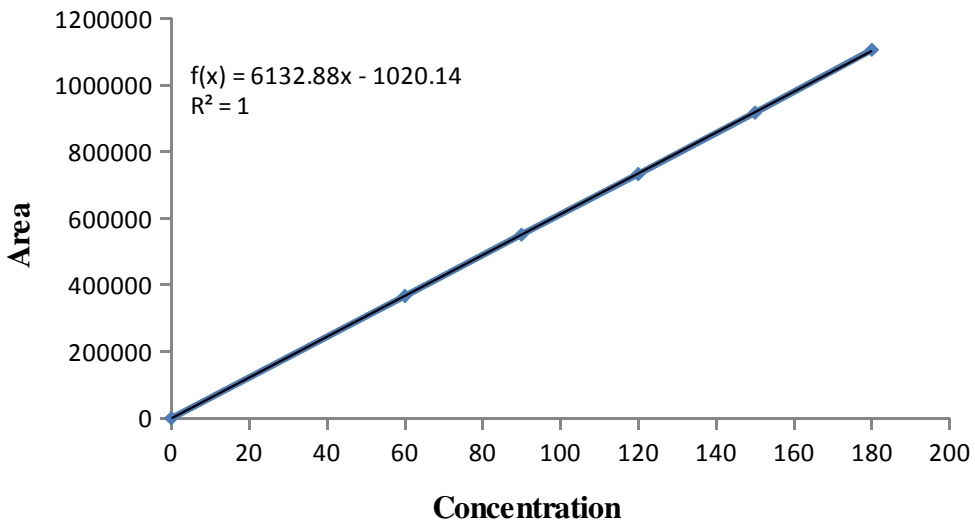

Fig. 16: Calibration plot of Chlordiazepoxide

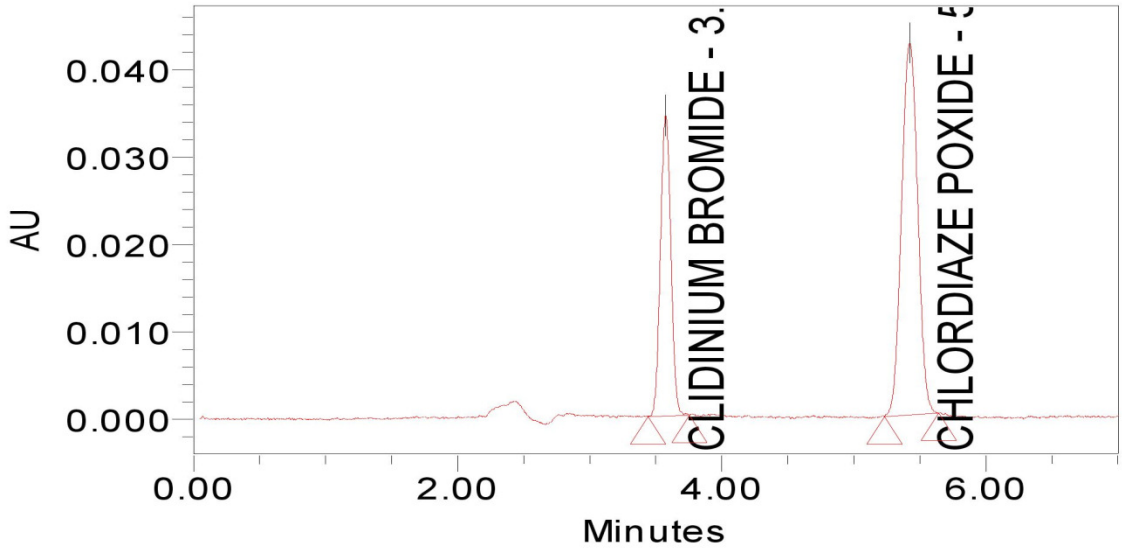

Fig. 17: Chromatogram of Clidinium bromide and Chlordizepoxidefor Linearity-1

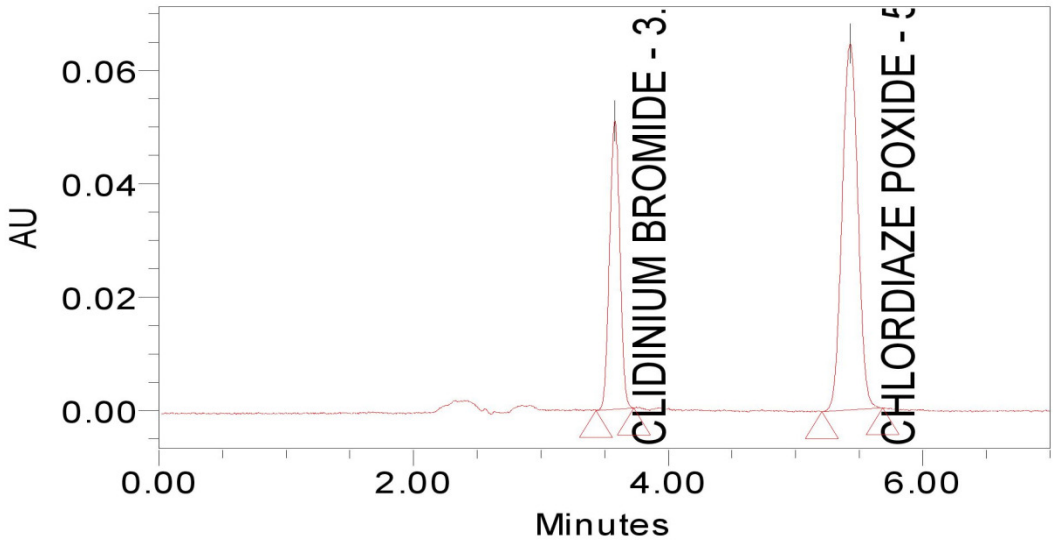

Fig. 18: Chromatogram of Clidinium bromide and Chlordizepoxidefor Linearity-2

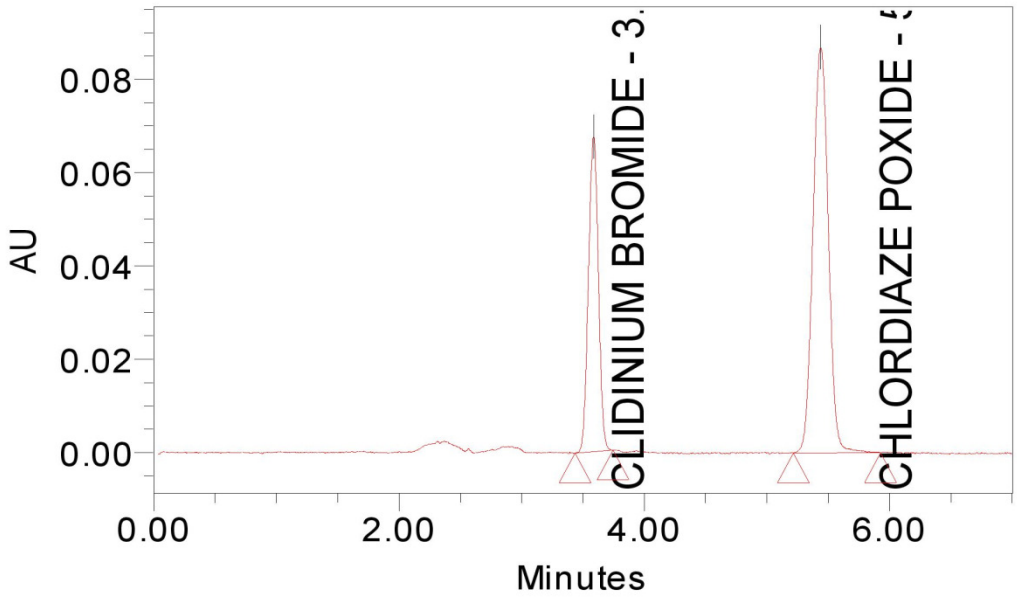

Fig. 19: Chromatogram of Clidinium bromide and Chlordizepoxidefor Linearity-3

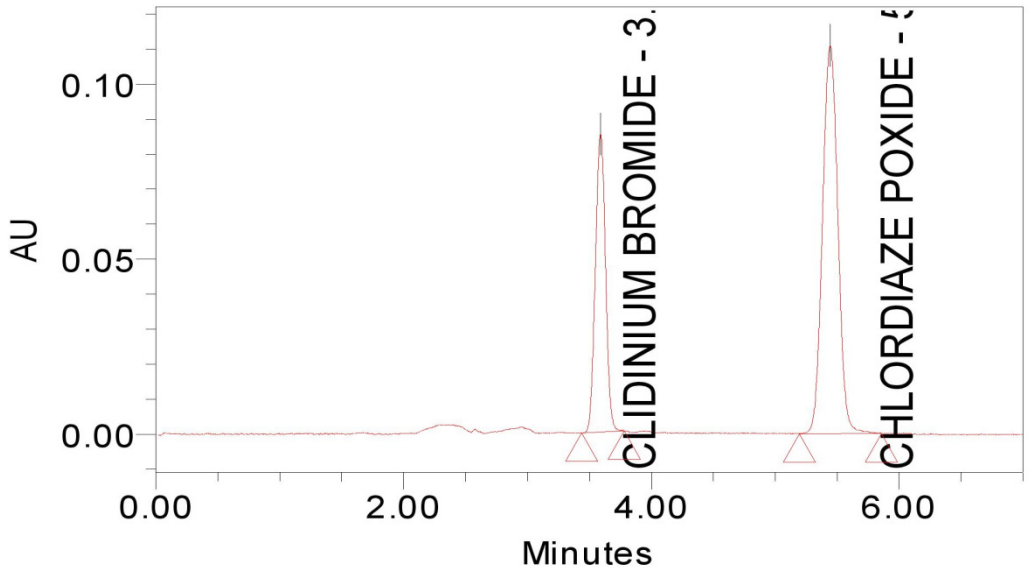

Fig. 20: Chromatogram of Clidinium bromide and Chlordizepoxidefor Linearity-4

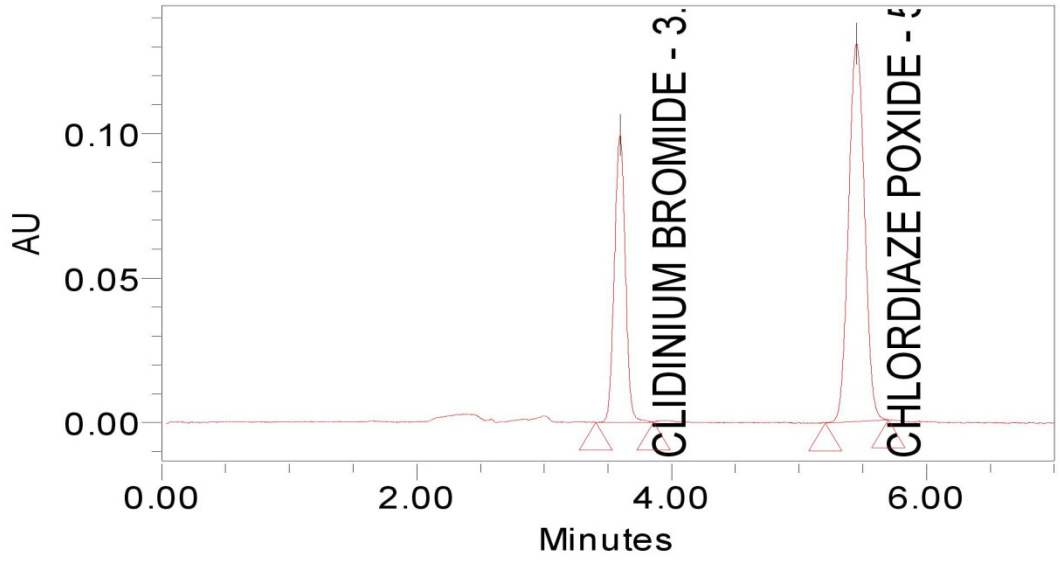

Fig. 21: Chromatogram of Clidinium bromide and Chlordizepoxidefor Linearity-5

Acceptance criteria:

