“RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF DICLOFENAC SODIUM IN SOFT GELATIN CAPSULE DOSAGE FORM”

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Submitted by

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TAMIL NADU

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CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled, **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF DICLOFENAC SODIUM IN SOFT GELATIN CAPSULE DOSAGE FORM",** submitted by student bearing **Reg. No. 261530203** to **"The Tamil Nadu Dr. M. G. R. Medical University"**, Chennai, for the partial fulfillment of the degree of **MASTER OF PHARMACY in Pharmaceutical Analysis,** was evaluated by us during the examination held on…….

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DECLARATION

The work presented in this dissertation entitled, "**RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF DICLOFENAC SODIUM IN SOFT GELATIN CAPSULE DOSAGE FORM**", was carried out by me, under the direct supervision of **Dr.V.SEKAR, M. Pharm., Ph.D.,** Professor, Head of the Department, Department Of Pharmaceutical Analysis, J.K.K. Nattraja College of Pharmacy, Komarapalayam. I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

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DEDICATED TO

ALMIGHTY,

MY BELOVED PARENTS, WIFE, GUIDE,

TEACHERS

&

FRIENDS

LIST OF ABBREVIATIONS USED

B.P	-	British Pharmacopeia
°C	-	Degree Centigrade
FDA	-	Food and Drug Administration
GC	-	Gas Chromatography
HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
ICH	-	International Conference Of Harmonization
I.P	-	Indian Pharmacopeia
IR	-	Infrared Spectroscopy
IUPAC	-	International Union of Pure and Applied Chemistry
LC	-	Liquid Chromatography
LC-MS	-	Liquid Chromatography-Mass Spectroscopy
LOD	-	Limit of detection
LOQ	-	Limit of quantitation
NMT	-	Not more than
Nm	-	Nanometer
NLT	-	Not less than
M	-	Molar
Max	-	Maximum
mg	-	Milligram

Min	-	Minute
Ml	-	milli litres
Mm	-	millimetre
M.W	-	Molecular weight
ODS	-	Octyl decyl silane
Ppm	-	Parts Per Million
RSD	-	Relative Standard Deviation
RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
S.D	-	Standard Deviation
TLC	-	Thin Layer Chromatography
t_R	-	Retention time
µg	-	Microgram
µl	-	Microlitre
µg	-	Microgram
US FDA	-	United States Food and Drug Administration
US NF	-	United States National Formulary
USP	-	United States Pharmacopeia
UV	-	Ultra-Violet
% v/v	-	Percentage volume per volume
Wt	-	Weight
%w/w	-	Percentage weight per weight
ʎ	-	Lambda

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1. INTRODUCTION

Analytical chemistry is concerned with the determination of the chemical composition of matter qualitatively and quantitatively. It is the multidisciplinary branch of science. Analytical chemistry has got extensive applications in various disciplines of chemistry such as inorganic, organic, and physical and biochemistry.Related sciences such as environmental science, agricultural science, oceanography, forensic science have the application of analytical chemistry. For instances monitoring of environmental pollutants like SO_2, CO, CO_2 is done by flurosecence or infrared spectroscopy.Analysis of dissolved oxygen and chlorine in water is carried out by potentiometry or colorimetry. (S. M. Khopkar, 2008).

Analytical chemistry has got a pivot role to play in determination of quality of medicines as quality is important and essential aspect to be considered in every product or service. Quality is more vital in medicines as it is related to life. A compromise in pharmaceutical quality is nothing but playing with the life of consumer. Application of analytical chemistry in pharmacy is termed as pharmaceutical analysis. The terms quality, quality control, qualityassuarance, total quality management are correlated. The ultimate goal of all these is to provide a product with good quality, safety, efficiency, purity, strength and identity. (Devis John, 1999).

General terms associated with chemical analysis

- **Analytical technique**

  It is the fundamental scientific phenomenon that is proved useful for providing information on the composition of substances.

- **Analytical method**

  The method is the specific application of technique to solve the analytical problem.

- **Protocol**

  The most specific description of a method is known as protocol.

- **Procedure**

  A procedure is written instructions for carrying out a method. The "standard methods" developed by the ASTM (American Society for Testing Materials) and the AOAC (Association of Official Analytical Chemists). It only provides the general steps to be followed. (Willard *et al.*, 1986.)

Pharmaceutical analysis is broadly classified into two branches, namely qualitative analysis and quantitative analysis.

A. **Qualitative analysis**: It deals with the identification and establishment of the identity of analyte among elements or compounds present in the sample.

B. **Quantitative analysis**: It deals with the quanification of the analyte present in the matter of sample. (S. Ravi Shankar, 2006).

The determination of the quality and of quantity of the sample is done the application of analytical methods.

Analytical methods are classified into two types

- Instrumental methods.
- Non-instrumental methods.

Non – instrumental methods of analysis

The physico-chemical properties are utilised in these methods of analysis to determine the contents or the composition of the substance. (Dr. A. V. Kasture and *et al.,* 2010)

Table No: 1.1 Classification of Non-instrumental methods (S. M. Khopkar, 2008)

S.No.	Method of analysis	Physico – chemical property
1	Volumetric analysis(acid-base titrations)	Neutralisation reaction
2	Redox titrations	Oxidation and reduction reactions
3	Complexometric titrations	Formation of complex
4	Solvent extraction	Extraction
5	Gravimetric analysis	Measurement of the weight precipitated.

Instrumental methods

The physical properties of the components are considered in these methods of analysis.

Analytical instrumentation plays an important role in production and evaluation of new products. The instrumentation provides lower detection limits which are required to assure safety, efficacy and quality of product of interest. (Willard *et al.*1986.)

Instruments used in chemical analysis do not give direct quantitative data but supplies information which is converted into suitable form which correlates with structure or content.

The instrument does the job in various steps which are as follows

- Generation of signal
- Transformation of signal into measurable form
- Amplification
- Presentation of signal (Read out system) (Dr. A. V. Kasture and *et al.,* 2010).

In some instrumental procedures the sample is destroyed (**destructive methods**), where as in others it remains unchanged(**non –destructive**)and may be used in subsequent studies.

The choice of an instrumental procedure for the determination of a specific element or compound really involves two choices:

1) Instrument to be used.

2) The chemical system (Gurudeep R. Chatwal and Sham K. Anand, 2008)

Advantages of instrumental methods

- A small amount of asample is sufficient for analysis.
- Determination is considerably fast.
- Complex mixture can be analysed with or without separation
- Precise, accurate, reliable results are obtained.

Limitation of instrumental methods

- Instrumental methods are expensive.
- Requirement of trained personnel for handling of instruments.
- Instrumental methods may not be specific.

- Sensitivity and accuracy depends upon the type of instrument.
- Frequent checking of results is necessary. (Dr. A. V. Kasture and *etal.,* 2010).

Classification of instrumental analysis

- Molecular analysis.
- Elemental analysis.

Principle types of chemical instrumentation

- Spectroscopic techniques.
- Electrochemical techniques.
- Chromatographic techniques
- Miscellaneous techniques
- Hyphenated techniques. (Gurudeep R. Chatwal and Sham K. Anand, 2008), (Willard *et al.,* 1986.)

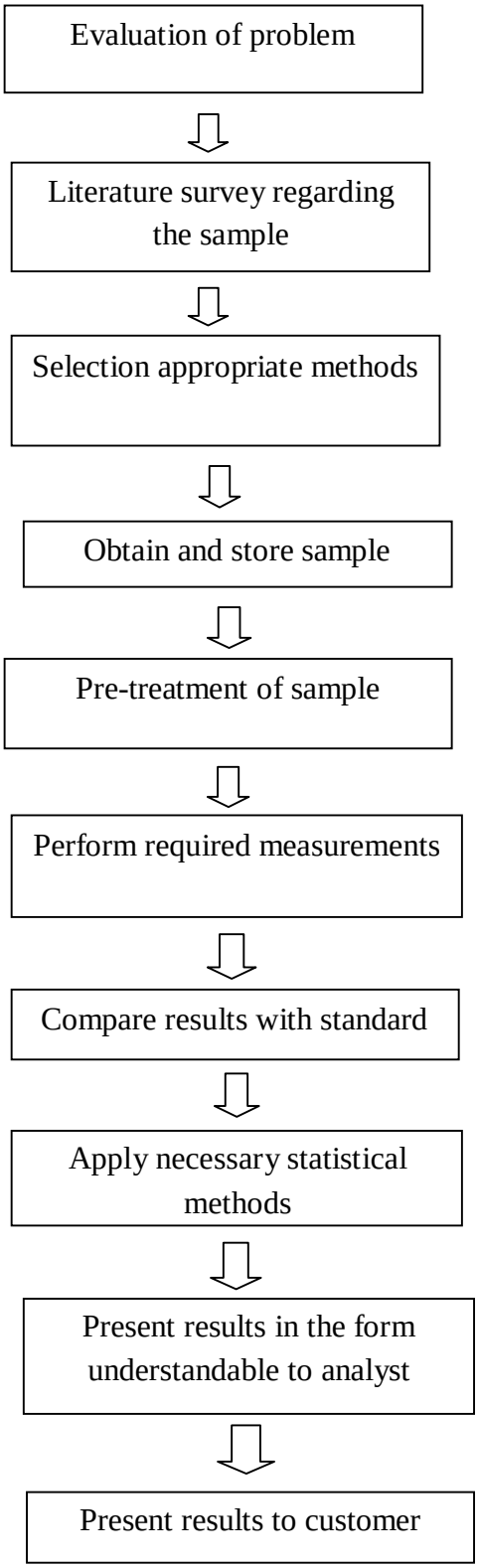
Table No: 1.2 Different types of Instrumental methods

S. No	Instrumental Technique	Instrumental Method	Physical property
1	Spectroscopy	UV-visible spectrophotometry	Absorption and emission of radition
		Florescence and phosphorescence spectrophotometry	Emission of radiation
		Infra red	Absorption of

		spectrophotometry	radiation
		Raman spectroscopy	Scattering of radiation
		x-ray spectroscopy	Diffraction of radiation
		Radio chemical techniques	Radio activity
		Nuclear magnetic resonance	Absorption of radiation
		Electron spin resonancespectroscopy	Absorption of radition
		Atomic spectrometry (emissionand absorption)	Emission and absorption of radiation
2	Electrochemical	Potentiometry	Electrical potential
		Polarography	Electrical current
		Amperometric techniques	Electrical current
		Coulometry	Quantity of electricity
		Electrogravimetry	Electrical potential
		Conductance	Conductivity

3	Chromatographic	Gas chromatography	Partition
		High performance of liquid chromatography	Adsorption or partition
4	Miscellaneous	Thermal analysis	Thermal properties
		Mass spectroscopy	Mass to charge ratio
		Refractometry and interferometry.	Refraction of radiation
		Polarimetry	Rotation of radiation
		Circular dichorism	Rotation of radiation
5	Hyphenated	GC – MS	Partition and mass to charge ratio
		GC – IR	Partition and absorption of radiation
		ICP – MS	Mass to charge ratio
		MS - MS	Mass to charge ratio

- Major steps in analytical method development (Willard *et al.,* 1986.)



Types of errors

- Random (indeterminate) Errors.
- Systematic (determinate) Errors. (Willard *et al.*, 1986.)

CHROMATOGRAPHY

The term Chromatography (in Greek, khromatos means colour and Graphos means written) means colourwriting**.** The term chromatography and its principles were discovered in 1903 by ***Mikhiltswett***. Chromatography is the separation of mixture of components into individual components by using a stationary phase and a mobile phase. (B.K. Sharma, Chromatography, 2007)

Chromatography is method of separating mixtures and identifying their components i.e. it's a separation method that exploits the differences in partitioning behaviour of analyse between a mobile phase and a stationary phase to separate components in a mixture. Components of a mixture may be interacting with the stationary phase based on charge (ion-ion-interactions, ion-dipole-interactions), Vander Waal's forces, relative solubility or adsorption (hydrophobic interactions, specific affinity).

Chromatography may be preparative or analytical. Preparative chromatography seeks to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography normally operates with smaller amounts of material and seeks to measure the relative proportions of analytes in a mixture. The two are not mutually exclusive.

Classification of chromatographic methods

- Gas – solid chromatography.
- Gas – liquid chromatography.
- Solid – liquid chromatography.
- Liquid- liquid chromatography. (B.K. Sharma, Chromatography, 2007

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High-performance liquid chromatography (HPLC) is the fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids.

The HPLC offers the following advantages.

- ❖ Speed (many analysis can be accomplished in 20 min or less).
- ❖ Greater sensitivity (various detectors can be employed).
- ❖ Improved resolution (wide variety of stationary phases).
- ❖ Reusable columns (expensive columns but can be used for many analysis).
- ❖ Ideal for the substances of low viscosity.
- ❖ Easy sample recovery, handling and maintenance.
- ❖ Instrumentation leads itself to automation and quantification (less time and less labour).
- ❖ Precise and reproducible.
- ❖ Integrator itself does calculations. (P.D.Sethi, 2001)

Applications of HPLC

- To purify synthetic or natural products
- To characterize metabolites
- To assay active ingredients, impurities, degradation products and in dissolution assays
- In pharmacodynamics and pharmacokinetic studies

TYPES OF HPLC TECHNIQUES

Based on modes of chromatography

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elution technique

- Isocratic separation
- Gradient separation

Based on the scale of operation

- Analytical HPLC
- Preparative HPLC

PRINCIPLES OF SEPARATION

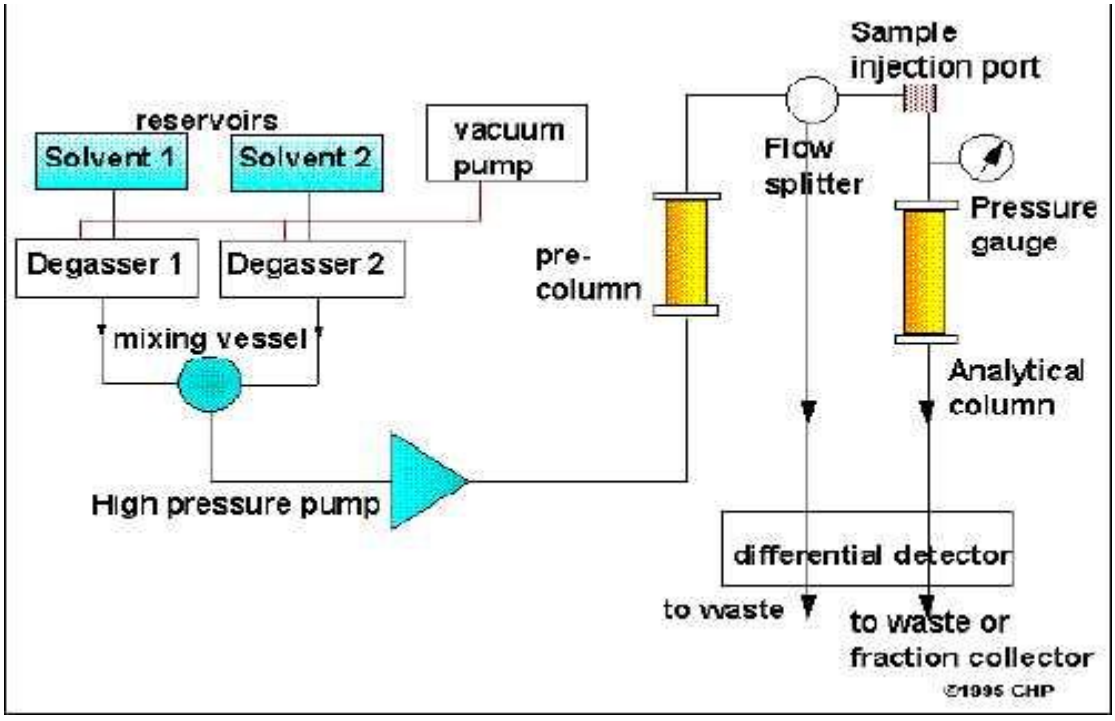
Adsorption chromatography employs high-surface area particles that adsorb the solute molecules. Usually a polar solid such as a silica gel, alumina or porous glass beads and a non-polar mobile phase such as heptane, octane or chloroform are used in adsorption chromatography. In adsorption chromatography, adsorption process is described by competition model and solvent interaction model.