Correlation Coefficient should be not less than 0.9990

Result:

The relationship between the concentration and the peak response of Clidinium bromide and Chlordiazepoxide was linear in the specific range and the regression coefficient was found to be 0.999 for bothClidinium bromide and Chlordiazepoxide

7.2.4 PRECISION:

Precision is a measure of the reproducibility of the whole analytical method under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. The precision is then expressed in terms of relative standard deviation. Acceptance criteria for the precision of the method is the %RSD should not be more than 2%.The results for the precision were shown in following ***table no 10***.And ***figure no 22-27.***

Table 10 **Method Precision:**

S.no	Clidinium bromide		Chlordiazepoxide	
	Peak area	**RT**	**Peak area**	**RT**
1	380537	3.581	733626	5.414
2	380607	3.585	733307	5.418
3	380412	3.584	733012	5.419
4	380102	3.583	733006	5.419
5	380742	3.583	733903	5.422
6	380766	3.583	733977	5.423
Mean	380527.6		733471	
SD	60739		191.88	
RSD %	0.06		0.02	

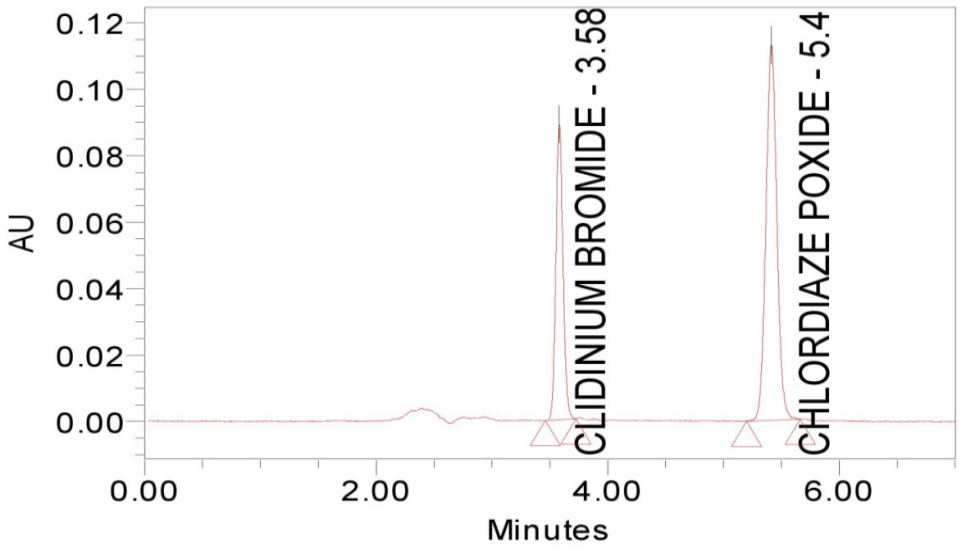

Fig. 22: Chromatogram of Clidinium bromide and Chlordizepoxidefor Precision-1

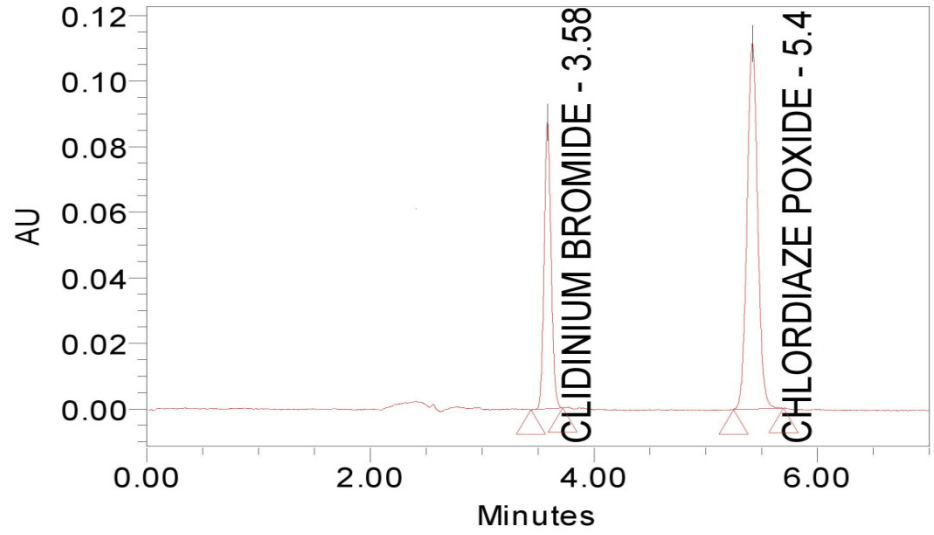

Fig. 23: Chromatogram of Clidinium bromide and Chlordizepoxidefor Precision-2

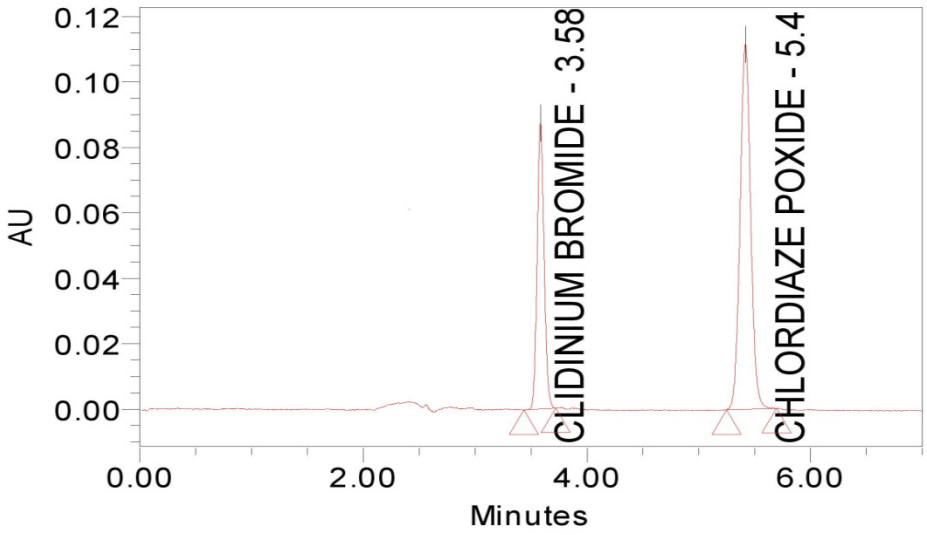

Fig. 24: Chromatogram of Clidinium bromide and Chlordizepoxidefor Precision-3

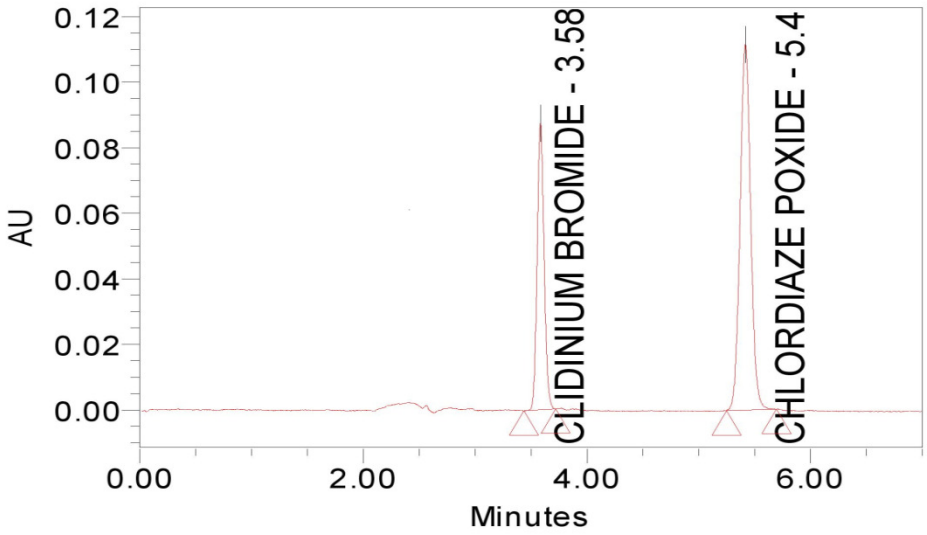

Fig. 25: Chromatogram of Clidinium bromide and Chlordizepoxidefor Precision-4

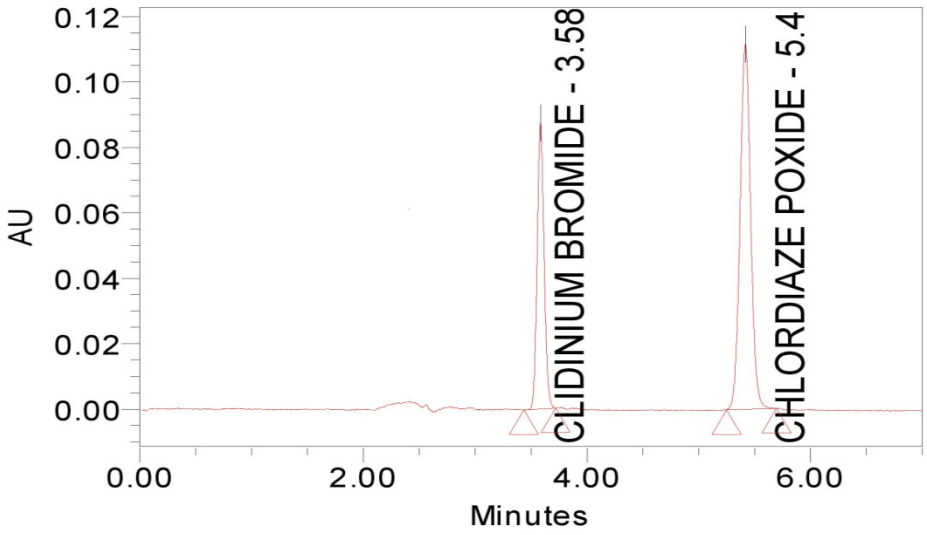

Fig. 26: Chromatogram of Clidinium bromide and Chlordizepoxidefor Precision-5

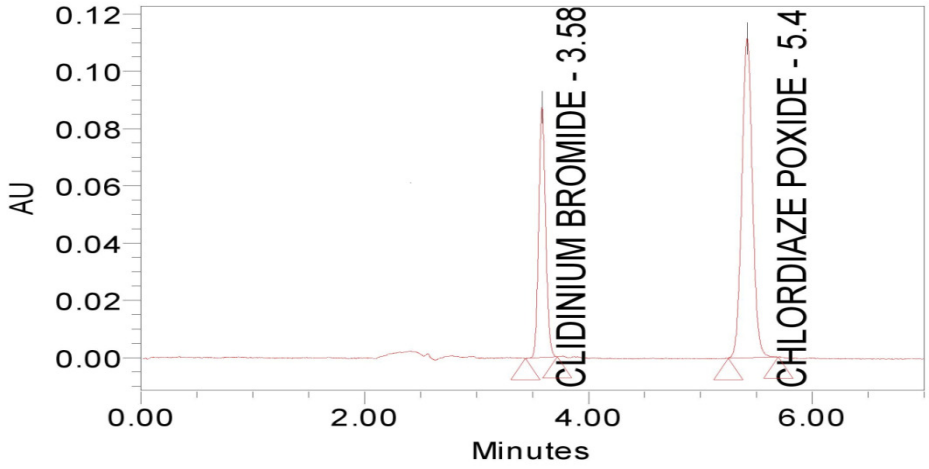

Fig. 27: Chromatogram of Clidinium bromide and Chlordizepoxidefor Precision-6

Acceptance Criteria:

% RSD of the sample replicate should not be more than 2.

Result

The % RSD for Clidinium bromide and chlordiazepoxidewas found to be 0.06 and 0.02 respectively. The % RSD value indicates a good degree of precision within the specified range.

7.2.5 ACCURACY

Accuracy of the method was determined by recovery experiments. To the formulation, the reference standards of the drug were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage relative standard deviation of the recovery werecalculated and shown in ***table no 11-12.*** And ***figure no 28-36***.

Table11:**Accuracy for Clidinium Bromide**

AMOUNT OF SPIKED	Peak Area	Amount of added (mg)	Amount recovered (mg)	% Recovery	Average recovery	% RSD
50%	190405	1.25	1.24	100.80	100.53	0.45
	190497	1.25	1.24	100.80		
	190659	1.24	1.24	100.00		
100%	380225	2.5	2.48	100.80	100.40	0.39
	380833	2.47	2.46	100.40		
	380206	2.5	2.5	100.00		
150%	571440	3.75	3.74	100.26	100.53	0.26
	571664	3.73	3.71	100.53		
	571077	3.74	3.71	100.80		

Table 12: **Accuracy for Chlordiazepoxide**

AMOUNT OF SPIKED	Peak Area	Amount of added (mg)	Amount recovered (mg)	% Recovery	Average recovery	% RSD
50%	366024	2.50	2.49	100.40	100.67	0.23
	366893	2.48	2.46	100.81		
	366878	2.49	2.47	100.80		
100%	733451	5.0	4.97	100.60	100.40	0.19
	733244	4.98	4.96	100.40		
	733399	4.99	4.98	100.20		
150%	1108041	7.5	7.49	100.13	100.26	0.13
	1106428	7.48	7.45	100.40		
	1109336	7.49	7.47	100.26		

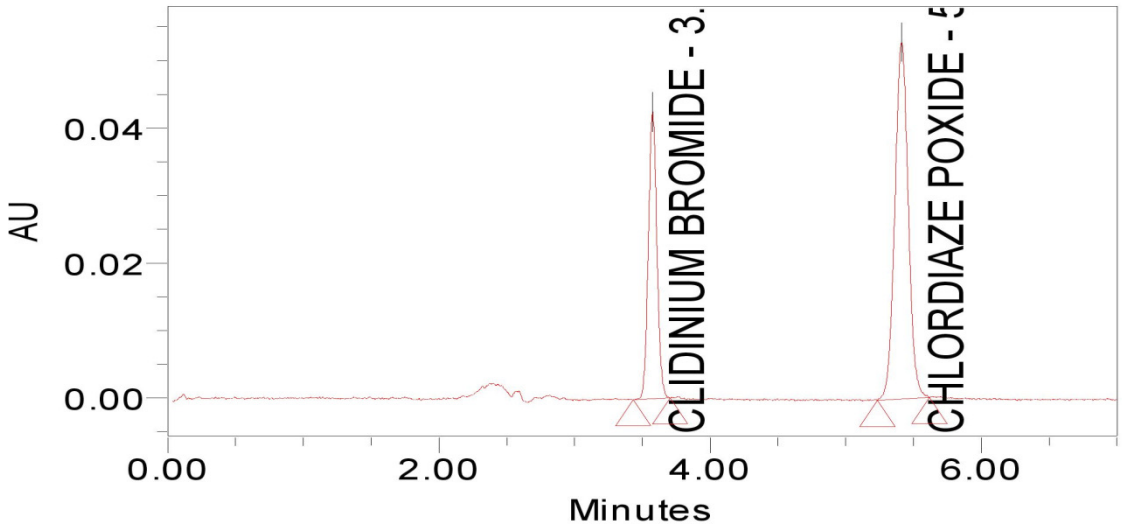

Fig. 28: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 50%spiking-1

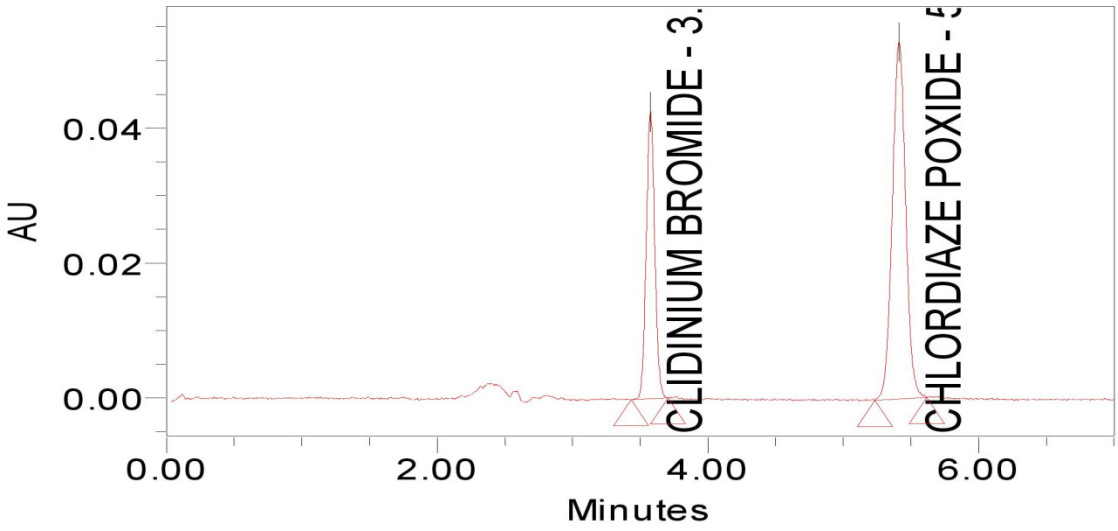

Fig. 29: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 50%spiking-2

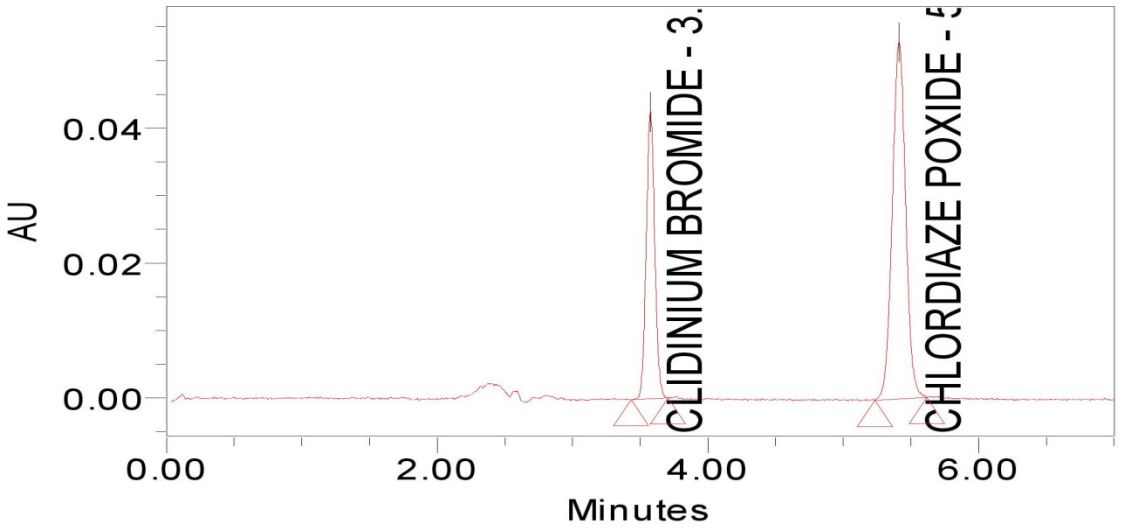

Fig. 30: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 50%spiking-3

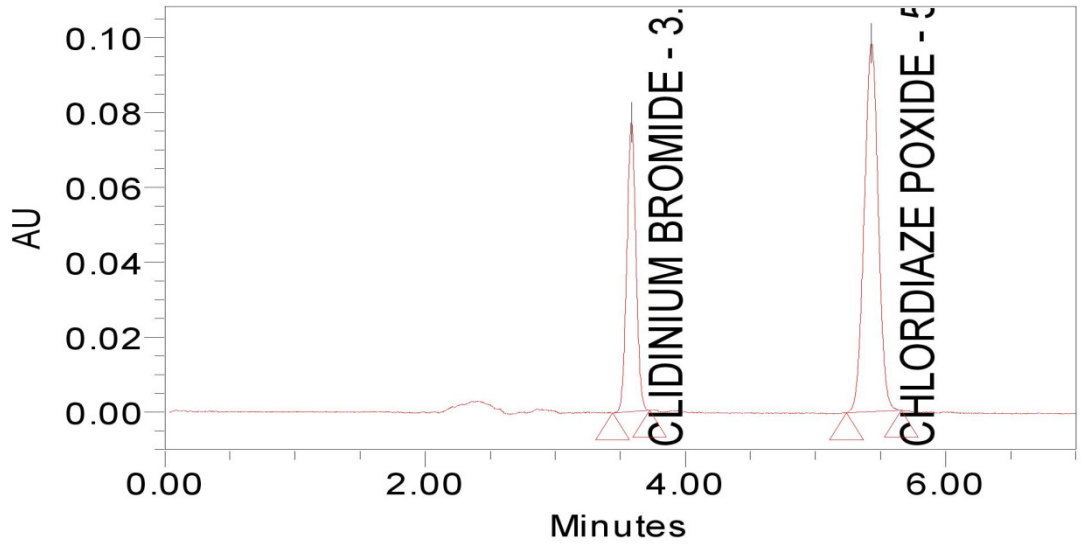

Fig. 31: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 100%spiking-1

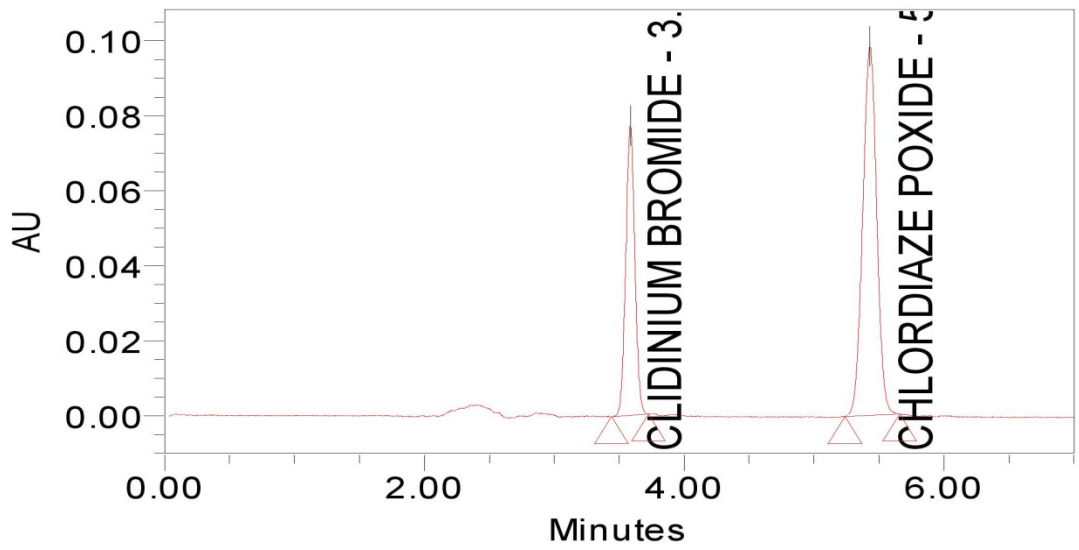

Fig. 32: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 100%spiking-2

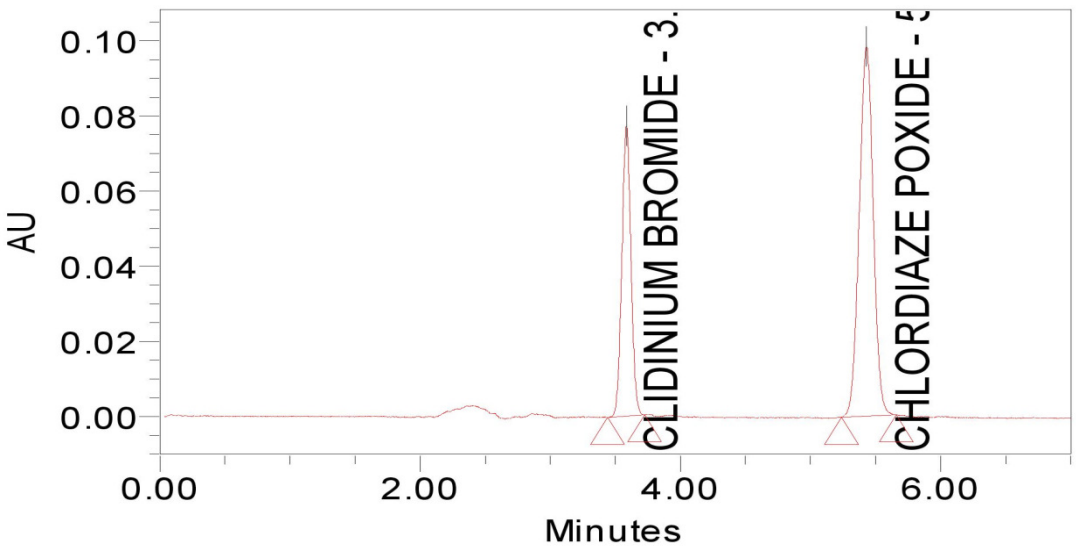

Fig. 33: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 100%spiking-3

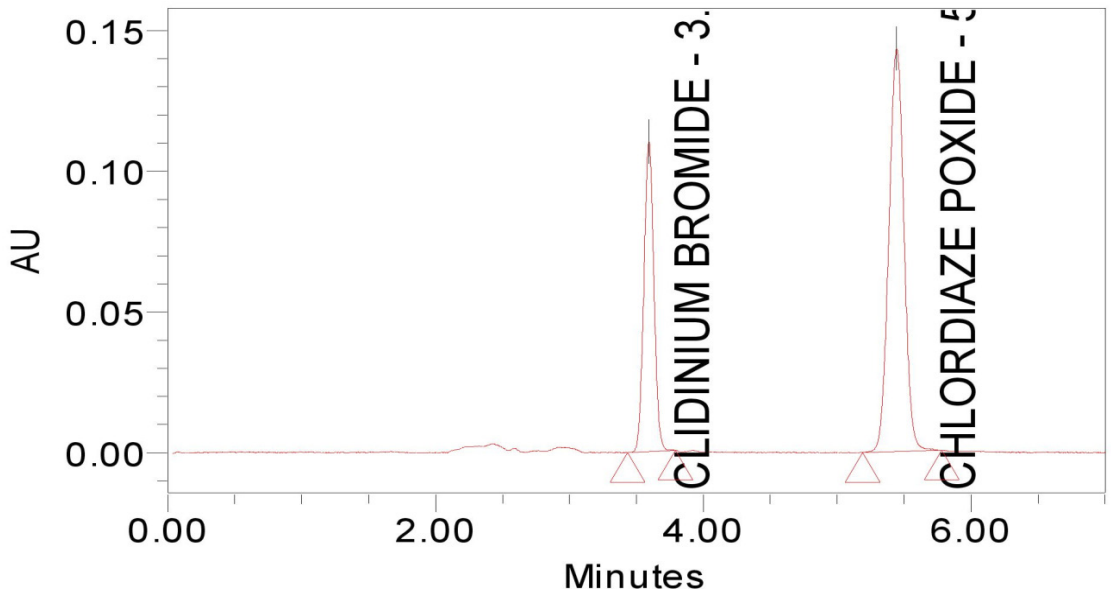

Fig. 34: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 150%spiking-1

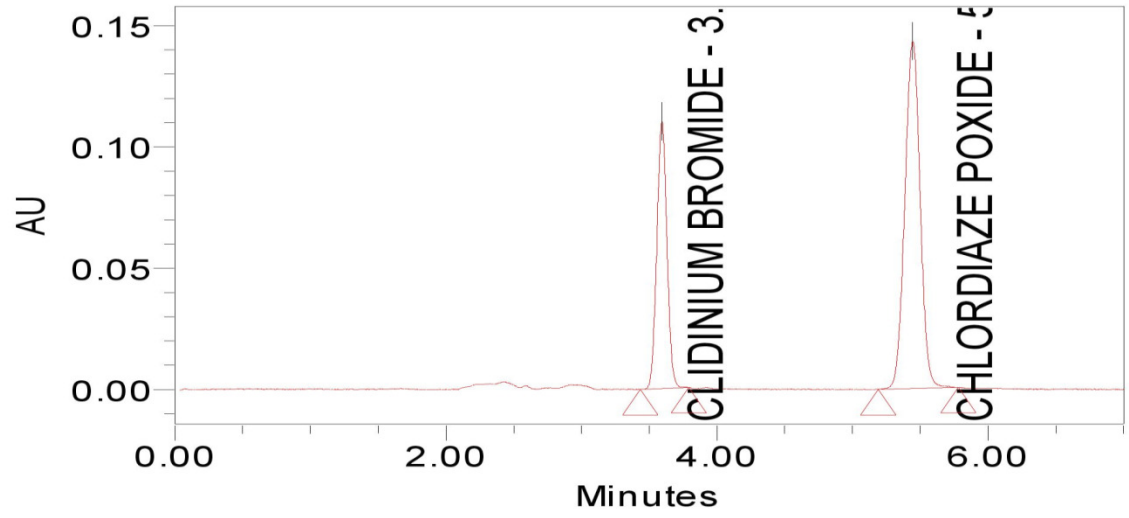

Fig. 35: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 150%spiking-2

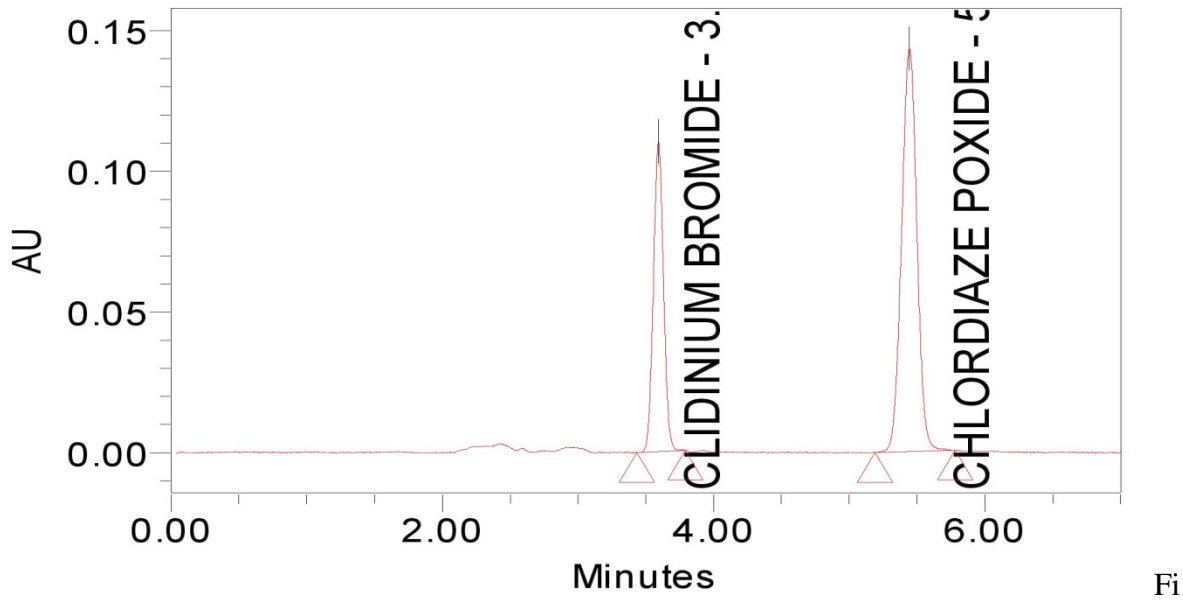

Fig. 36: Chromatogram of Clidinium bromide and Chlordiazepoxidefor 150%spiking-3

Acceptance criteria:

The mean percentage recovery of the Clidinium bromide and Chlordiazepoxideat each spike level should be not less than 98.0% and not more than 102.0%.