In partition chromatography, the solid support is coated with a liquid stationary phase. The relative distribution of solutes between the two liquid phases determines the separation. The stationary phase can either be polar or non polar. If the stationary phase is polar and the mobile phase is non polar, it is called normal phase

partition chromatography. If the opposite case holds, it is called reverse-phase partition chromatography. In normal phase mode, the polar molecule partition preferentially in to the stationary phase and are retained longer than non-polar compounds. In reverse phase partition chromatography, the opposite behavior is observed.(Willard *et al.*, 1986)

INSTRUMENTATION OF HPLC

Figure no 1.1 typical diagram of HPLC



SOLVENT DELIVERY SYSTEM (B.K.Sharma, Instrumental methods of chemical analysis, 2005)

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.

Types of pumps in HPLC

1. Syringe pump (screw driven)
2. Reciprocating pump
    - Single piston reciprocating pump
    - Dual piston reciprocating pump
    - Reciprocating diaphragm pump
3. Pneumatic pump
    - Direct pressure pump
    - Amplifier pump

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.

MOBILEPHASE

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. The following points should also be considered when choosing a mobile phase

1. It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.

2. Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.

3. The mobile phase should have a p^H 2.5 and p^H 7.0 to maximise the life time of the column.

4. Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible.

5. Minimise the absorbance of buffer. Since trifluoroacetic acid, acetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products with outchromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, which does not absorb above 200 nm.

6. Use volatile mobile phases when possible, to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, formic acid, acetic acid, and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220 nm.

Table No: 1.3

PHYSICAL PROPERTIES OF COMMON HPLC SOLVENTS

Solvent	MW	BP	RI (25^oC)	UV Cut-off (nm)	Density g/ml (25^oC)	Viscosity cP (25^oC)	Dielectric Constant
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21

Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethyl acetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2Cl_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
Water	18.0	100	1.333	170	0.998	1.00	78.5

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 0.45µm filter, vacuum degassing with an air-soluble membrane, helium purging ultra sonification, 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.

COLUMNS

The heart of the HPLC system is the column. The choice of column packing material and mobile phases depends on the physical properties of the drug. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C_8 or C_{18} column and determine if it provides good separations. If this column does not provide good separation or the mobile phase is unsatisfactory, alternate methods or

columns should be explored. In Normal phase mode it contains Silanolgroups (hydroxyl). Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading. Reverse phase mode contains the following groups

C_{18} - Octadecylsilanecolumn (ODS)

C_8 - Octyl column

C_4 - Butyl column

CN - Cyano column

NH_2 - Amino column

SAMPLE INTRODUCTION SYSTEM

Two means for analyte introduction on the column are injection into 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.

DETECTORS

Detectors used depend upon the property of the compounds to be separated. Optical detectors are most frequently used.

- **UV-Ultraviolet Detector:** The most commonly used detector in LC is the ultraviolet absorption detector. Two types of detectors are available. One is a fixed wavelength detector which operates at 254nm where most drug compounds absorb. The other is variable wavelength detector which can be operated from 190 to 600nm

- **RI – Refractive Index (Universal analyte detector):** Solvent must remain the same throughout separation. Very temperature sensitive. Sometimes difficult to stabilize baseline.

- **FD – Fluorescence detector:** Excitation wavelength generates fluorescence emission at a higher wavelength. Analytes must have fluorophore group. Sensitive and selective. Results dependent upon separation conditions.

- **MS – Mass Spectroscopic detector:** Mass to charge ratio (m/z) allows specific compound identification. Several types of ionization techniques include electro spray, atmospheric pressure chemical ionization, electron impact. The detector usually contains low volume cell through which the mobile phase passes carrying the sample components.

- **PDA - Photo Diode Array Detector:** This is recent one which is similar to UV detector which operates from 190-600nm. Radiations of all wavelengths falls on detector simultaneously. The resulting spectra is a 3D or a three dimensional plot of response time and wavelength.

- **Conductivity Detector:** Based on electrical conductivity, the response is recorded. The detector is used when the compounds have conducting ions like anions and cations.

- **Amperometric Detector:** This detector is used when compounds have functional groups which can be either oxidized or reduced. The diffusion current recorded is directly proportional to concentration of compound to be eluted.

INJECTORS

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where sample introduction is done with the help of auto samplers and microprocessors.

In liquid chromatography, liquid samples may be injected directly and solid samples need to be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference and loss in efficiency. It is always best to remove particles from the sample by filtering or centrifuging since continuous injections of particulate material will eventually cause blockage of injection devices or columns.

Sample sizes

Typical sample mass with 4.6 mm ID columns range from the nanogram level up to about 2 mg diluted in 20 ml of solvent. In general, it should be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds or both, may often be extracted, or eliminated, by selective detection.

STATISTICAL PARAMETERS

Linear regression

Once linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r' then the best straight line through the data points has to be estimated. This can be often done by visual inspection of graph but in many cases it is far more sensible to evaluate the best straight line by linear regression.

Equation of Straight line

$$y = mx + c$$

Where, 'y', the dependent variable was plotted as result of changing 'x', the independent variable.

Slope (m), at any point of the line is given by formula

$$m = (y_2 - y_1)/ (x_2 - x_1)$$

Correlation coefficient (r)

The correlation co-efficient is used as a measure of the correlation between two variables. When variables x and y are correlated rather than being functionally related. The person correlation co efficient is one of the most convenient method to calculate. This is given by

Correlation(r) = $[N\Sigma XY - (\Sigma X)(\Sigma Y) / \sqrt{([N\Sigma X^2 - (\Sigma X)^2][N\Sigma Y^2 - (\Sigma Y)^2])}]$

where,

N = Number of values or elements

X = First Score

Y = Second Score

ΣXY = Sum of the product of first and Second Scores

ΣX = Sum of First Scores

ΣY = Sum of Second Scores

ΣX^2 = Sum of square First Scores

ΣY^2 = Sum of square Second Scores

The maximum, value of r is 1. When this occurs, there is exact correlation between the two variables. When r is zero, there is complete independence of the variables. The minimum value of r is -1. A negative correlation co-efficient indicates that the assumed dependence is opposite to what exists and therefore a positive co-efficient for the revered relation. The fit must be poor when 'r' becomes smaller than 0.98 and very poor when less than 0.9.

Standard deviation

It is commonly used in statistics as a measure of precision and is more meaning full than is the average deviation. It may be thought of as a root mean square deviation of values from their average and is expressed mathematically as

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

Where

S = Standard deviation

If N is large (50 or more) then of course it of immaterial whether the term in the denomination is N - 1 or N.

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = Deviation of a value from the mean

N = Number of observations

Percentage relative standard deviation (% RSD)

It is also known as co efficient of variation. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\text{R.S.D (\%)} = \frac{\text{S.D}}{x} \times 100$$

Where

S.D = Standard deviation

Variance

The variance is defined as S^2 and is more important in statistics than S. However, the latter is much more commonly used in chemical data.

Standard error of mean (S.E)

The standard error of mean can be defined as the value obtained by the division of standard deviation by square root of number of observations. It is mathematically expressed as,

$$\text{S.E.} = \frac{\text{S.D}}{\sqrt{n}}$$

Where,

S.D = Standard deviation.

n = number of observations.

HPLC METHOD DEVELOPMENT (B.K.Sharma, Instrumental methods of chemical analysis, 2005)

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible and it should allow the use of sophisticated tools such as computer modelling. During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C_{18} column with UV detection.

SAMPLE AND ANALYTE INFORMATION

This information is useful for the selection of appropriate sample preparation procedures as well as the initial detection and chromatographic modes. If critical data are not available (e.g., pKa, solubility), separate studies should be initiated as soon as possible. The chemical structure of the analyte furnishes data on molecular weight and the nature of the functional groups. Particular attention should be directed to acidic, basic, aromatic, or reactive functional groups from which estimates of pKa, solubility, chromophoric, or stability data can be inferred.

SELECTION OF CHROMATOGRAPHIC MODE

Reversed-phase chromatography (RPC), the most common mode for small organic molecules. Note that ionisable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents. 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

SAMPLE PREPARATION

Samples occur in various forms

- Solutions ready for injection
- Solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
- Solids must be dissolved or extracted
- Samples that require pre-treatment to remove interferences and/or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and/or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvent is close to that of the mobile phase since this minimizes baseline upset and other problems.

Some samples require a partial separation (pre-treatment) prior to HPLC, because of need to remove interferences, concentrate sample analytes or eliminate "column killers". In many cases the development of an adequate sample pre-treatment can be challenging for achieving a good HPLC separation.

CHOICE OF THE COLUMN

Selection of the column is the first and the most important step in method development. Some of the important parameters to be considered while selecting chromatographic column

- 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

Table No: 1.4 COLUMN SELECTION FLOW CHART

Sample	Chromatographic mode	Column choice
Ionisable (cationic or anionic)	Reverse Phase-ion pair (allows neutral and charged compounds to be simultaneously analyzed)	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
	Ion suppression	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
	Anionic Ion Exchange	Strong Anion exchange
	Cationic Ion exchange	Strong Cation exchange

Sample	Chromatographic mode	Column choice
Neutral	Normal phase	Increasing polarity of bonded phases diol CN < NH_2 < Silica < Alumina.
	Reverse phase	Increasing polarity of bonding phase C-18 < C-8 < Phenyl < C-2 < TMS < CN

SELECTION OF SOLVENT DELIVERY SYSTEM

Chromatographic separation with isocratic elution i.e. all constituents of the mobile phase is mixed and pumped together as a single solvent, is always preferable however, gradient elution is powerful tool in achieving separation between closely eluting compounds or compounds having widely differing in polarities.

SELECTION OF MOBILE PHASE

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all 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 mobile phase-stationary phase. For a given

stationary phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile phase 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 following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer and its strength
- pH of the buffer or pH of the mobile phase
- Mobile phase composition

BUFFERS IF ANY AND ITS STRENGTH

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.

The retention also depends on the molar strengths of the buffer. Molar strength is inversely proportional to the retention times. Ideally the strength of the buffer shall be opted between 0.01M to 0.2M. After selecting the strength of the buffer, it can be varied by about 10 - 20% and the effect of variation was studied and it should be rugged for atlseast 2% variation in strength.

p^H of the mobile phase

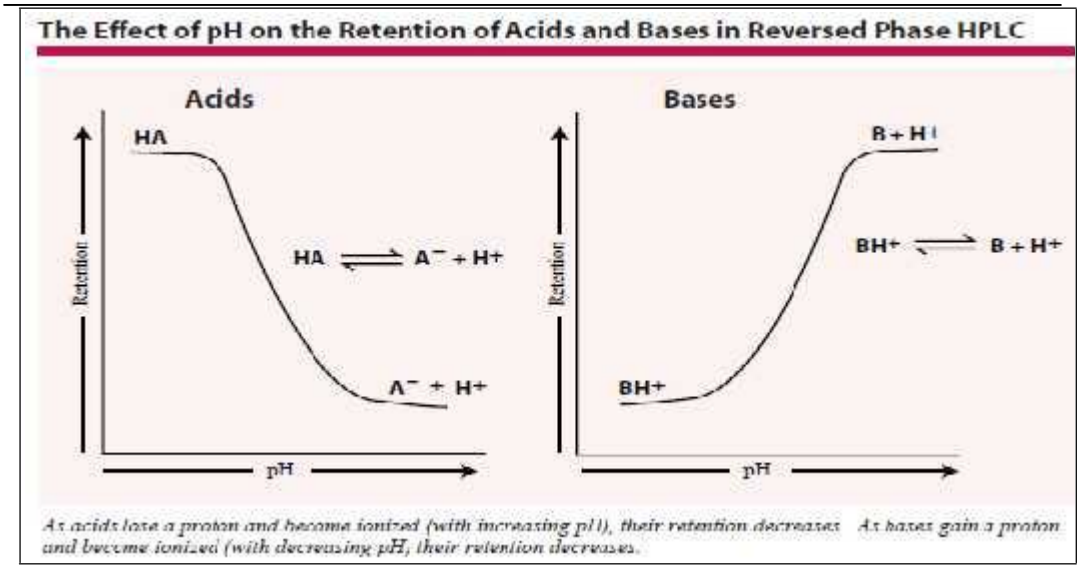
It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This

is due to the fact that the silioxane linkages are cleaved below pH 2.0, while pH values above 8.0 the silica may dissolve.

$p^H$of the buffer

p^H plays an important role as it controls the elution properties by controlling the ionization characteristics. In RP-HPLC the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. So, acid shows decrease in retention with increasing pH while base show increase in retention.

Figure no 1.2



SELECTION OF BUFFER

Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Almost all of the pH related change in retention occurs for pH values within ±1.5 units of pKa value. Out side this range the compound is either ionized or unionized, and its retention doesn't change much with pH.

The relationship between RP-HPLC retention and mobile phase pH is more complicated for compounds that contain multiple acidic and/or basic groups. Buffer

strength of 10-50 mM are generally adequate. The buffers showing UV absorbance below 220 nm were preferable.