Result

From the Accuracy table it was found that % Recovery of the drug was found to be in the range of 100.40-100.53 % and 100.26-100.67 % for Clidinium bromide and Chlordiazepoxide respectively. This indicates that the method was accurate.

7.2.6 RUGGEDNESS

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts. Acceptance criteria for ruggedness,the %RSD should not be more than 2%. Results of this study were shown in **table no 13-14** and **figure no 37-48.**

***Table13:*Intraday Precision and Intermediate precision Analyst - 1**

Parameter	Inj	Clidiniumbromide			Chlordiazepoxide		
		Rt	AUC	%ASSAY	Rt	AUC	%ASSAY
Intraday Precision Data Analyst-1	1	3.593	571440	99.78	5.447	1108041	100.42
	2	3.592	571664	99.14	5.448	1106428	100.26
	3	3.592	571077	99.56	5.450	1109336	100.00
	4	3.594	571988	99.60	5.450	1109177	100.32
	5	3.594	571855	99.41	5.445	1100070	100.65
	6	3.594	571184	99.80	5.444	1105351	98.94
	Mean		571534	99.55		1106401	100.10
	R.S.D		0.06			0.31	

Table14: **Intraday Precision and Intermediate precision Analyst - 2**

Parameter	Inj	Clidiniumbromide			Chlordiazepoxide		
		Rt	AUC	%ASSAY	Rt	AUC	%ASSAY
Intraday Precision Data Analyst-2	1	3.553	571540	100.05	5.407	1112045	100.12
	2	3.572	571464	99.21	5.418	1113421	99.60
	3	3.552	571177	99.88	5.410	1112332	101.49
	4	3.564	571688	100.15	5.400	1114179	100.89
	5	3.574	571555	98.86	5.385	1110073	100.38
	6	3.584	571284	99.78	5.374	1112355	100.44
	Mean		571451	99.65		1112401	100.49