Table No: 1.5 COMMONLY USED BUFFERS FOR RP-HPLC

Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	2.1 7.2 12.3	1.1 – 3.1 6.2 – 8.2 11.3 – 13.3	200
Formic acid*	3.8	2.8 – 4.8	210
Acetic acid*	4.8	3.8 – 5.8	210
Citrate	3.1 4.7 5.4	2.1 – 4.1 3.7 – 5.7 4.4 – 6.4	230
Tris	8.3	7.3 – 9.3	205
Triethylamine*	11.0	10.0 – 12.0	200
Pyrrolidine	11.3	10.3 – 12.3	200

* Volatile buffers

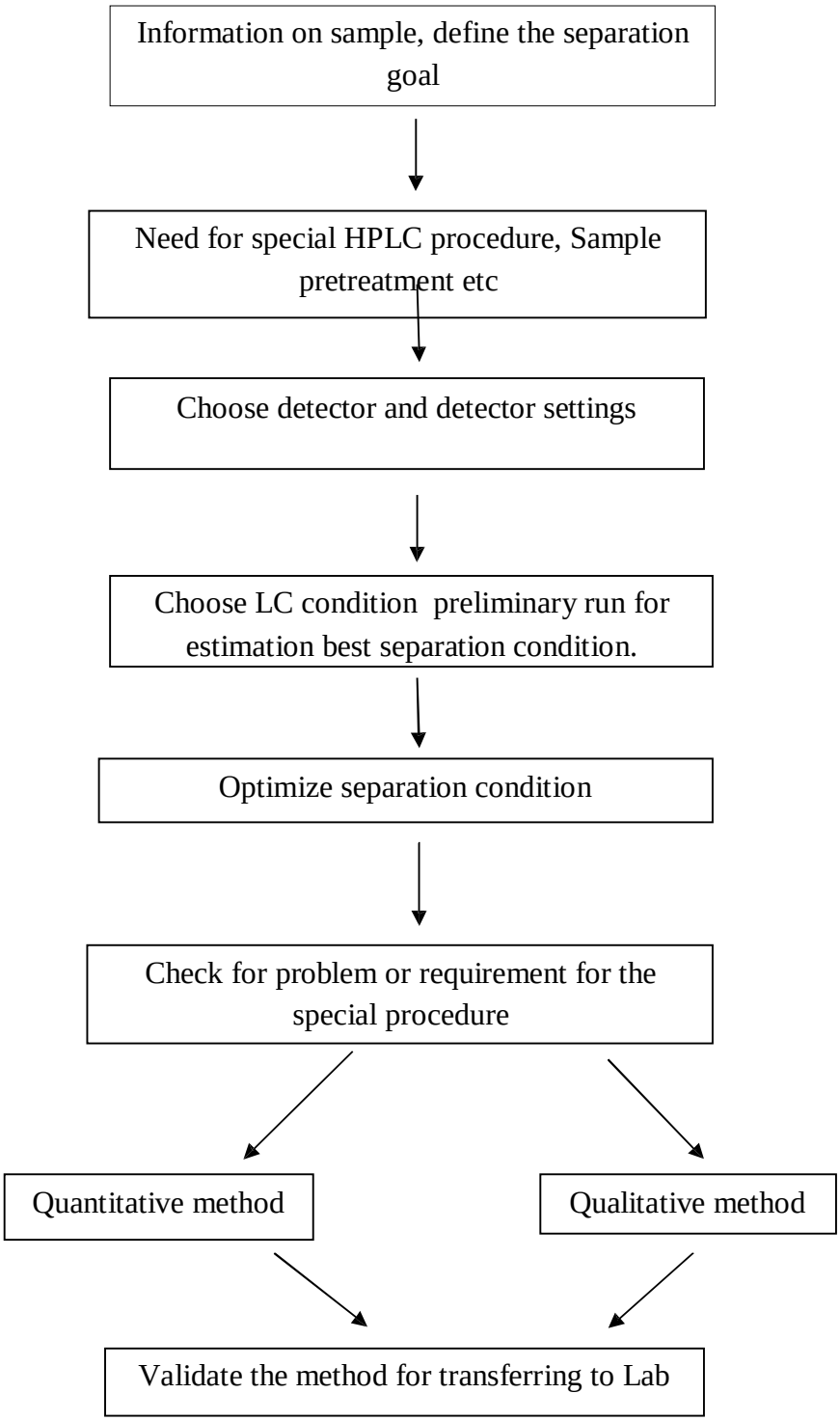
SELECTION OF FLOW RATE

Generally flow rate shall not be more than 2.0 ml/min. The flow rate shall be selected based on the following data.

- Retention time
- Column back pressure
- Resolution between the peaks
- Peak symmetries

The flow rate which gives least retention times, good peak symmetries, least back pressures and better separation will be selected.

Development of HPLC method



PERFORMANCECALCULATIONS

SYSTEM SUITABILITY PARAMETERS

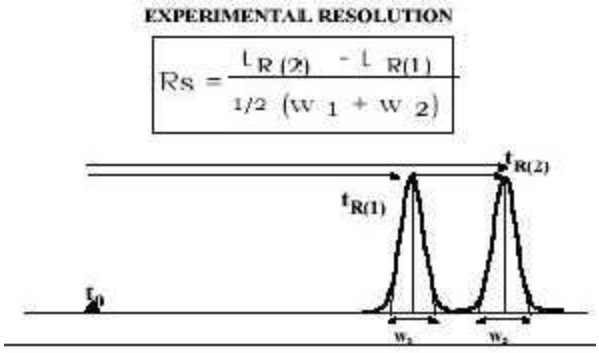
1) Retention time (t_R)
2) Resolution (R_S)
3) Capacity factor ($k`$)
4) Selectivity (α)
5) Number of Theoretical plates (N)
6) HETP
7) Asymmetry factor
8) Tailing factor

1) Retention time

Chromatographic retention is to measure the time between the injection point and maximum of the detector response for correspondent compound. This parameter called “retention time” is inversely proportional to the eluent flow rate.

2) Resolution

The goal of most HPLC analyses is the separation of one or more analytes in the sample from all other components present. Resolution (Rs) is a measure of the degree of separation of two adjacent analytes. Resolution of two adjacent band is defined as the distance between band peaks divided by the average band width. Retention and band width are measured in units of time.



Where,

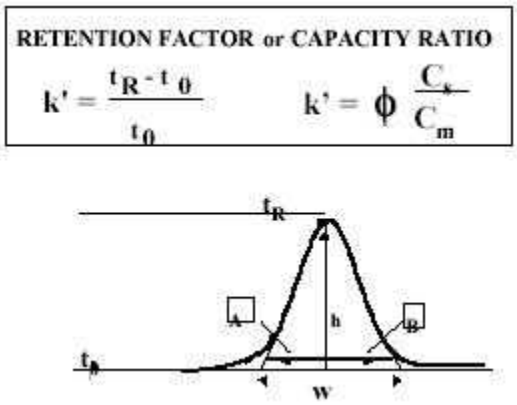
Rt_1 and Rt_2 are the retention times of components 1 and 2

W_1 and W_2 are peak widths of components 1 and 2.

$R_S \geq 2.0$ is a desirable target for method development.

3) Capacity factor (K')

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



The retention factor k is given by the equation

$$k = (t_R - t_0) / t_0$$

Where,

't_R' is the band retention time

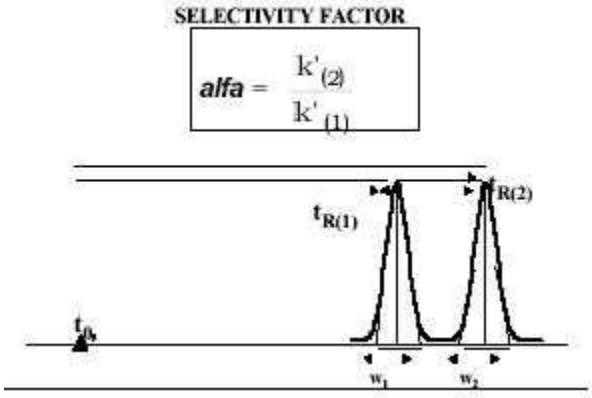
't_0' is the column dead time.

4) Selectivity (α)

The selectivity α is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the following formula.

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak, respectively.



COLUMN EFFICIENCY & PLATE NUMBER

The number of theoretical plates or plate number is a measure of column efficiency. An efficient column produces sharp peaks and can separate many sample components in a relatively short time. Theoretical plates (N) are defined as the square of the ratio of the retention time divided by the standard deviation of the peak (σ).

$$N = \left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{4t_R}{w_b}\right)^2 = 16\left(\frac{t_R}{w_b}\right)^2.$$

Another way to express efficiency of column is by calculating height equivalents of theoretical plates (HETP).

$$H = L/N$$

Where H = HETP; L = Length of the column; N = number of theoretical plates.

Lower the HETP, higher is the efficiency of the column, i.e., higher the theoretical plates more efficient the column.

7) Peak asymmetry

Peak asymmetry factor as can be used as a criterion of column performance. The peak half width, divided by the corresponding front half width, a gives the asymmetry factor.

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

Where,

'b', is the distance at 50% peak height between leading edge to the perpendicular drawn from the peak maxima

'a', is the width of the peak at half the peak height.

8) Tailing factor

The tailing factor T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

In some cases, values less than unity may be observed. As peak asymmetry increases, integration, hence precision becomes less reliable.

According to USP (2000) Peak-tailing factor can be calculated by using the formula

$$T = W_{0.05}/2\ f$$

Where,

'$W_{0.05}$' is the width of the peak at 5% height and

'f' is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 50% of the peak height from the base line.

Table No: 1.6 System Suitability Parameters specifications

S. No.	Parameter	Specifications
1	Capacity Factor (k')	o.5 < k < 20
2	Theoretical Plates	>2000
3	Tailing factor	<2
4	Resolution	>2
5	Selectivity	>2

ANALYTICAL METHOD VALIDATION

Method validation can be defined as per ICH as, "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".

SPECIFICITY/SELECTIVITY

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces distinguishable responses for a single analyse of number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective.

LINEARITY

Linearity of an analytical procedure is its ability (with in a given range) to obtain test results which are directly proportional the concentration (amount) of analyte in the sample. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be

evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.

RANGE

Range is the interval between the upper and lower concentration of the analyte in the sample for which it has a suitable level of precision, accuracy and linearity.

ACCURACY

Accuracy is the measure of the closeness of the experimental value to that of the true value. Accuracy should be established across the specified range of the analytical procedure.

A. Assay

1.1 Drug Substance

Several methods of determining accuracy are available

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material)

b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined.

c) Accuracy may be inferred once precision, linearity and specificity have been established.

1.2. Drug Product

Several methods for determining accuracy are available

a) Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analysed have been added.

b) In cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined.

c) Accuracy may be inferred once precision, linearity and specificity have been established.

2. Impurities (Quantitation)

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities. In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

PRECISION

Precision is the measure of how close the data values are to each other for a series of measurements under the same analytical conditions obtained from multiple sampling of the same homogeneous sample. Precision may be considered at three levels.

A. Repeatability

1. Injection Repeatability

The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC

instrument under the chromatographic conditions and day tested. The specification, as the coefficient of variation in % or relative standard deviation (RSD), set here will determine the variation limit of the analysis. The tighter the value, the more precise or sensitive to variation one can expect the results. This assumes that the chromatograph does not malfunction after the system suitability testing has been performed. The set of four duplicate samples were injected sequentially. Variations in peak area and drift of retention times are noted.

Recommendations

As part of methods validation, a minimum of 10 injections with an RSD of 2% is recommended. With the methods for release and stability studies, an RSD of 2% for precision of the system suitability tests for at least five injections (n=5) for the active drug either in drug substance or drug product is desirable. For low-level impurities, higher variations may be acceptable.

2. Analysis Repeatability

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

B. Intermediate precision

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

C. Reproducibility

As defined by ICH, reproducibility expresses the precision between laboratories as in collaborative studies. Multiple laboratories are desirable but not always attainable because of the size of the firm.

LIMIT OF DETECTION

Limit of detection is the lowest concentration of analyte in a sample which can be detected, but not necessarily quantitated, as an exact value under the stated experimental conditions.

A. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

B. Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

C. Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as

$$DL = \frac{3.3\ \sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

LIMIT OF QUANTIFICATION

Limit of quantification is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the

quantification limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

A. Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

B. Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

C. Based on the Standard Deviation of the Response and the Slope

The quantification limit (QL) may be expressed as

$$QL = \frac{10\,\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

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

RUGGEDNESS

Ruggedness is the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental

conditions, operators and materials. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

ROBUSTNESS

Robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness can be partly assured with good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study.

Examples of typical variations are

- Stability of analytical solutions
- Extraction time
- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate.
- Different columns (different lots and/or suppliers)

2. LITERATURE REVIEW

1. Nayak, Diptish Ku, Kumar, VankarKaushik, Patnaik, Arabinda, Vol.4, No.4, pp 1595-1600, Oct-Dec 2012 . "Simultaneous estimation of Rabeprazole sodium and diclofenac sodium by RP-HPLC method in combined dosage-form", International Journal of Pharm Tech Research,2(2):1488 . The solvent used is pH 6.8 phosphate buffer. Linearity range was 2-10µg/ml and 5-25 µg/ml for Rabeprazole sodium and Diclofenac sodium respectively. The methods were validated with respect to linearity, precision and accuracy.
2. P.Palanisamy, B.Jayakar, M.Kumar, R.Margret Chandra, B.S.Venkateshwralu Development Of A Rp Hplc Method For The Estimation Of Diclofenac Sodium, Vitamin B, Vitamin B6 and Vitamin B12 Insoft Gelatin Capsule Dosage Form. IJPTP, 2014, 5(3),1002-1013.The Diclofenac Sodium, the separation was carried out in Phenomenex ODS C 18 column (150 x 4.6mm; 5µm) using mobile phase consisting of mixture of 400mL potassium hydrogen phosphate (pH 3.0) and 600mL of Acetonitrile. The flow rate was 1.0mL/min and effluent was detected at 254nm. The retention time of Diclofenac sodium was 5.4 min. The percentage recovery was within the range between 100.00% and 101.86% for Diclofenac Sodium. The linearity range was found to be 20-400µg/mL (r2=1) for Diclofenac Sodium.
3. B.Gowramma*, S. Rajan, S. Muralidharan, S. N. Meyyanathan and B. Suresh. " A Validated RP-HPLC Method For Simultaneous Estimation Of Paracetamol And Diclofenac Potassium In Pharmaceutical Formulation" . Vol.2, No.1, pp 676-680, Jan-Mar 2010 . The method was carried out on a Phenomenex LUNA C18 (25 cm x 4.6 mm i.d., 5 µ) column with a mobile phase consisting of acetonitrile: sodium dihydrogen ortho phosphate (adjusted to pH 3.5 using orthophosphoric acid) in the ratio of 70:30 v/v at a flow rate of 1.0 mL/min. Detection was carried out at 278 nm. Aceclofenac was used as an internal standard. The retention times of paracetamol,

diclofenac potassium and aceclofenac were 5.9, 9.4 and 3.12 min, respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation and solution stability. The proposed method can be used for the estimation of these drugs in combined dosage forms.