	%R.S.D		0.03			0.12	

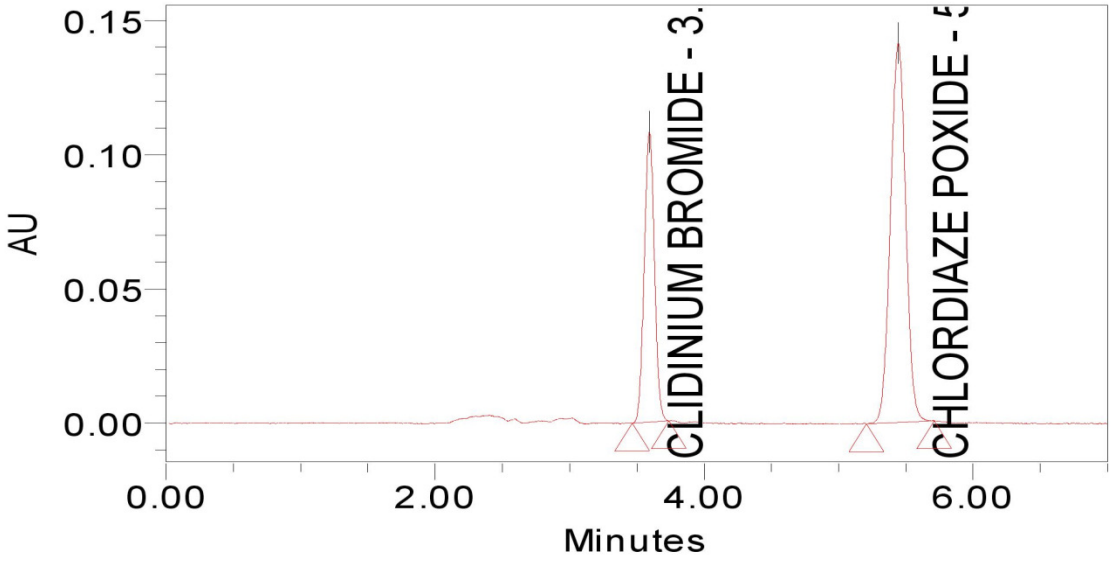

Fig. 37: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -1

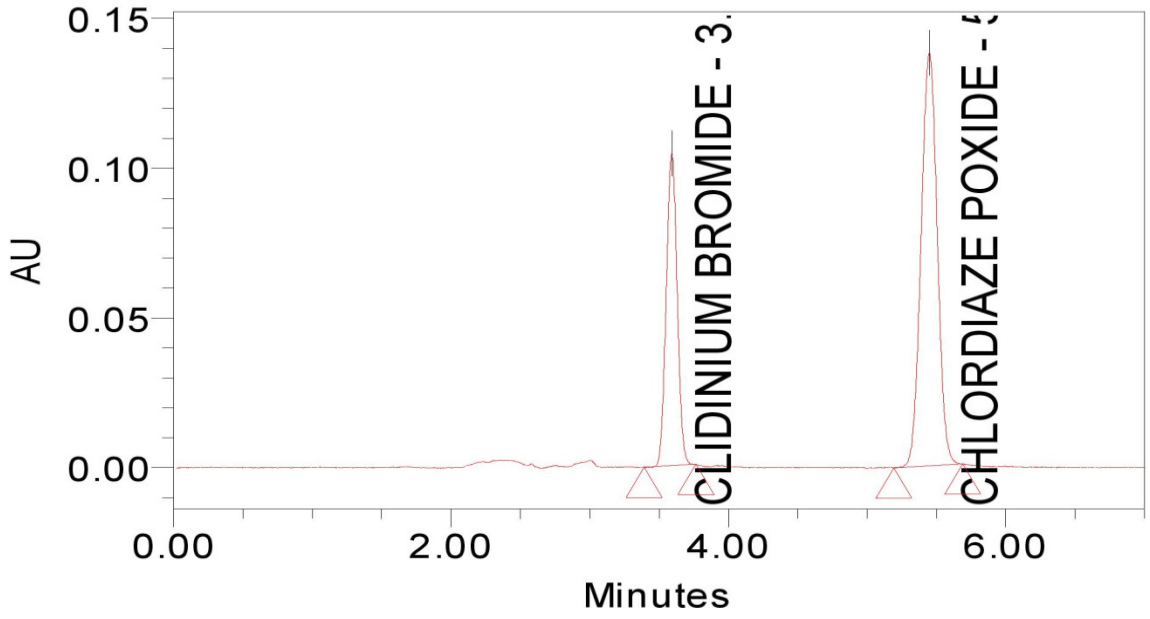

Fig. 38: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -2

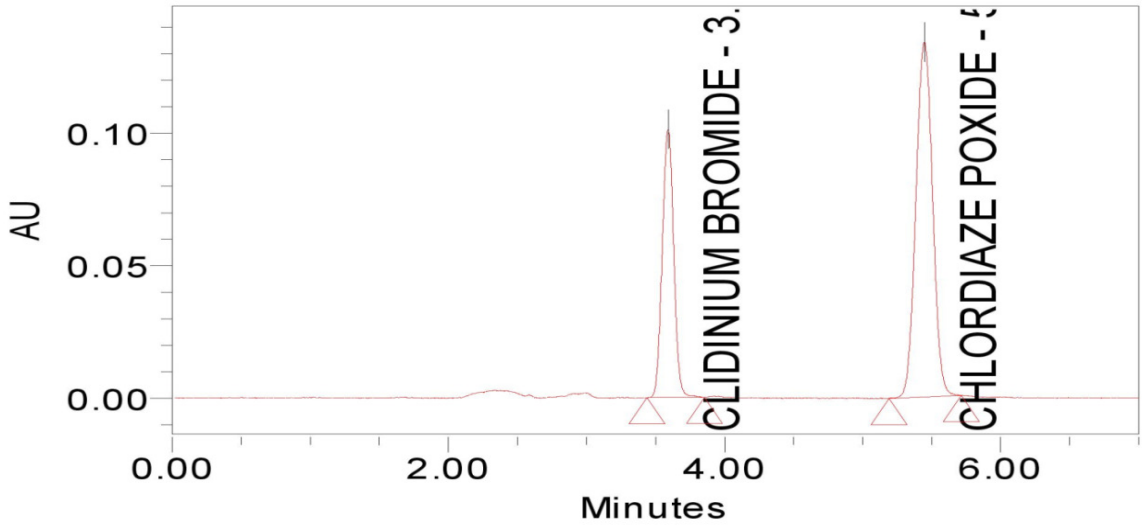

Fig. 39: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -3

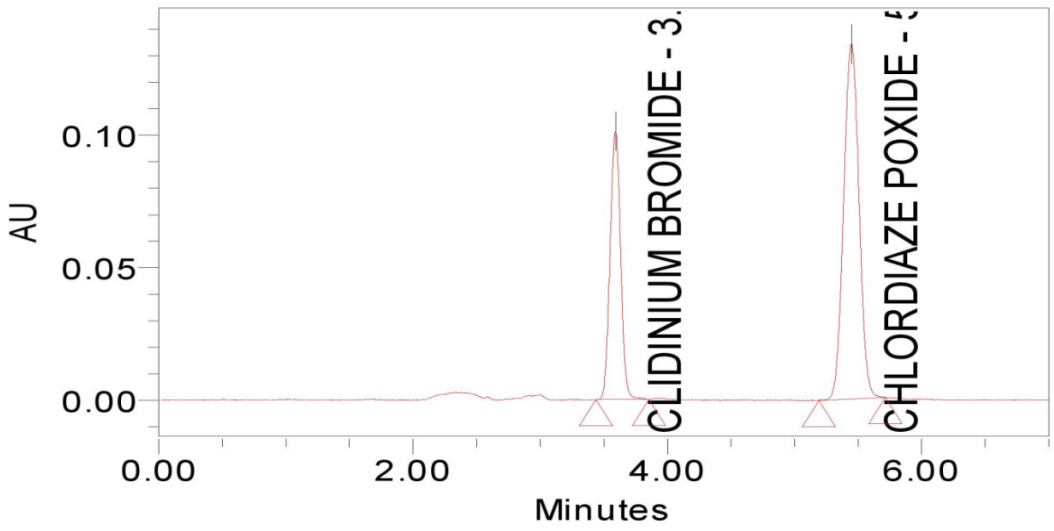

Fig. 40: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -4

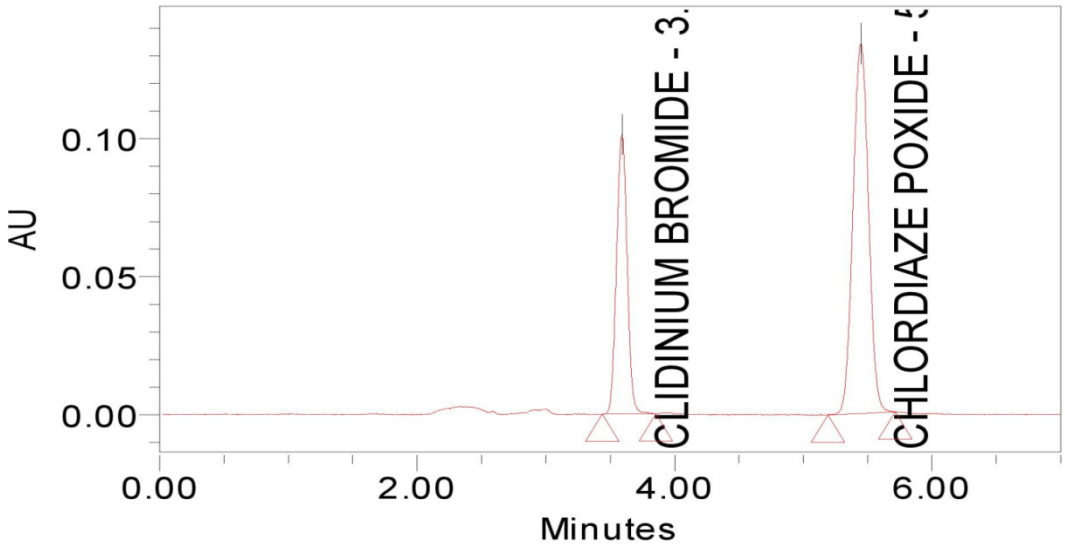

Fig. 41: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -5

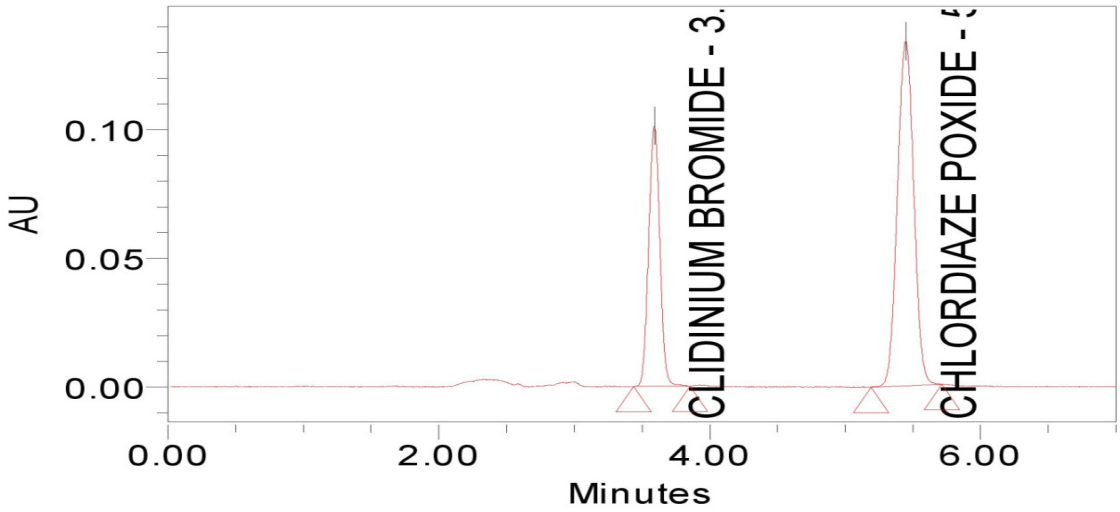

Fig. 42: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -6

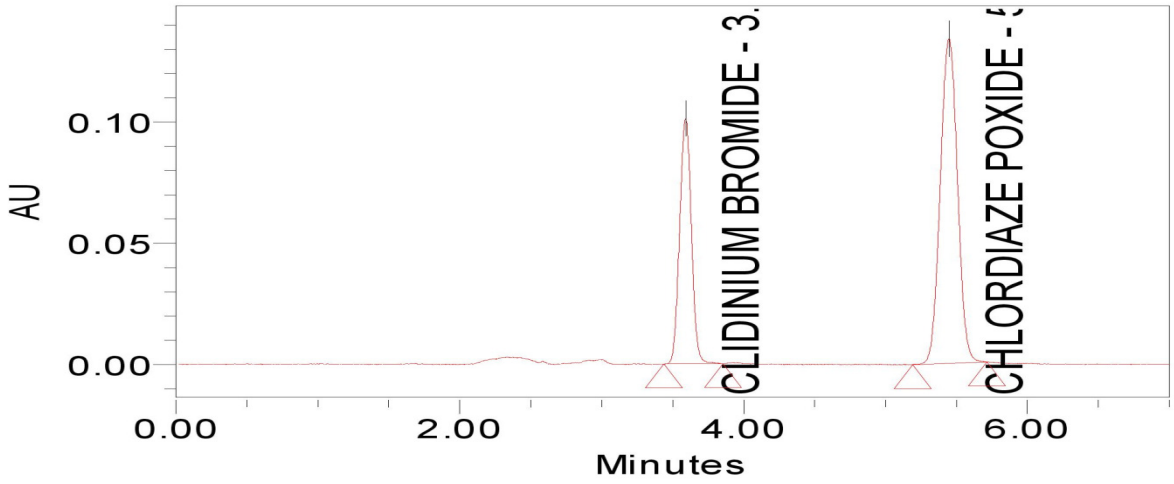

Fig. 43: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -1

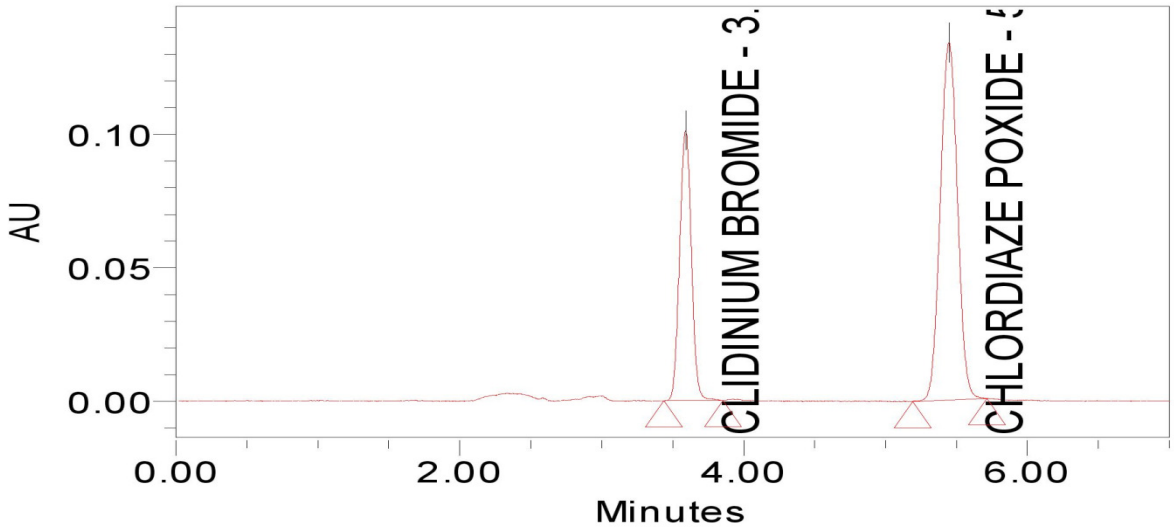

Fig. 44: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -2

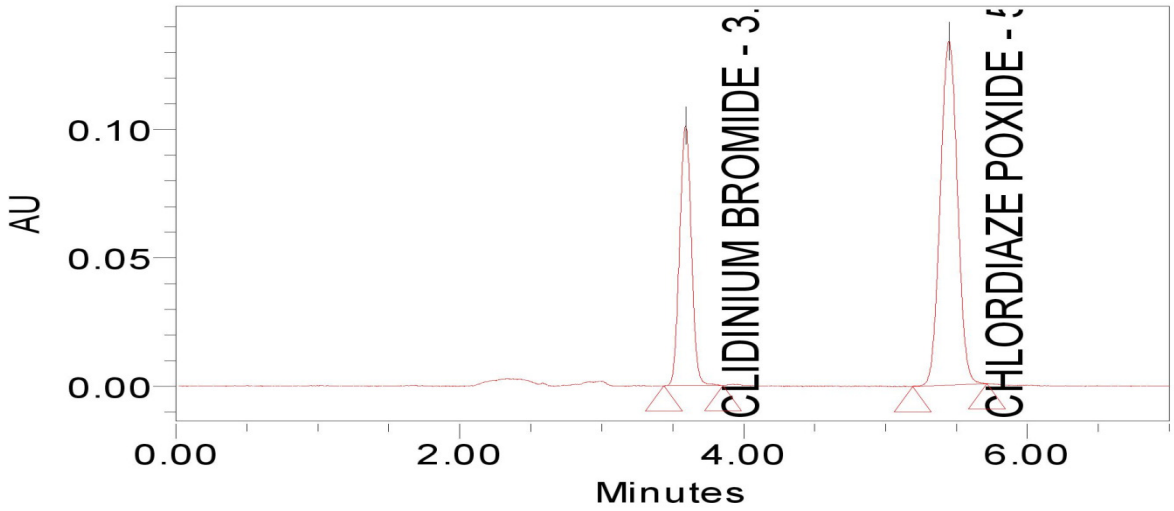

Fig. 45: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -3

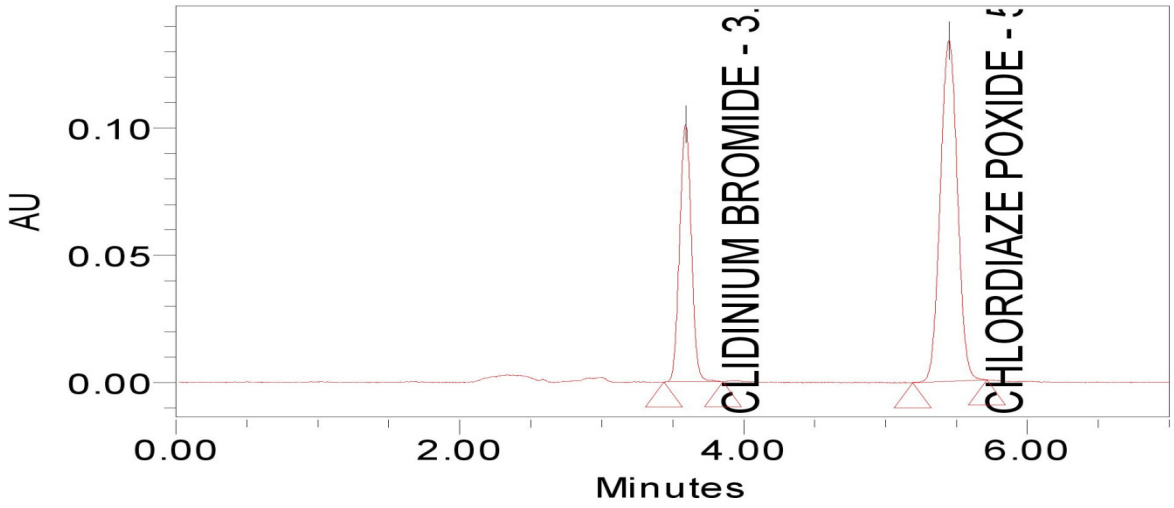

Fig. 46: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -4

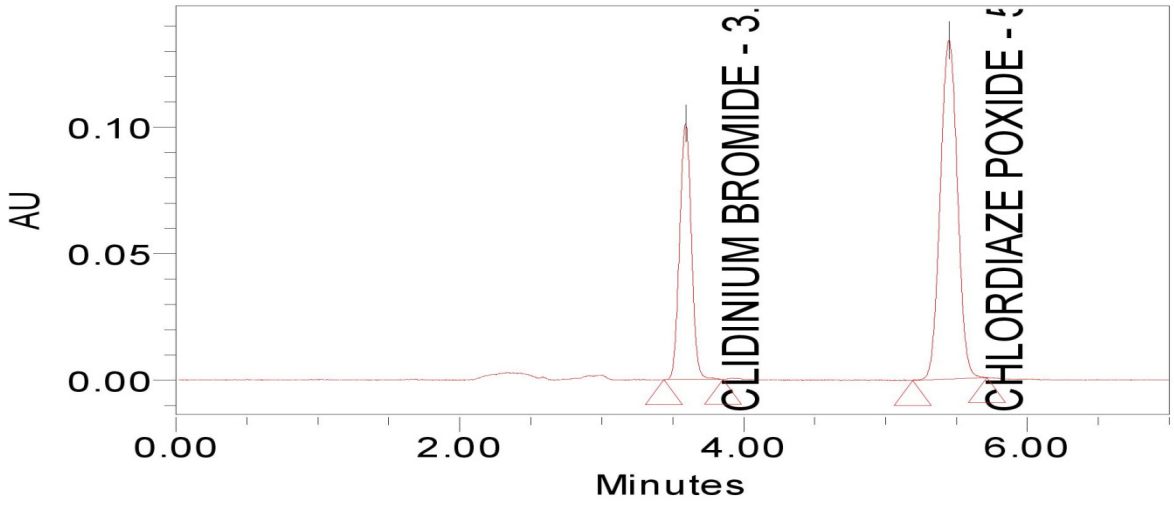

Fig. 47: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -5

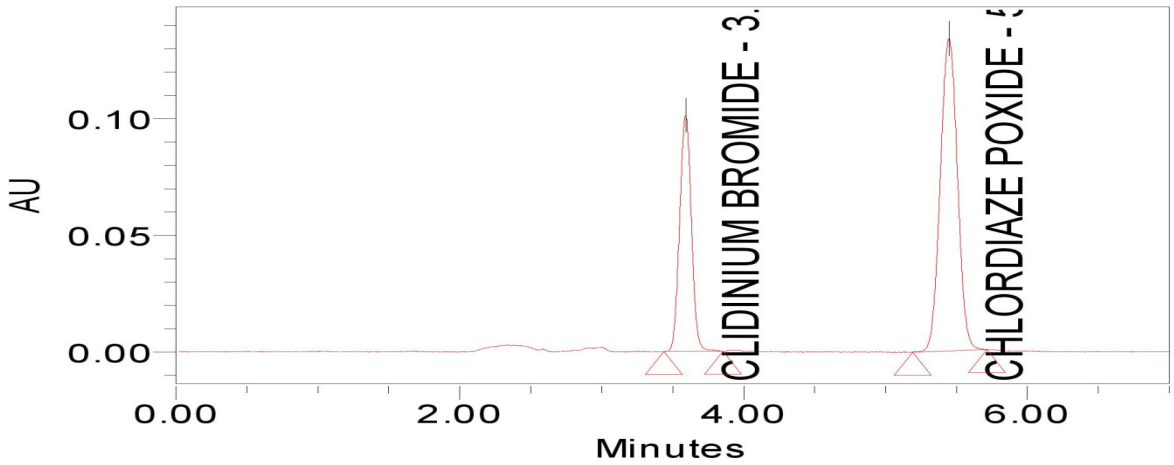

Fig. 48: Chromatogram of Clidinium bromide and Chlordiazepoxidefor ruggedness -6

Acceptance criteria:

%RSD not more than 2%

Result:

Individual % RSD of Assay was found to be 0.25 % and 0.60 %. The Relative standard deviation of % Assay of Analyst 1 found to be for Clidinium bromide 0.06 % and Chlordiazepoxide 0.31 % and the sample passed the test. The relative standard deviation of %

Assay of Analyst 2 found to be for Clidinium bromide 0.03 % and Chlordiazepoxide0.12 % respectively.

7.2.7 ROBUSTNESS:

The robustness study was carried out for variation flow rate and temperature