4. Terasa Kubala, Baldev Gambhir & S. Ian Borst. A Specific Stability Indicating Hplc Method to Determine Diclofenac Sodium in Raw Materials and Pharmaceutical Solid Dosage Forms .Pages 749-757 | Published online: 20 Oct 2008 . This method is specific, accurate and stability indicating. The method employs a reverse-phase octylsilane (C18) column with a mobile phase composed of acetonitrile/methanol/pic B-6 (25:25:50) and detection at 229 nm. The method resolves six principal related compounds with quantitation in the range 0.3-1.5%. Assay recoveries by spiking commercial formulations with diclofenac sodium were 99.64 ± 1.30%. Drug content in several commercial formulations are reported. Accelerated stability tests were conducted on raw materials and drug products and 1-(2,6-dichlorophenyl)-2-indolin-2-one was identified for the first time as a degradation product in solid dosage forms which are stressed under humidity and heat.
5. Satish, A.,Patel.,Kalpesh, M., Prajapati, "Development and Validation of RP-HPLC method for simultaneous Estimation of Chlorozoxazone and Diclofenacsodium combination",pharma tutor -1715. The chromatographic separation was performed on ACE 5 C18 column (150 mm × 4.6 mm i.d., 5 µm particle size). Mobile phase consisted of a mixture of phosphate buffer (0.02 M KH2PO4, pH adjusted to 3 using orthophosphoric acid), acetonitrile and methanol (30: 30: 40, v/v/v) at a flow rate of 1.0 ml/min. The detection wavelength was set at 279 nm. The proposed method was validated for linearity, accuracy, precision, LOD and LOQ. The calibration curve was linear over the range of 2-50 µg/ml for Chlorzoxazone and 2-50 µg/ml for Diclofenac sodium. The retention times were 2.8 min for Chlorzoxazone and 6.3 min for Diclofenac sodium. The mean recoveries were 101.1 ± 0.47 and 100.8 ± 0.77 for Chlorzoxazone and Diclofenac sodium, respectively.

The method has been successfully applied to determine the content of both drugs from the synthetic mixture. Hence, the method can be easily adopted for quality control analysis of both drugs in mixture.

6. Venkata Raveendra Babu Vemula, Pankaj Kumar Sharma, Asian J Pharm Clin Res, Vol 6, Suppl 3, 2013, 186-189. RP-HPLC Method Development And Validation For Simultaneous Estimation Of Diclofenac And Tolperisone In Tablet Dosage Form. Chromatography was carried out isocratically at 30°C ± 0.5°C on an XDB C-18 column (4.6 x 150mm, 5µ particle size) with a mobile phase composed of acetonitrile -phosphate buffer pH-3.4 (30:70% v/v) at a flow rate of 1.0 mL/min. Detection was carried out using a PDA detector at 260 nm.
7. Modi, M.V., M.M. Patel, Patel, C.N., Bharadia, P.D., "Development and validation of new RP-HPLC method for the estimation of Diclofenac Sodium and Famotidine in bulk and pharmaceutical dosage form", Inventi impact: pharm Analysis and Quality Assurance, 2011,11:154. Chromatography was performed on a Varian C18 column (250 mm x 4.6 mm i.d., 5 µm particle size), column with mobile phase containing acetonitrile,methanol and water in the ratio of 25:30:45 v/v.The flow rate was 1.0 ml/min and the eluent was monitored at 283 nm. The selected chromatographic conditions were found to effectively separate Diclofenac sodium (RT- 2.432 min) and Famotidine (RT- 5.082 min). Linearity for Diclofenac sodium and Famotidine were found in the range of 5-35µg/ml and 2-14µg/ml. The values obtained of LODs were 0.036µg/ml and 0.028µg/ml, LOQs were 0.11µg/ml and 0.088µg/ml for Diclofenac sodium and Famotidine, respectively. The proposed method was found to be fast, accurate, precise, reproducible and rugged and can be used for simultaneous analysis of Diclofenac sodium and Famotidine in combined pharmaceutical formulations.

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3. OBJECTIVE AND PLAN OFWORK

The drug analysis plays an important role in all aspects regarding the drug right the development to the therapeutic use of the drug. Industries manufacturing pharmaceutical must ensure that the raw material used and the final product obtained meets the required specification to fulfill this purpose they rely upon quantitative chemical analysis.

The Literature survey indicates that a very few methods were developed for the estimation of Diclofenac Sodium by RP-HPLC. So an attempt was made to develop a new RP-HPLC method which is more reliable, economical and flexible.

The objective of the present work is to develop a new method of estimation for Diclofenac Sodium in all formulations. So an attempt was made to develop and validate a simple, precise, accurate, linear and rapid RP-HPLC method as per ICH guidelines for the estimation of Diclofenac Sodium in pure pharmaceutical dosage forms and to apply the developed method to determine the validation of compounds.

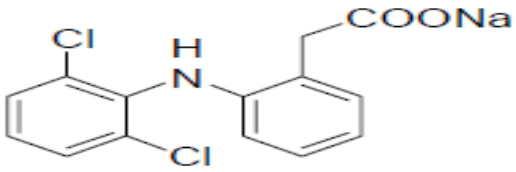
Plan of Work

- To obtain thorough knowledge of Practical RP-HPLC method.
- To establish the initial chromatographic conditions for method development of assay for Diclofenac Sodium .
- To validate the developed method a per the Q2 specifications of ICH guidelines

4. DRUG PROFILE

Name : Diclofenac Sodium

Chemical structure



Chemical Formula : $C_{14}H_{10}Cl_2NNaO_2$

IUPAC name : Sodium 2-[(2,6-dichlorophenyl)-amino] phenylacetate.

Molecular weight : 318.1

Description : White to off-white, hygroscopic, crystalline powder.

Solubility : Freely soluble in methanol; soluble in ethanol; sparingly soluble in water; practically insoluble in chloroform and in ether.

Melting point : About 280ºC

Drug category : Cyclo-oxygenase inhibitor; analgesic; anti-inflammatory

Mechanism of action

The primary mechanism responsible for its anti-inflammatory, antipyretic, and analgesic action is thought to be inhibition of prostaglandin synthesis by inhibition of cyclooxygenase (COX). It also appears to exhibit bacteriostatic activity by inhibiting bacterial DNA synthesis.

Inhibition of COX also decreases prostaglandins in the epithelium of the

stomach, making it more sensitive to corrosion by gastric acid. This is also the main side effect of diclofenac. Diclofenac has a low to moderate preference to block the COX2-isoenzyme (approximately 10-fold) and is said to have, therefore, a somewhat lower incidence of gastrointestinal complaints than noted with indomethacin and aspirin.

Pharmacokinetics

Bioavailability	:	Approximately 50% bioavailability orally.
Metabolism	:	Hepatic, oxidative, primarily by CYP2C9, also by CYP2C8, CYP3A4, as well as conjugative by glucuronidation (UGT2B7)and sulfation; no active metabolites exist
Half life	:	1.2-2.0 hours (35% of the drug enters enterohepatic recirculation)
Excretion	:	40% biliary 60% urine
Dosage form	:	Capsule
Route	:	oral

Medical uses

Diclofenac is used to treat pain, inflammatory disorders, and dysmenorrhea

Adverse effects

Indigestion, gas, stomach pain, nausea, vomiting; diarrhea, constipation; headache, dizziness, drowsiness; stuffy nose; itching, increased sweating; increased blood pressure; or. swelling or pain in your arms or legs.

5. MATERIALS AND INSTRUMENTS USED

Instrumentation

a) **System**: HPLC Aglilent -1260 series
b) Detector: PDA
c) Injector: Autosampler
d) Column: C18, 15 cm X 4.6 mm, 5 µm
e) Henna p^H meter
f) Gelman sicence vaccum pump
g) Ultra sonicator
h) Millipore – solvent filtration unit
i) Shimadzu electronic balance

The HPLC system used comprised of degasser, quarternary pump, auto sampler, thermostated column compartment, photo diode array detector.

Chemicals and Reagents used

- Milli Q Water: HPLC grade, supplied by Thomas baker chemicals Ltd., Mumbai, India.
- Potassium dihydrogen orthophosphate: AR grade, Supplied by Qualigens Fine Chemicals Ltd., Mumbai, India.
- Acetonitrile: HPLC grade, Supplied by Spectrochem, Mumbai, India.
- Orthophosphoric acid: AR grade, Supplied by Qualigens Fine Chemicals Ltd., Mumbai, India.

Information regarding Drug sample

Dosage form of sample : Soft gelatin Capsule

No. of Drugs in combination : 1

Label claim

Diclofenac Sodium BP : 50 mg

Solubility : Freely soluble in methanol & Acetonitrile; soluble in ethanol; sparingly soluble in water; practically insoluble in chloroform and in ether.

Manufactured by : Caplin point laboratories ltd

6. METHOD DEVELOPMENT AND OPTIMIZATION

SOLUBILITY

According to literature review collected Diclofenac Sodium are freely soluble in methanol & Acetonitrile; soluble in ethanol; sparingly soluble in water; practically insoluble in chloroform and in ether. The solubility of there drugs in Acetonitrile and water was checked. Finally, The mixture of Acetonitrile and Buffer (6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid.) in the ratio of 60:40 v/v was chosen for present work.

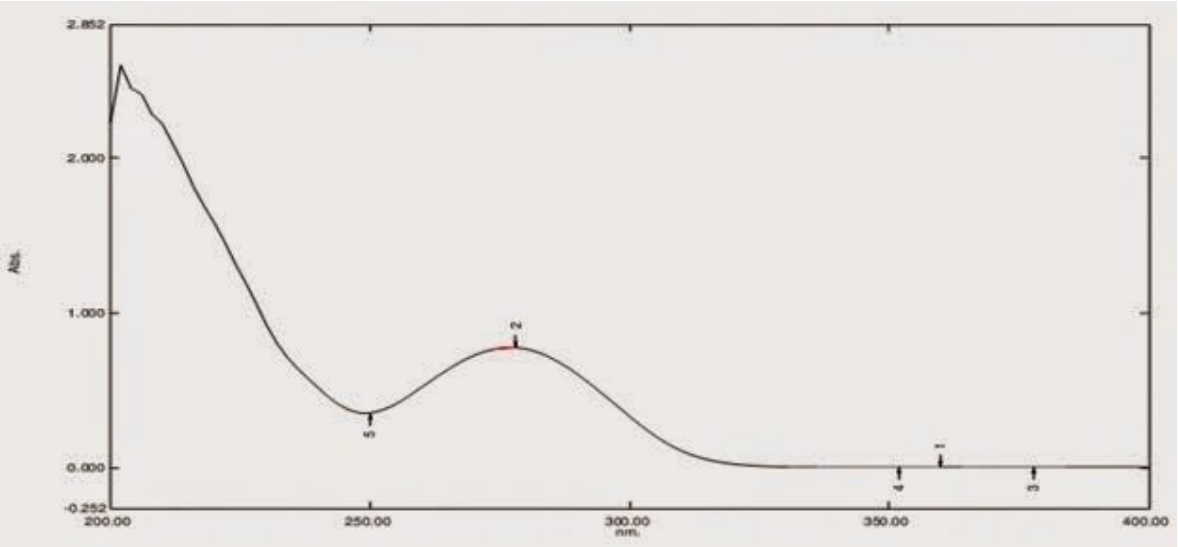
SELECTION OF CHROMATOGRAPHIC METHOD FOR SEPARATION

Selection of the method depends upon the nature of sample (ionic/ionisable/neutral), its molecular weight and solubility. Most of the drugs are polar in nature and hence reversed phase HPLC is preferred over the normal phase HPLC method. The drug combinations concerned for the present study are also polar. Hence reversed phase HPLC was selected for separation of the drug combination because of its suitability.

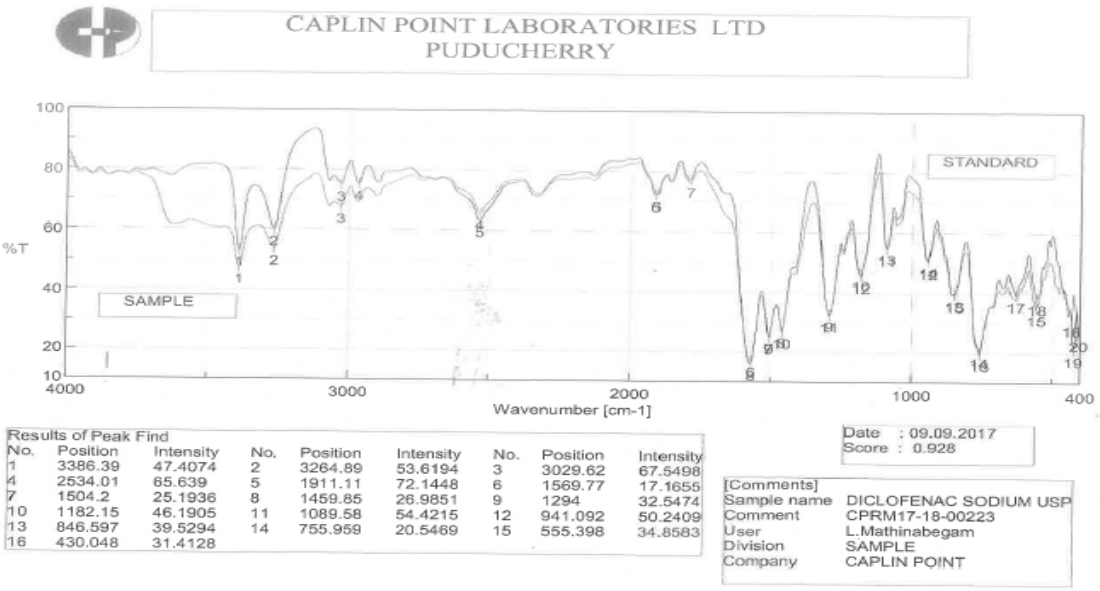
SELECTION OF DETECTION OF WAVELENGTH (λ_{max})

Selection of detection of wavelength is a critical step in the analytical method. The spectrum of diluted solutions was scanned over the range of 200 – 400 nm in spectrum mode.