The of variation flow rates was shown in **table no15-16**andresults are shown in **figure no49-52.**

Table no.15: Flow rate changes for Clidinium bromide and Chlordiazepoxide

Clidinium bromide				Chlordiazepoxide		
Flow rate	**Retention time**	**Peak area**	**Plate count**	**Retention time**	**Peak area**	**Plate count**
1.2	4.465	483250	9055	6.789	936068	9245
1.8	2.988	325035	7093	4.536	622136	7245

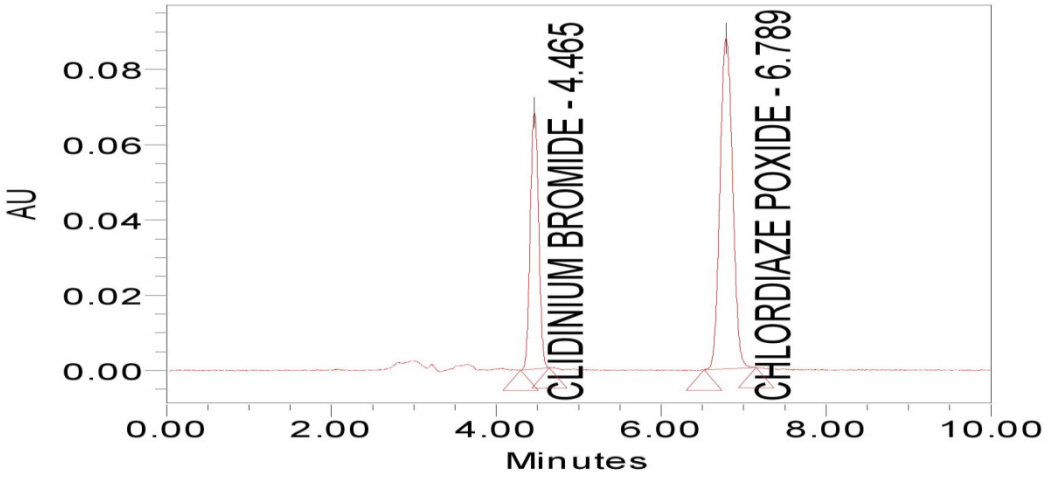

Fig. 49: Flow rate changes (Flow rate 1.2mL)

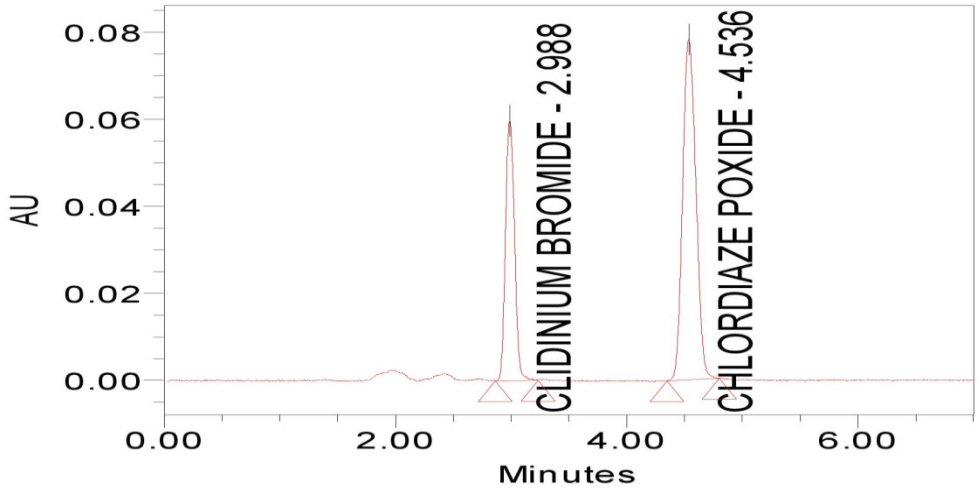

Fig. 50: Flow rate changes (Flow rate 1.8mL)

Table: 16: Temperature changes for Clidinium bromide and Chlordiazepoxide

Clidinium bromide				**Chlordiazepoxide**		
Temperature (ºC)	**Retention time**	**Peak area**	**Plate count**	**Retention time**	**Peak area**	**Plate count**
28	3.582	385490	8034	5.438	752227	8150
30	3.563	395511	8921	5.644	750899	8454

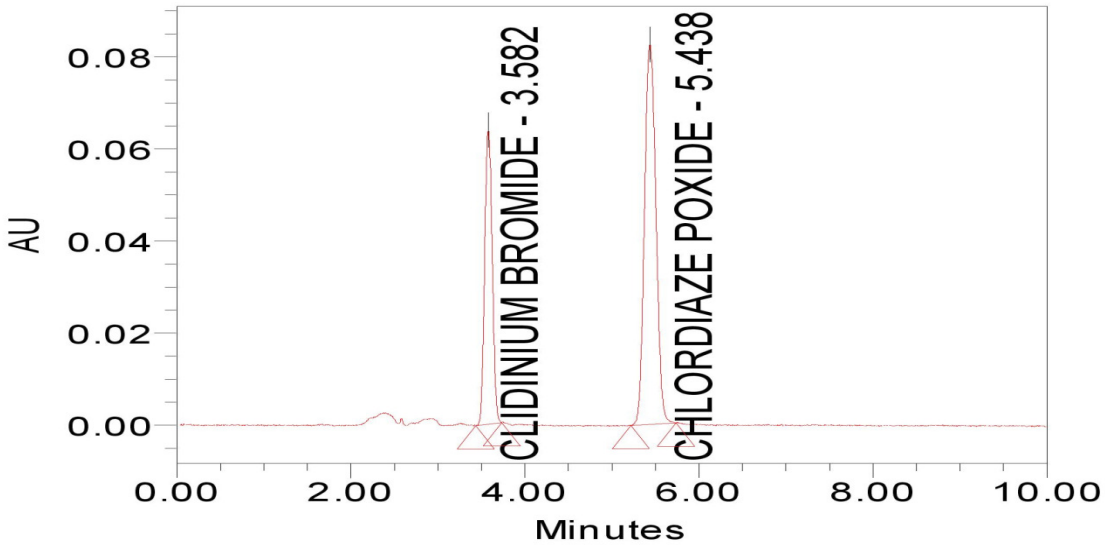

Fig. 51: Temperature changes (Temperature 28^0c)