UV SPECTRA OF DICLOFENAC SODIUM



Identification by IR Spectroscopy



Results of Peak Find

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3386.39	47.4074	2	3264.89	53.6194	3	3029.62	67.5498
4	2534.01	65.639	5	1911.11	72.1448	6	1569.77	17.1655
7	1504.2	25.1936	8	1459.85	26.9851	9	1294	32.5474
10	1182.15	46.1905	11	1089.58	54.4215	12	941.092	50.2409
13	846.597	39.5294	14	755.959	20.5469	15	555.398	34.8583
16	430.048	31.4128						

Date : 09.09.2017
Score : 0.928

[Comments]

Sample name	DICLOFENAC SODIUM USP
Comment	CPRM17-18-00223
User	L.Mathinabegam
Division	SAMPLE
Company	CAPLIN POINT

OPTIMIZED CHROMATOGRAPHIC CONDITIONS FOR ASSAY

Column	: Inertsil ODS-3 C18, 25 cm x 4.6 mm, 5 µm
Flow Rate	: 1.0 mL/minute
Pump mode	: Isocratic
Detector wavelength	: 254 nm
Injection volume	: 20 µL
Column Temperature	: 25.0˚C

Mobile phase composition: Acetonitrile and Buffer (6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid) in the ratio of 60:40 v/v.

Diluent: Mobile phase

Prepare a mixture Acetonitrile and Buffer (6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid.) in the ratio of 60:40 v/v. , mix well. Filter the solution through 0.45 µm nylon filter and sonicate for 10 minutes

Methodology adopted

- Mobile phase selection
- Preparation of buffer solution
- Preparation of mobile phase
- Preparation of diluent
- Preparation of standard stock solution
- Preparation of standard solution
- Preparation of sample solution
- Setting the instrumental parameters before performing the analysis for
    1. Detector
    2. Pump

- Development of chromatogram and determination of retention time.

PREPARATION OF SOLUTIONS

Preparation of buffer solution

Weigh and dissolve 6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid.

Selection of mobile phase

The mobile phase system consisting of Acetonitrile and Buffer (6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid.) in the ratio of 60:40 v/v was found to be effective in the separation of Diclofenac Sodium in pure form as well as in dosage forms.

Preparation of Mobile Phase

Prepare a mixture 400 volume of Buffer and 600 volume of Acetonitrile, mix well. Filter the solution through 0.45 µm nylon filter and sonicate for 10 minutes.

Preparation of Diluent

Prepare a mixture 400 volume of Buffer (6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid.) and 600 volume of Acetonitrile, mix well. Filter the solution through 0.45 µm nylon filter and sonicate for 10 minutes.

[Note: Perform the tests in the dark or under golden fluorescent or other low-actinic light and use low actinic glassware's in the performance of the following procedure.]

Procedure for preparation of analytical solution:

Preparation of standard solution (50.0 mcg/mL of Diclofenac Sodium):

Weigh accurately about 50 mg of Diclofenac Sodium working standard and transfer into 50 mL volumetric flask, add 30 mL of diluent and sonicate for 5 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Transfer 5 mL of the above solution through pipette into 100 mL volumetric flask and dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

Preparation of sample solution (50.0 mcg/mL of Diclofenac Sodium):

Accurately weigh and transfer 5 capsules (equivalent to 250 mg of Diclofenac Sodium) into 250 mL volumetric flask. Add about 25 mL of water and sonicate for 20 minutes to disperse the capsules shell. Add about 120 mL of diluent and sonicate for 20 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Transfer 5 mL of the above solution through pipette into 100 mL volumetric flask and dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

7. METHOD VALIDATION

Validation of analytical method is a process to establish that the performance characteristics of the developed method meet the requirement of the intended analytical application. (Lloyd R. Synder, 1997) (ICH Harmonized Tripartite Guidelines, 2005)

SYSTEM SUITABILITY PARAMETERS

System suitability is the test to ensure that the methods can generate results of acceptable accuracy and precision. (Lloyd R. Synder, 1997)

Standard solutions of Diclofenac Sodium were prepared as per test method and five replicate injections were made to study system suitability parameters.

Parameters studied

Tailing Factor.

Number of theoretical plates (N).

Retention time.

Relative standard deviation .

Typical analytical parameters used in method validation include

1. Specificity
2. Linearity and Range
3. Precision
4. Accuracy
5. Ruggedness
6. Limit of detection
7. Limit of quantitation
8. Selectivity

SPECIFICITY

The specificity of the method can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials. (Lloyd R. Synder, 1997) (ICH Harmonized Tripartite Guidelines, 2005)

Procedure:

Inject the blank, placebo, standard and sample preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Injection sequence

Table No:7.1

Particulars	Number of Injection
Blank	1
Placebo preparation	1
Standard preparation	5
Sample preparation	2
Bracketing standard	1

Acceptance criteria:

- No any peak should be obtained in the retention time of Diclofenac Sodium from the blank and placebo chromatograms.

System suitability results:

Table No:7.2

S.No.	Parameter	Results obtained	Acceptance criteria
01	Tailing factor	1.30	**NMT 2.0**
02	Theoretical Plates	8494	**NLT 2000**
03	% RSD	0.068	**NMT 2.0 %**

Specificity Results:

Table No:7.3

S.No.	Name of the solution	Results obtained	Acceptance criteria
01	Blank	No peak found in the retention time of Diclofenac Sodium	No any peak should be found in the retention time of Diclofenac Sodium
02	Placebo	No peak found in the retention time of Diclofenac Sodium	No any peak should be found in the retention time of Diclofenac Sodium

LINEARITY AND RANGE

Linearity is the measure of how well a calibration plot of response Vs concentration approximates a straight line. Linearity can be assessed by performing the single measurement s at several analyte concentrations. The data are then processed using a linear least square regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity. (Lloyd R. Synder, 1997). (ICH Harmonized Tripartite Guidelines, 2005).

Ability to obtain test results which are directly proportional to the concentration of analyte. For an establishment of the linearity 60%, 80%, 100%, 120%, and 160% of standard and sample concentrations shall be used.

Preparation of Linearity from standard:

Standard stock solution (1000 mcg/mL of Diclofenac Sodium):

Weigh accurately about 50 mg of Diclofenac Sodium working standard and transfer into 50 mL volumetric flask, add 30 mL of diluent and sonicate for 5 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well.

60 % Linearity standard concentration (30.0 mcg/mL of Diclofenac Sodium):

Transfer 3.0 mL of above standard stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

80 % Linearity standard concentration (40.0 mcg/mL of Diclofenac Sodium):

Transfer 4.0 mL of above standard stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

100 % Linearity standard concentration (50.0 mcg/mL of Diclofenac Sodium):

Transfer 5.0 mL of above standard stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

120 % Linearity standard concentration (60.0 mcg/mL of Diclofenac Sodium):

Transfer 6.0 mL of above standard stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution

through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

160 % Linearity standard concentration (80.0 mcg/mL of Diclofenac Sodium):

Transfer 8.0 mL of above standard stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

Preparation of Linearity solution from Sample:

Sample stock solution (1000 mcg/mL of Diclofenac Sodium):

Accurately weigh and transfer 5 capsules (equivalent to 250 mg of Diclofenac Sodium) into 250 mL volumetric flask. Add about 25 mL of water and sonicate for 20 minutes to disperse the capsules shell. Add about 120 mL of diluent and sonicate for 20 minutes to dissolve. Cool and dilute up to the volume with diluent.

60 % Linearity sample concentration (30.0 mcg/mL of Diclofenac Sodium):

Transfer 3.0 mL of above sample stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

80 % Linearity sample concentration (40.0 mcg/mL of Diclofenac Sodium):

Transfer 4.0 mL of above sample stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

100 % Linearity sample concentration (50.0 mcg/mL of Diclofenac Sodium):

Transfer 5.0 mL of above sample stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution

through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

120 % Linearity sample concentration (60 mcg/mL of Diclofenac Sodium):

Transfer 6.0 mL of above sample stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

160 % Linearity sample concentration (80.0 mcg/mL of Diclofenac Sodium):

Transfer 8.0 mL of above sample stock solution through pipette into 100 mL volumetric flask. Dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

Injection sequence:

Table No:7.4

Different Linearity Standard and Sample preparations	Number of Injection
Blank	1
Standard preparation	5
60 % Linearity standard and sample concentration	2
80 % Linearity standard and sample concentration	2
100 % Linearity standard and sample concentration	2
120 % Linearity standard and sample	2

concentration	
160 % Linearity standard and sample concentration	2
Bracketing Standard	1

Acceptance criteria:

- Correlation co-efficient between concentrations and its area should be more than 0.998.
- Report the Regression co-efficient.
- Report the residual sum of squares.
- Report the intercept.
- Report the slope of the regression line.
- Report the linearity graph.

LINEARITY REPORT:

The linearity response of Diclofenac Sodium was determined across the range are given in the following tables. The result shown in the table and its graphical

Table No:7.5

S.No.	Parameter	Results obtained	Acceptance criteria
01	Tailing factor	1.30	**NMT 2.0**
02	Theoretical Plates	8203	**NLT 2000**
03	%RSD	0.082	**NMT 2.0 %**

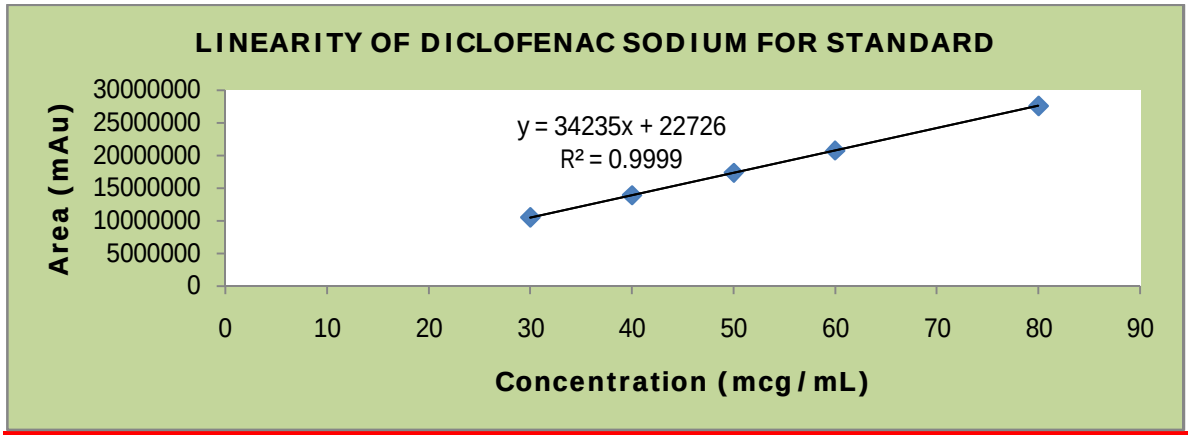
Linearity data obtained from 60 % to 160 % for Standard:

Table No:7.6

Linearity level concentration in %	Concentration in (mcg/mL)	Standard area -1	Standard area-2	Average area
60	30	10510134	10517466	10513800
80	40	13888541	13913995	13901268
100	50	17375825	17338125	17356975
120	60	20736432	20764499	20750466
160	80	27622578	27628150	27625364

.Linearity graph obtained for Standard:

Table No:7.7



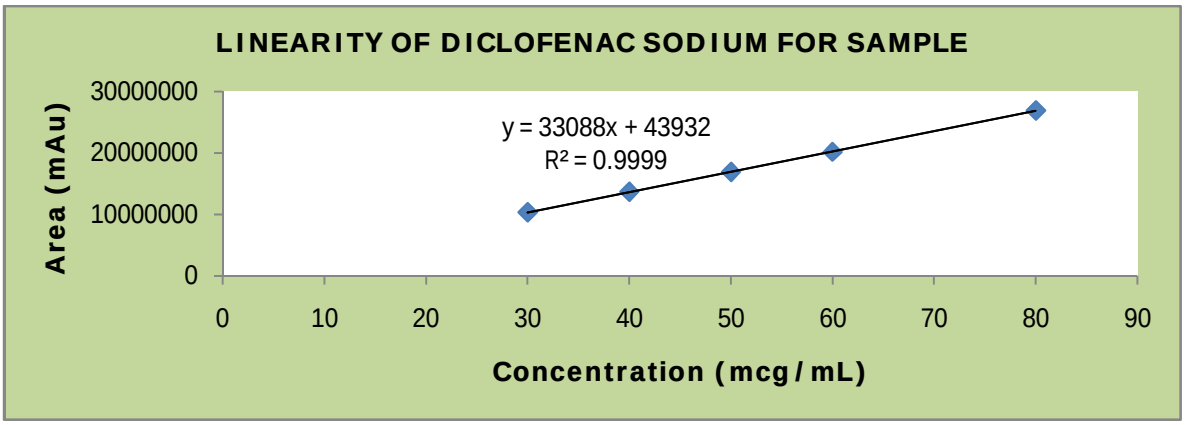
Linearity data obtained from 60% to 160% for Sample:

Table No:7.8

Linearity level concentration in %	Concentration in (mcg/mL)	Sample area -1	Sample area-2	Average area
60	30	10380500	10389220	10384860
80	40	13779199	13647543	13713371
100	50	16938975	16959321	16949148
120	60	20221767	20214573	20218170
160	80	26979686	26940612	26960149

Linearity graph obtained for Sample:

Table No:7.9



Linearity results for Standard and Sample:

Table No:7.10

S. No.	Parameter	Results obtained		Acceptance criteria
		Standard	Sample	
01	Correlation coefficient	0.99999	0.99997	**NLT 0.998**
02	Regression coefficient	0.99999	0.99993	**Informative**
03	Residual sum of squares	1224830679.61	11055132717.03	**Informative**
04	Intercept	227263.8243	439328.1622	**Informative**
05	Slope of regression line	342352.1284	330880.9892	**Informative**

PRECISION

(ICH Harmonized Tripartite Guidelines 2005) Precision is the measure of the repeatability of result.

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements under the prescribed conditions.

SYSTEM PRECISION:

Determines the closeness of agreement of the same homogenous standard preparations under the prescribe conditions.

Procedure:

Inject the blank, standard preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Injection sequence:

Table No:7.11

Particulars	Number of Injection
Blank	1
Standard preparation	6

Acceptance criteria:

- The Tailing factor of the peak due to Diclofenac Sodium obtained from five replicates standard solution injections should be not more than 2.0.
- Theoretical plates of the peak obtained for five replicates standard solution injections of Diclofenac Sodium should be not less than 2000.
- The relative standard deviation of the area obtained for Five replicate standard solution of Diclofenac Sodium should be not more than 2.0%.
- The relative standard deviation of the retention time obtained for five replicate standard solution of Diclofenac Sodium should be not more than 1.0%.

SYSTEM PRECISION REPORT:

System suitability results:

Table No:7.12

S.No.	Parameter	Results obtained	Acceptance criteria
01	Tailing factor	1.30	**NMT 2.0**
02	Theoretical Plates	8483	**NLT 2000**
03	% RSD of area for five standard injections	0.055	**NMT 2.0 %**
04	% RSD of retention time for five standard injections	0.03	**NMT 1.0 %**

System precision Result in standard solution:

Table No:7.13

S. No.	Retention time	Standard area	Tailing factor	Theoretical plates
01	6.827	16847470	1.31	8472
02	6.830	16824096	1.30	8472
03	6.830	16846315	1.29	8476
04	6.830	16835165	1.29	8485
05	6.830	16846787	1.30	8498
06	6.833	16836738	1.29	8492
Mean	**6.830**	**16839429**	**1.30**	**8483**
Std dev	**0.002**	**9238.39**	**NMT 2.0**	**NLT 2000**
(RSD	**0.03**	**0.055**		
Limit	**NMT 1.0 %**	**NMT 2.0 %**		

METHOD PRECISION (REPEATABILITY):

Determines the closeness of agreement of the same homogenous sample under the prescribe conditions. Performing assay of Diclofenac Sodium Soft gelatin Capsules 50 mg a minimum of six sample preparations from a single batch shall be made and analyze separately.

Procedure:

Inject the blank, standard and sample preparations based on “Injection sequence” detailed below and measure the corresponding area.

Injection sequence:

Table No:7.14

Particulars	Number of Injection
Blank	1
Standard preparation	6
Sample preparations – 1,2,3,4,5,6	2
Bracketing standard	1

Acceptance criteria:

- Assay obtained for each six sample preparations should be between 90.0 and 110%
- The RSD obtained for the assay results for six sample preparations should be not more than 2.0 %.

METHOD PRECISION (REPEATABILITY) REPORT:

The Method precision was determined by preparing six sample solutions of Diclofenac Sodium from Diclofenac Sodium Soft gelatin Capsules 50 mg as per the procedure included in the protocol.

System suitability Result in standard solution:

Table No:7.15

S.No.	Parameter	Results obtained	Acceptance criteria
01	Tailing factor	1.29	**NMT 2.0**
02	Theoretical Plates	8472	**NLT 2000**
03	%RSD	0.357	**NMT 2.0 %**

Method Precision results:

Table No:7.16

No. of Sample preparation	Samples Area			Results obtained % of drug	Acceptance criteria
	Sample -1	**Sample -2**	**Average**	**% of drug**	
01	16614927	16856159	16735543	**99.56**	**90.0% - 110.0%**
02	16576480	16608884	16592682	**98.86**	
03	16594408	16582746	16588577	**98.74**	
04	17001470	17012613	17007042	**101.24**	
05	17041613	17046783	17044198	**101.40**	
06	16408934	16414578	16411756	**97.80**	
			Mean	**99.60**	
			Std. Dev	**1.446**	
			RSD	**1.452**	**NMT 2.0 %**

ACCURACY (ICH Harmonized Tripartite Guidelines, 2005)

Accuracy is defined as closeness of measured value to the true value

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Preparation of analytical solution:

Preparation of standard solution (50.0 mcg/mL of Diclofenac Sodium):

Weigh accurately about 50 mg of Diclofenac Sodium working standard and transfer into 50 mL volumetric flask, add 30 mL of diluent and sonicate for 5 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Transfer 5 mL of the above solution through pipette into 100 mL volumetric flask and dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate.

Placebo spiked with 50 % standard preparation (25.0 mcg/mL of Diclofenac Sodium):

Weigh accurately 5 Diclofenac Sodium Placebo capsules and 125 mg of Diclofenac Sodium Working standard and transfer into 250 mL volumetric flask. Add about 25 mL of water and sonicate for 20 minutes to disperse the capsules shell. Add about 120 mL of diluent and sonicate for 20 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Transfer 5 mL of the above solution through pipette into 100 mL volumetric flask and dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 100 % standard preparation (50.0 mcg/mL of Diclofenac Sodium):

Weigh accurately 5 Diclofenac Sodium Placebo capsules and 250 mg of Diclofenac Sodium Working standard and transfer into 250 mL volumetric flask. Add about 25

mL of water and sonicate for 20 minutes to disperse the capsules shell. Add about 120 mL of diluent and sonicate for 20 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Transfer 5 mL of the above solution through pipette into 100 mL volumetric flask and dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 150 % standard preparation (75.0 mcg/mL of Diclofenac Sodium):

Weigh accurately 5 Diclofenac Sodium Placebo capsules and 375 mg of Diclofenac Sodium Working standard and transfer into 250 mL volumetric flask. Add about 25 mL of water and sonicate for 20 minutes to disperse the capsules shell. Add about 120 mL of diluent and sonicate for 20 minutes to dissolve. Cool and dilute up to the volume with diluent and mix well. Transfer 5 mL of the above solution through pipette into 100 mL volumetric flask and dilute up to the volume with diluent. Mix and filter the solution through 0.45 µm nylon filter and collect the solution in HPLC vial after discarding about first 2 mL of the filtrate. Prepare the sample preparation in triplicate.

Procedure:

Inject the blank, standard and placebo spiked standard preparations based on **"Injection sequence"** detailed below and measure the corresponding area.

Injection sequence Table:

Table No:7.17

Particulars	**No. of injections**
Blank	1
Standard preparation	6
Placebo spiked standard solution 50% - preparations 1, 2 & 3	Each 3

Placebo spiked standard solution 100% - preparations 1, 2 & 3	Each 3
Placebo spiked standard solution 150% - preparations 1, 2 & 3	Each 3
Bracketing Standard	1

Calculations for Diclofenac Sodium accuracy

Step 1: Standard added in mg (Working standard solution)

$$= \frac{\text{Standard wt in mg}}{50} \times \frac{5}{100}$$

Step 2: Placebo spiked standard added in mg at 100%

$$= \frac{\text{Wt of spiked standard in mg}}{250} \times \frac{5}{100}$$

Step 3: Placebo spiked standard recovered in mg

$$= \frac{\text{Placebo spiked standard area X standard added in mg (Working standard solution)}}{\text{Average area of working standard solution}}$$

Step 4: Recovery in percentage

Placebo spiked standard recovered in mg

=---------- X 100

Placebo spiked standard added in mg

Acceptance criteria:

In each concentration, the Diclofenac Sodium working standard spiked with placebo should be recovered between 98.0 % and 102.0 %.

ACCURACY REPORT:

Diclofenac Sodium Working standard was spiked with the proposed weight of placebo between said ranges and studied for its recovery. This study was performed as per the procedure included in the protocol.

System suitability Results:

Table No:7.18

S.No.	Parameter	Results obtained	Acceptance criteria
01	Tailing factor	1.28	**NMT 2.0**
02	Theoretical Plates	8168	**NLT 2000**
03	%RSD	0.065	**NMT 2.0 %**

Accuracy Results:

Table No:7.19

Accuracy Level in %	Standard added in mg	Standard recovered in mg	% Recovered	Acceptance criteria
50	0.024926	0.024852	99.70	**98.0 % - 102.0 %**
	0.024900	0.024952	100.21	
	0.024982	0.024782	99.20	
100	0.049798	0.049287	98.97	
	0.049822	0.049370	99.09	
	0.049766	0.049355	99.17	
150	0.074670	0.075058	100.52	
	0.074724	0.074829	100.14	
	0.074728	0.073916	98.91	

RUGGEDNESS (ICH Harmonized Tripartite Guidelines, 2005)

Defined by USP, The Ruggedness is the degree of reproducibility of test results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory and from analyst to analyst.

Procedure

Working standard solutions and working sample solution were prepared by different analyst on different days. Solutions were injected as per the test method and chromatograms were recorded.

Inject the blank, standard and sample solution preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Injection sequence:

Table No:7.20

Particulars	Number of Injection
Blank	1
Standard preparation	5
Sample preparations – 1, 2, 3 (Analyst 1 and 2)	Each 2
Bracketing Standard	1

Acceptance criteria:

- Assay in % obtained for each six sample preparations should be between 90.0 and 110 %
- The RSD obtained for the assay results for six sample preparations should be not more than 2.0 %.

INTERMEDIATE PRECISION-(RUGGEDNESS) REPORT:

The Intermediate precision was determined by preparing six sample solutions of Diclofenac Sodium Soft gelatin Capsules 50 mg by a different analyst.

System suitability results:

Table No:7.21

S.No.	Parameter	Results obtained	Acceptance criteria
01	Tailing factor for	0.97	**NMT 2.0**
02	Theoretical Plates	3613	**NLT 2000**
03	%RSD	0.365	**NMT 2.0%**

- **Intermediate Precision Results:**

Table No:7.22

Analyst-01

No. of Sample preparation	Samples Area			Results obtained	Acceptance criteria
	Sample -1	**Sample -2**	**Average**	**Percentage of drug(%)**	
01	136605033	136576585	136590809	99.46	**90.0% - 110.0%**
02	135078128	135292523	135185326	98.36	
03	137355003	139342029	138348516	100.68	
				99.5	
				1.16	
				1.17	**NMT 2.0 %**

Table No:7.23

Analyst-02

No. of Sample preparation	Samples Area			Results obtained	Acceptance criteria
	Sample -1	**Sample -2**	**Average**	**Percentage of drug(%)**	
04	135391567	135866633	135629100	98.82	**90.0% - 110.0%**
05	133686975	133716375	133701675	97.4	
06	134637569	134608473	134623021	98	
				98.07	
				0.71	
				0.73	**NMT 2.0 %**

ROBUSTNESS (ICH Harmonized Tripartite Guidelines, 2005)

Robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variation in method parameters. It provides information about the reliability of method.

Determination

The robustness of an analytical method is determined by analysis of aliquots of homogenous lots by differing physical parameters like flow rate and column temperature.

Change in flow rate plus (1.2 mL/minute):

For Chromatographic conditions, follow the method of analysis except by changing the flow to 1.2 mL / minute instead of 1.0 mL / minute.

Change in flow rate minus (0.8 mL/minute):

For Chromatographic conditions, follow the method of analysis except by changing the flow to 0.8 mL / minute instead of 1.0 mL / minute.

Change in wavelength plus (256 nm):

For Chromatographic conditions, follow the method of analysis except by changing the wavelength to 256 nm instead of 254 nm.

Change in wavelength minus (252 nm):

For Chromatographic conditions, follow the method of analysis except by changing the wavelength to 252 nm instead of 254 nm.

Acceptance criteria:

- Assay obtained for each robustness parameter should be between 90.0 and 110.0 %. The combined RSD obtained for the assay result of an each robustness parameter and six assay results of method precision should be not more than 2.0 %.

ROBUSTNESS REPORT:

A deliberate plus and minus changes in the analytical method parameters such as in wavelength, and flow rate was altered and the assay analytical study done as per the protocol.

System suitability results:

Table No:7.24

S.No.	Parameter Name	Results obtained		
		Tailing factor	**Theoretical Plates**	**Area (RSD)**
01	Wavelength Plus (256 nm)	1.29	8171	0.094
02	Wavelength Minus (252 nm)	1.28	8166	0.149
03	Flow rate Plus (1.2 mL)	1.26	7676	0.216
04	Flow rate Minus (0.8 mL)	1.30	8830	0.100
Acceptance criteria		**NMT 2.0**	**NLT 2000**	**NMT 2.0%**

Robustness results obtained:

Table No:7.25

S.No	Parameter Name	Results obtained	Acceptance criteria
		Drug obtained in %	
01	Wavelength Plus (256 nm)	99.70	**90.0% - 110.0%**
02	Wavelength Minus (252 nm)	99.44	
03	Flow rate Plus (1.2 mL)	100.18	
04	Flow rate Minus (0.8 mL)	99.84	

Combined Method precision and Robustness results obtained:

Table No:7.26

S.No	Parameter Name	Results obtained Drug obtained drug in %	Acceptance criteria
01	Method precision - 1	99.56	**90.0% - 110.0%**
02	Method precision - 2	98.86	
03	Method precision - 3	98.74	
04	Method precision - 4	101.24	
05	Method precision - 5	101.40	
06	Method precision - 6	97.80	
07	Wavelength Plus (256 nm)	99.70	
08	Wavelength Minus (252 nm)	99.44	
09	Flow rate Plus (1.2 mL)	100.18	
10	Flow rate Minus (0.8 mL)	99.84	
Mean		99.68	
Std Dev		1.10	
% RSD		1.10	**NMT 2.0%**

STABILITY STUDIES (ICH Harmonized Tripartite Guidelines, 2005)

Stability of the sample and standard solutions used in HPLC method is required to generate reproducible and reliable results. The sample and standard solutions were subjected to short term stability studies at room temperature. Stability studies were carried out initially, at 8 hours and 16 hours, 24 hours time lapse of solution preparation. This study should be performed by injecting standard solution and sample solution at probable time points, and minimum not less than 24 hours shall be studied.

Note:

Maintain the chromatographic conditions and solutions preparation as per the procedure given in method of analysis and injections sequence for solution stability study is given in below table.

The maximum time for inject able usage of standard and sample solution is absolutely depends on the **X Hours** of solutions stability studied.

Injection sequence:

Table No:7.27

Particulars	**Number of Injections**
Blank (0 Hour)	1
Standard preparation (0 Hour)	1
Sample preparation (0 Hour)	1
Blank (X Hours)	1
Standard preparation (X Hours)	1
Sample preparation (X Hours)	1

Acceptance criteria:

Cumulative % RSD for area obtained between initial time point and various probable intervals time points should be not more than 2.0 %.

STABILITY OF ANALYTICAL SOLUTIONS:

Standard and sample solutions to be used in the analytical method are scrutinized for their solution's stability. This study was performed by injecting standard and sample solution for the period of 24 hours.