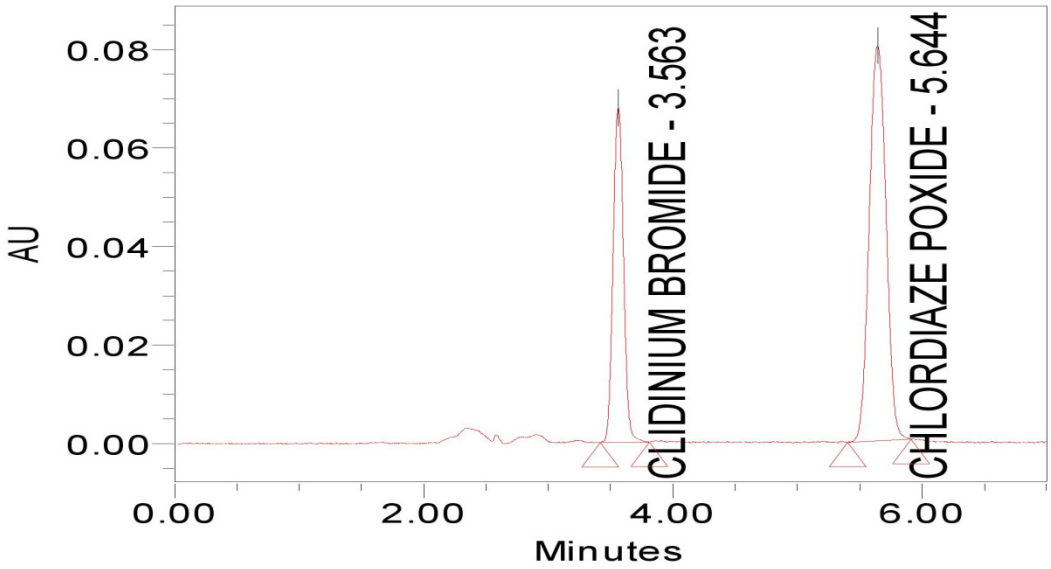

Fig. 52: Temperature changes (Temperature 30^0c)

Result:

When flow rate was altered RT has no changed significantly, when temperature was altered there was no change in the RT significantly. The method was found to be robust as per ICH Guidelines.

7.2.8 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ):

LOD and LOQ were calculated by using instrumental method.

Limit of detection:

Detection limit were calculated by the method based on the standard deviation ( ) and slope of the calibration plot, using the formula

$$\text{Limit of Detection} \quad \sigma \quad = \frac{\times 3.3}{S}$$

σ = Standard deviation

S = Slope of the calibration curve

Limit of quantification:

Detection limit were calculated by the method based on the standard deviation (σ) and slope of the calibration plot, using the formula

$$\text{Limit of Quantitation} \quad \sigma \quad = \frac{\times 10}{S}$$

σ = Standard deviation

S = Slope of the calibration curve

Table* 17: *LOD and LOQ

Sample	LOD	LOQ
Clidinium bromide	0.49 µg/ml	1.6 µg/ml
Chlordiazepoxide	0.8 µg/ml	2.8 µg/ml

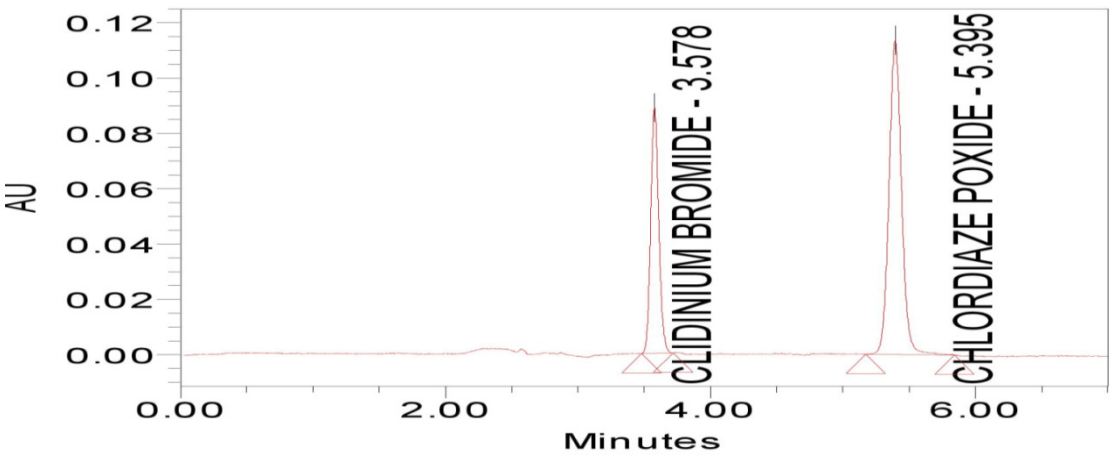

Fig. 61: LOD

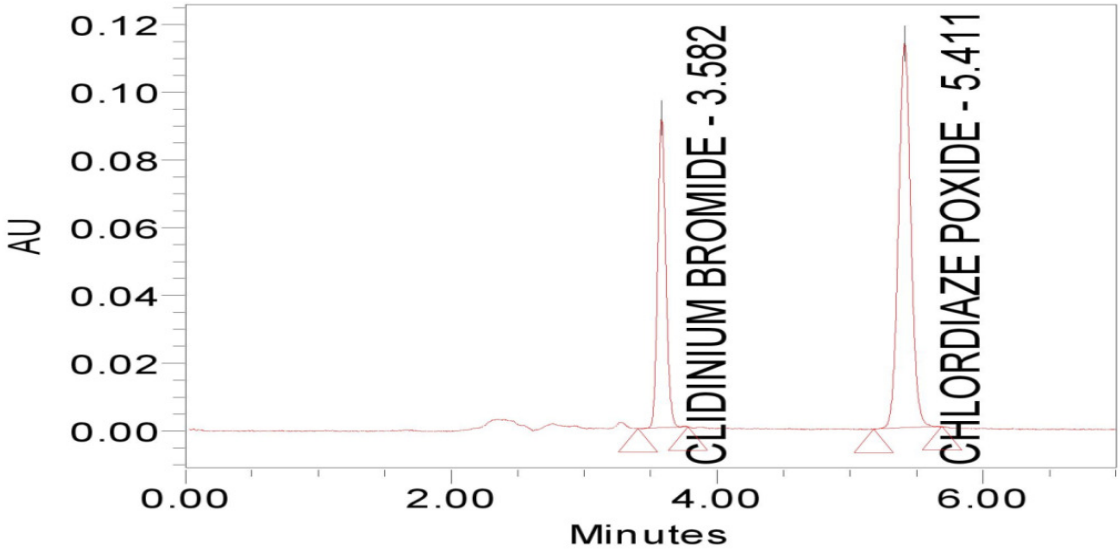

Fig. 62: LOQ chromatogram of Clidinium bromide and Chlordiazepoxide

Acceptance criteria:

LOD should not exceed LOQ.

Result:

TheLOD and LOQ of Clidinium bromide is 0.49 and 1.6

The LOD and LOQ of Chlordiazepoxide is 0.8 and 2.8.

8. SUMMARY AND CONCLUSION

In the present study, new RP-HPLC method for simultaneous estimation of Clidinium bromide and Chlordiazepoxide in bulk and pharmaceutical dosage form was developed. The developed method was validation for various parameters such as accuracy, precision, ruggedness, linearity, robustness, system suitability, specificity, limit of detection and limit of quantification as per ICH guidelines.

The results of the studies are summarized as follows

A) Method development:

- Trial 5 was optimized for the method development of deliberately changing the chromatographic conditions.
- Column used was OC C_8 (250 x 4.5mm, 5.0µm), mobile phase composition of Buffer pH 4.5 : Methanol (80:20), Flow rate 1.5 mL min^{-1}.UV detection was carried out at 240 nm.
- Assay percentage was found to be 100.05 % for Clidinium bromide and 100.1 % forChlordiazepoxide.

B) Validation Parameters:

- The calibration was linear with correlation coefficient 0.999 for Clidinium bromide and 0.999 forChlordiazepoxide.
- In precision it was found that % RSD is less than 2% which indicates that the proposed method has good reproducibility.
- The system suitability parameter indicates good resolution of both the peaks > 2.
- From the Accuracy was found that % Recovery of the drug was found to be in the range of 100.40-100.53% and 100.26-100.67% for Clidinium bromide and Chlordiazepoxide respectively.
- Robustness, When flow rate was altered RT has no changed significantly, when temperature was altered there was no change in the RT significantly.
- LOD and LOQ, the limit of detection (LOD) was 0.49 µg mL^{-1} for Clidinium bromide and 0.8 µg mL^{-1} for Chlordiazepoxide and the limit of quantitation (LOQ) was 1.6 µg mL^{-1} for Clidinium bromide and 2.8 µg mL^{-1} for Chlordiazepoxide.

Conclusion

The developed RP-HPLC method has been successfully applied for the simultaneous determination of Clidinium bromide and Chlordiazepoxide in pure drug and marketed formulation. The methods are found to be rapid, simple, accurate and convenient to adopt. The developed methods are completely validated with all validation parameters.The results indicate that this method are precise, accurate further more it is easy and convenient for routine drug analysis.

Plan for further study

The proposed method was simple, precise and accurate. Furthermore methods can be developed by the sophisticated instruments like GC-MS and LC-MS and stability studies can be carried by using RP-HPLC.

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10. APPENDIX

%	:	Percentage
µ mol	:	Micromole
µg	:	Microgram
^{14}C	:	Carbon - 14
^{3}H	:	Tritium
ACN	:	Acetonitrile
API	:	Active Pharmaceutical Ingredient
AUC	:	Area under Curve
CAS	:	Chemical Abstracts Service
cm	:	Centimetre
EGFR	:	Epidermal Growth Factor
FDA	:	Food and Drug Administration
Fig	:	Figure
GC	:	Gas chromatography
HETP	:	High equivalent theoretical plate
HPLC	:	High-performance liquid
HPTLC	:	High-performance thin layer
K	:	Tailing Factor
LC	:	Liquid chromatography
LC-MS	:	Liquid chromatography-Mass
LOD	:	Limit of detection
LOQ	:	Limit of quantification

mg	:	Milligram
Min	:	Minute
mm	:	Millimeter
MS	:	Mass Spectroscopy
NF	:	National Formulary
nm	:	Nanometer
NSCLC	:	Non - small Cell Lung Cancer
ODS	:	Octyldecylsilane
RI	:	Refractive Index
RI	:	Refractive index
RP-HPLC	:	Reversed-phase high-performance liquid
Rs	:	Resolution
RSD	:	Relative standard deviation
S	:	Separation from Peak
S/N	:	Signal to Noise ratio
SD	:	Standard Deviation
TLC	:	Thin layer chromatography
USFDA	:	United States Food and Drug Administration
USP	:	United States Pharmacopoeia
UV	:	Ultraviolet
v/v	:	Volume by volume
WHO	:	World Health Organization