System suitability results and Cumulative % RSD results obtained for Stability of standard solution:

Table No :7.28

S. No.	Time point	Standard solution area	Results obtained		
			Cumulative % RSD	Tailing factor	Theoretical plate
01	0^{th} hour	16777065	NA	1.33	7924
02	4^{th} hour	16781531	0.019	1.34	7984
03	8^{th} hour	16831375	0.179	1.33	7951
04	12^{th} hour	16849225	0.214	1.35	8012
05	16^{th} hour	16978143	0.484	1.35	7951
06	20^{th} hour	16988890	0.557	1.36	7936
07	24^{th} hour	17135284	0.785	1.36	7923
		Limit	**NMT 2.0 %**	**NMT 2.0**	**NLT 2000**

System suitability results and Cumulative % RSD results obtained for Stability of sample solution:

Table No:7.29

S. No.	Time point	Standard solution area	Results obtained		
			Cumulative % RSD	Tailing factor	Theoretical plate
01	0^{th} hour	16682977	NA	1.33	7922
02	4^{th} hour	16691317	0.035	1.34	7969
03	8^{th} hour	16697379	0.043	1.35	7940
04	12^{th} hour	16717797	0.089	1.36	8009
05	16^{th} hour	16688628	0.080	1.35	7950
06	20^{th} hour	16702950	0.074	1.35	7921
07	24^{th} hour	16695481	0.068	1.35	7905
Limit			**NMT 2.0 %**	**NMT 2.0**	**NLT 2000**

LIMIT OF DETECTION (ICH Harmonized Tripartite Guidelines, 2005)

Limit of detection is the lowest concentration of the analyte that can be detected by injecting decreasing amount, not necessarily quantity by the method, under the stated experimental conditions. The minimum concentration at which the analyte can be detected is determined from the linearity curve by applying the formula.

LOD = 3.3 SD/slope.

The lowest concentration of Diclofenac sodium hat can be detected was determined from standard curve was **0.77 µg/ml.**

LIMIT OF QUANTITATION (ICH Harmonized Tripartite Guidelines, 2005)

Limit of quantitation is the lowest concentration of the analyte in a sample that can

be estimated quantitatively by injecting decreasing amount of drug with acceptable precision and accuracy under the stated experimental conditions of the method. Limit of quantitation can be obtained from linearity curve by applying the following formula.

LOQ = 10 SD/ slope.

The lowest concentration at which peak can be quantified is called LOQ. It was found to be **2.34µg/ml** for Diclofenac Sodium.

Table No: 7.30

Sample	LOD	LOQ
Diclofenac sodium	0.77 µg/ml.	2.34µg/ml.

ASSAY RESULTS

PROCEDURE

Separately Blank, Standard and test preparation was injected into liquid chromatogram and the areas for major peaks were recorded by using the following formula.

Calculation

Sample area × sample dilution × purity of working standard × Average weight×100X
Convertion factor

Standard area × standard dilution × Label claim x100

Assay result for Replicate injection

Table No: 7.31

Inj. No.	Area of Diclofenac sodium	w/w % Diclofenac sodium Recovered
1	16735543	99.56
2	16592682	98.86
3	16588577	98.74
Mean	16638934	99.05
S.D	83691.0205	0.44287
%R.S.D	0.50	0.45

RESULT

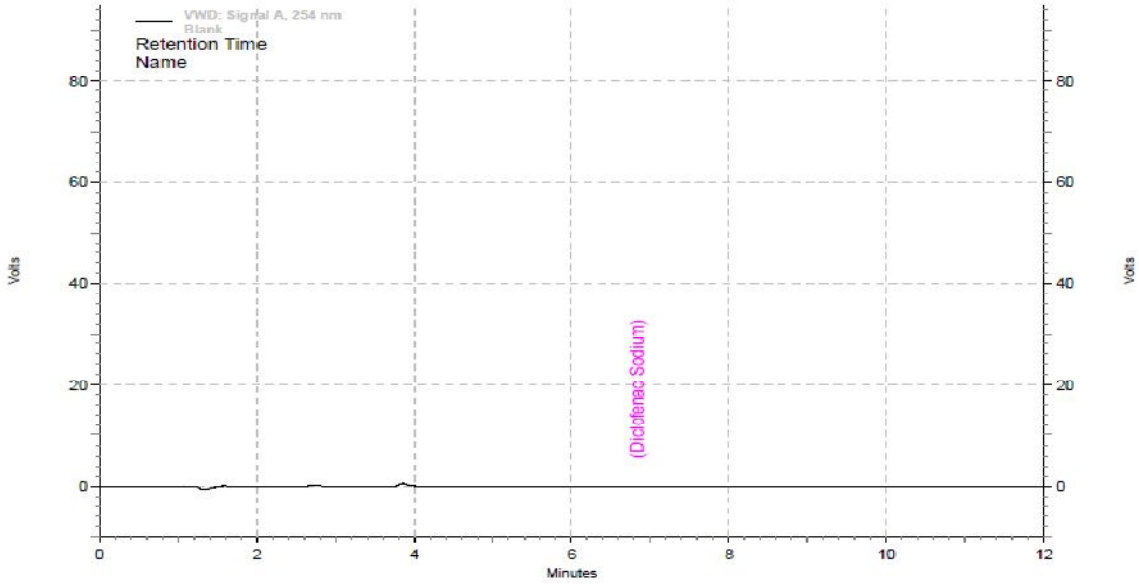
The mean recovery for assay results was found to be 97 – 99w/w % for Diclofenac sodium

8.CHROMATOGRAMS

Specificity (Blank, Standard & Sample)

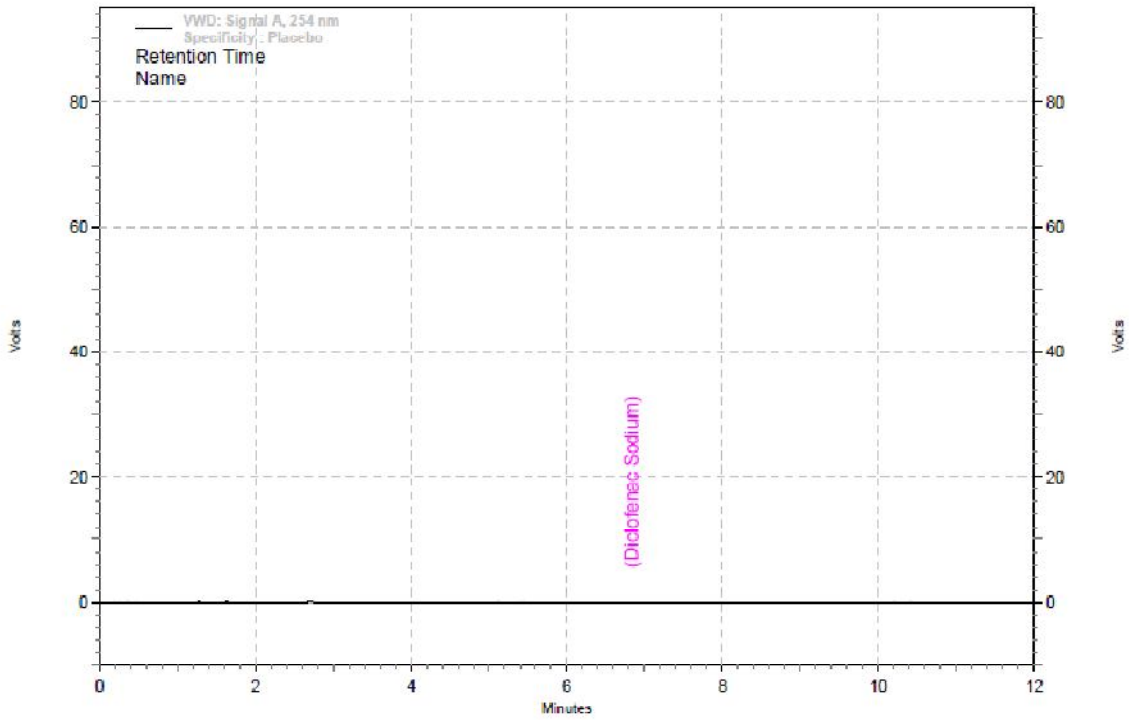
Chromatogram No – 8.1

Blank



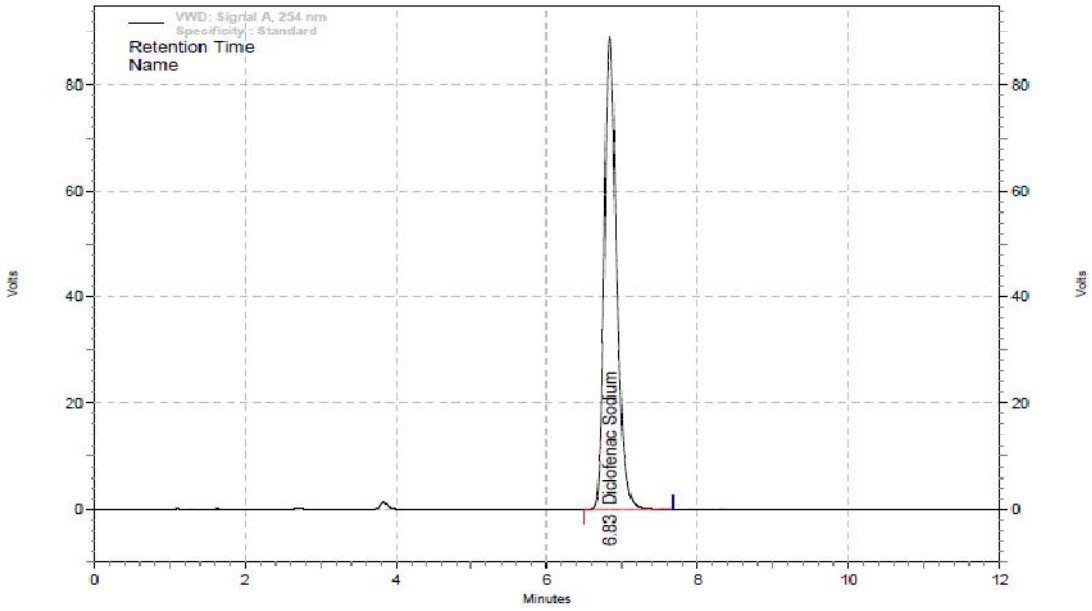
Chromatogram No 8.2

Placebo



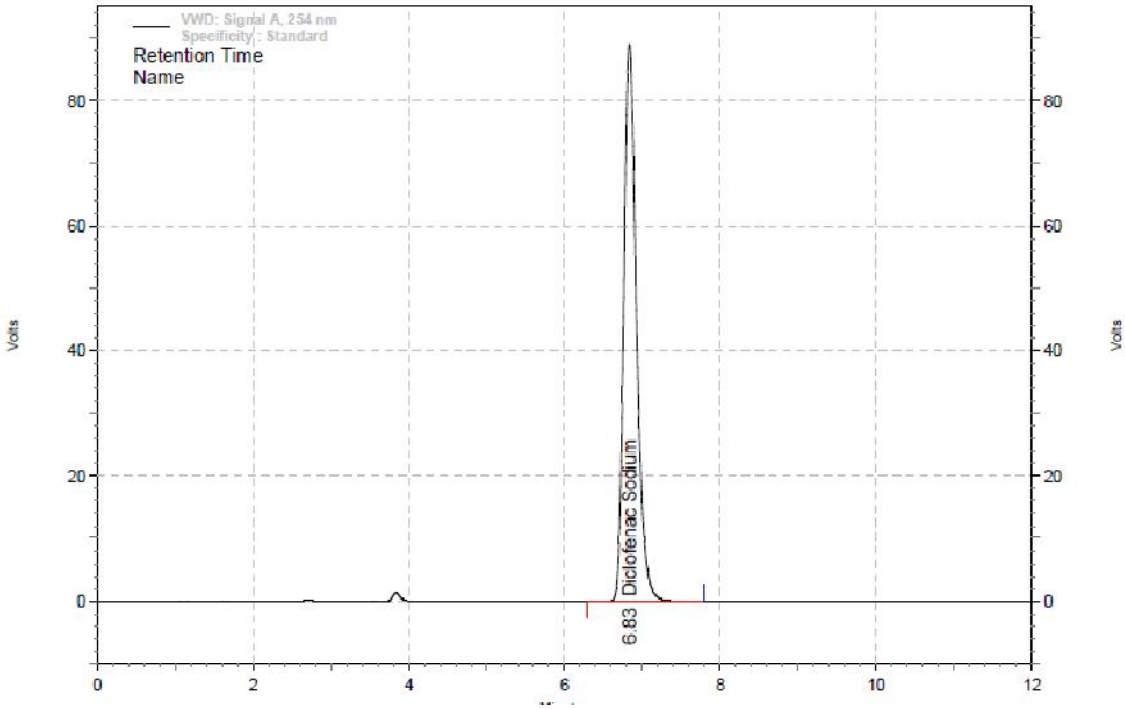
Chromatogram No – 8.3

Standard (Replicate no-1)



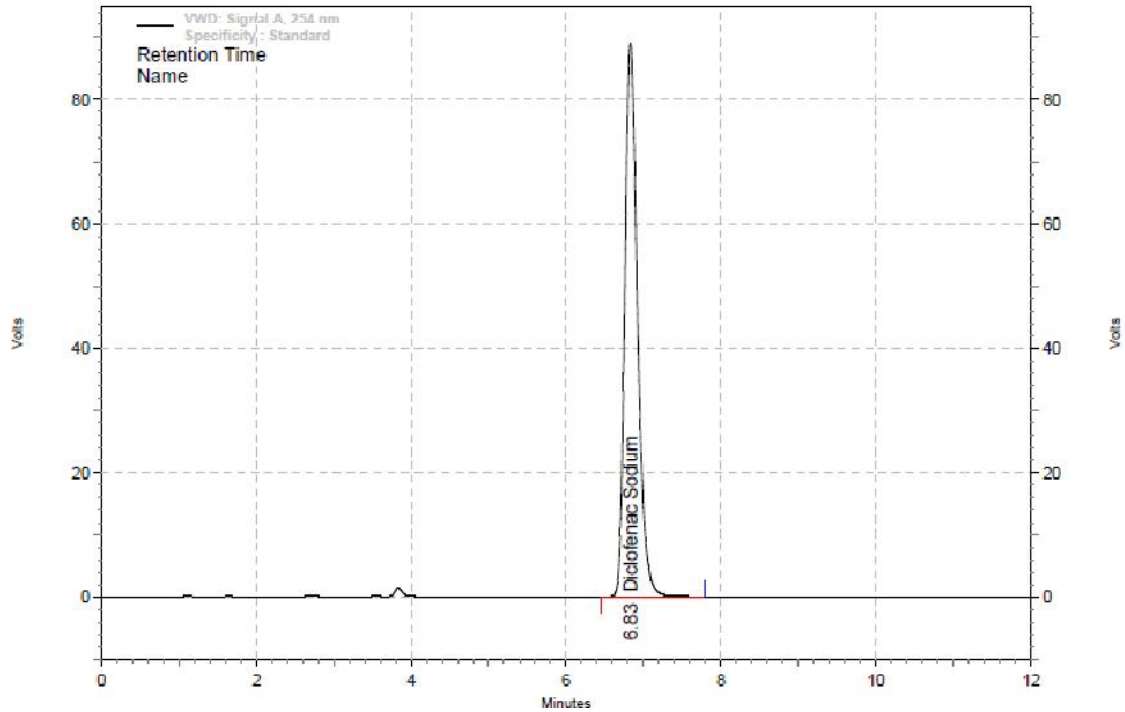
Chromatogram No – 8.4

Standard (Replicate no-2)



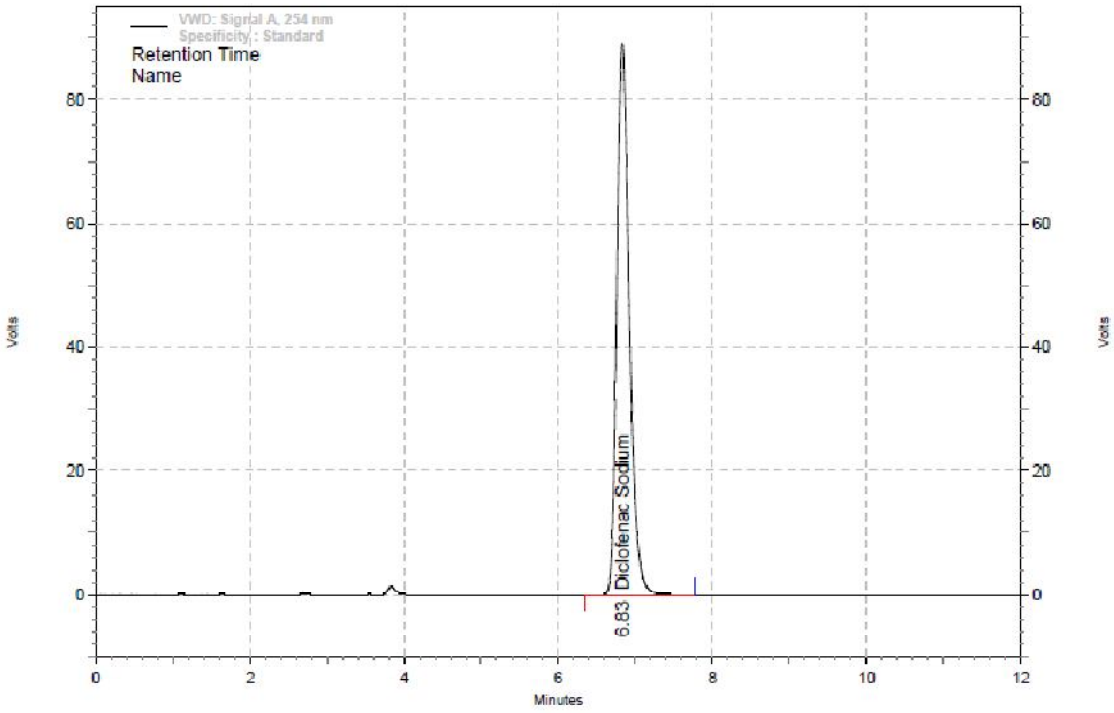
Chromatogram No – 8.5

Standard (Replicate no-3)



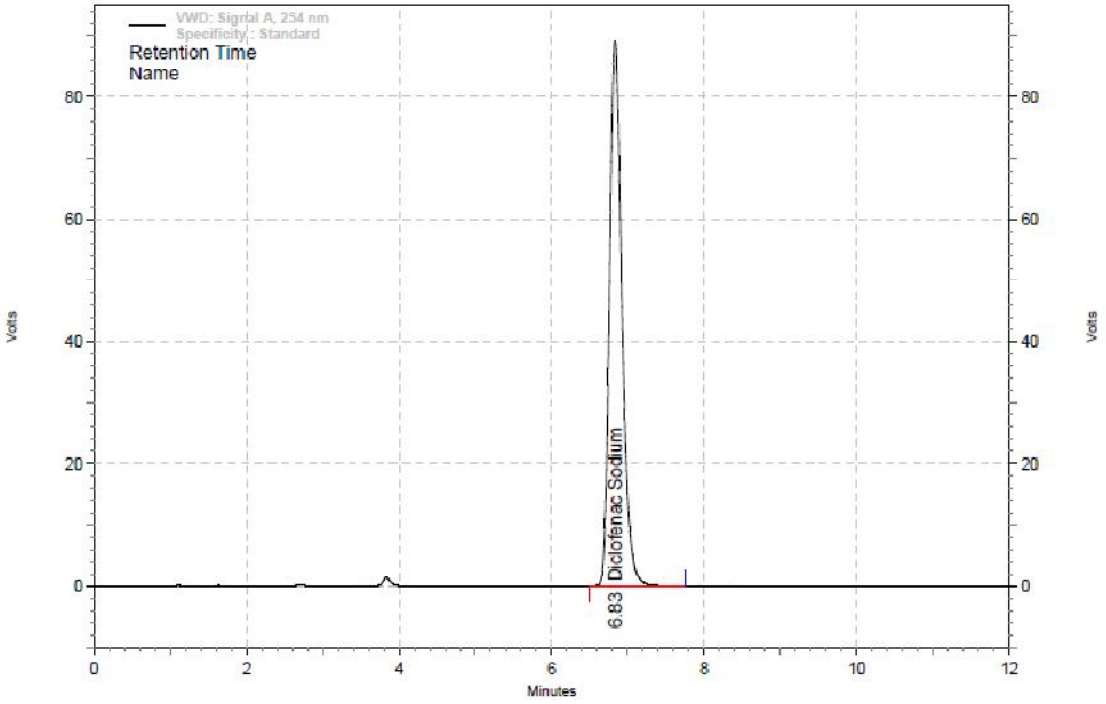
Chromatogram No – 8.6

Standard (Replicate no-4)



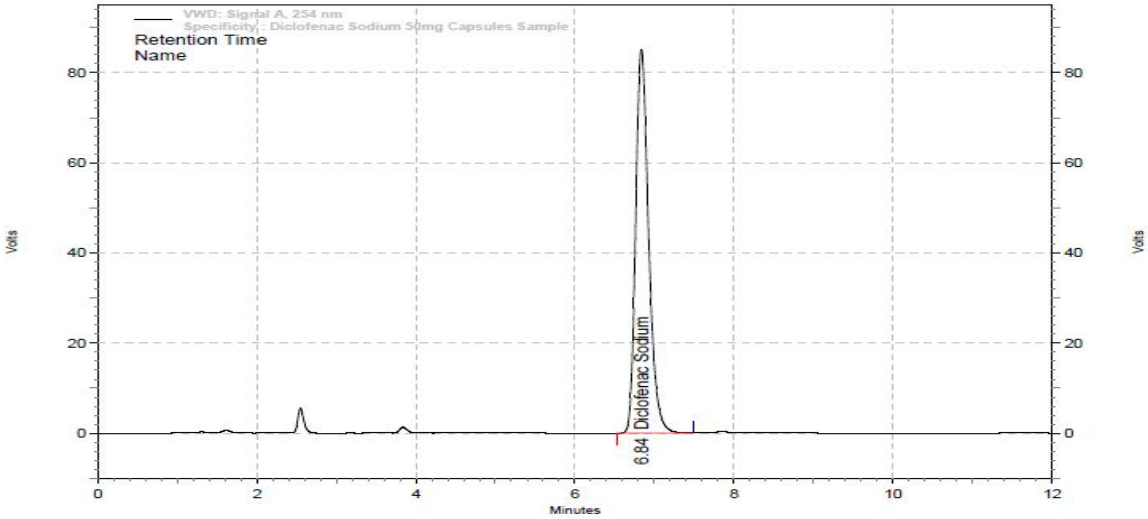
Chromatogram No – 8.7

Standard (Replicate no-5)



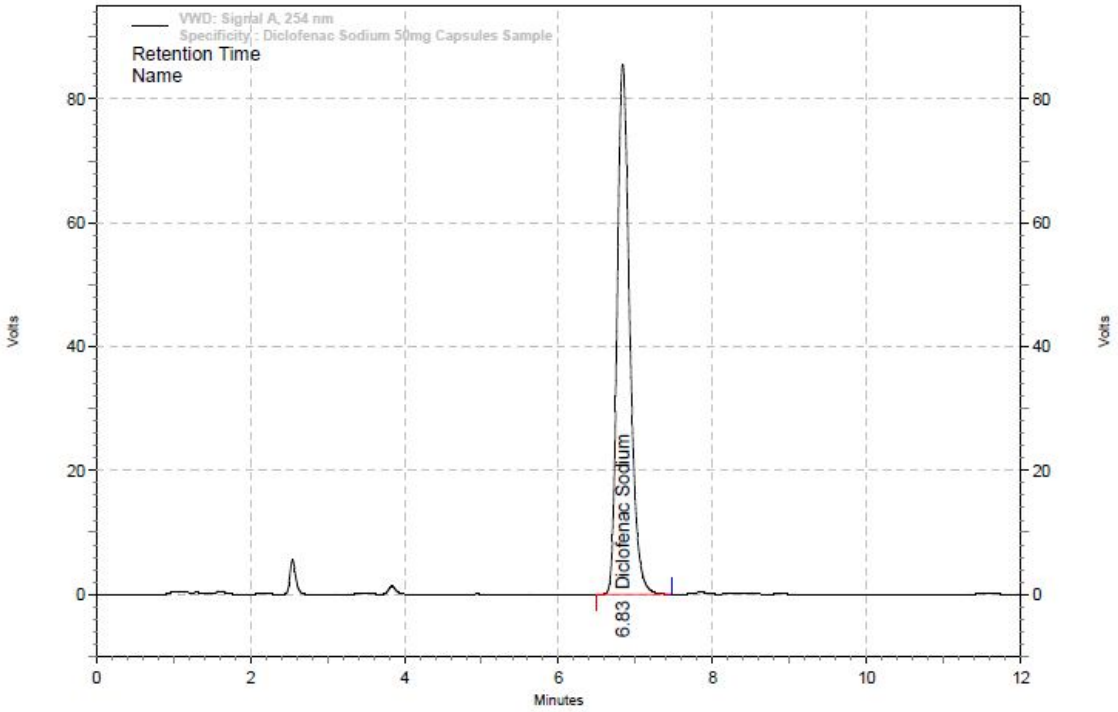
Chromatogram No – 8.8

Sample (Replicate no-1)



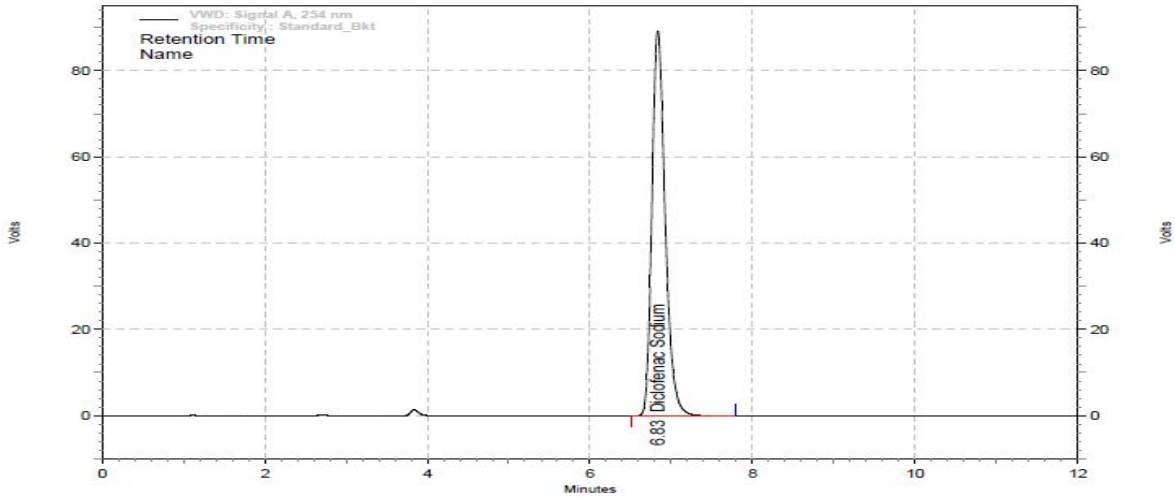
Chromatogram No – 8.9

Sample (Replicate no-2)



Chromatogram No – 8.10

Bracketing standard

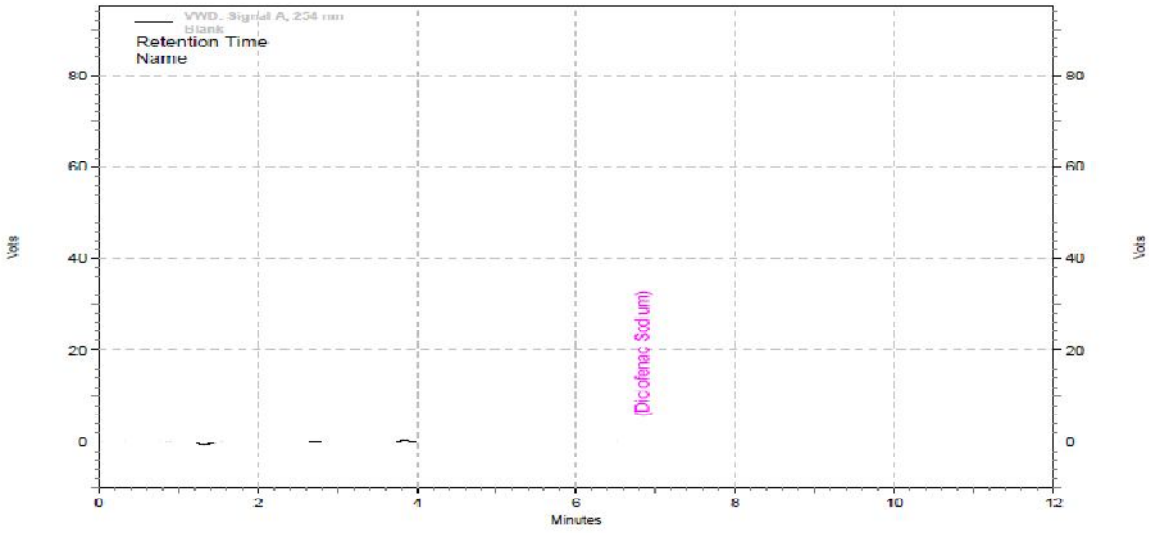


LINEARITY AND RANGE

(Blank, Standards, linearity samples 60% ,80%,100%120%160%)

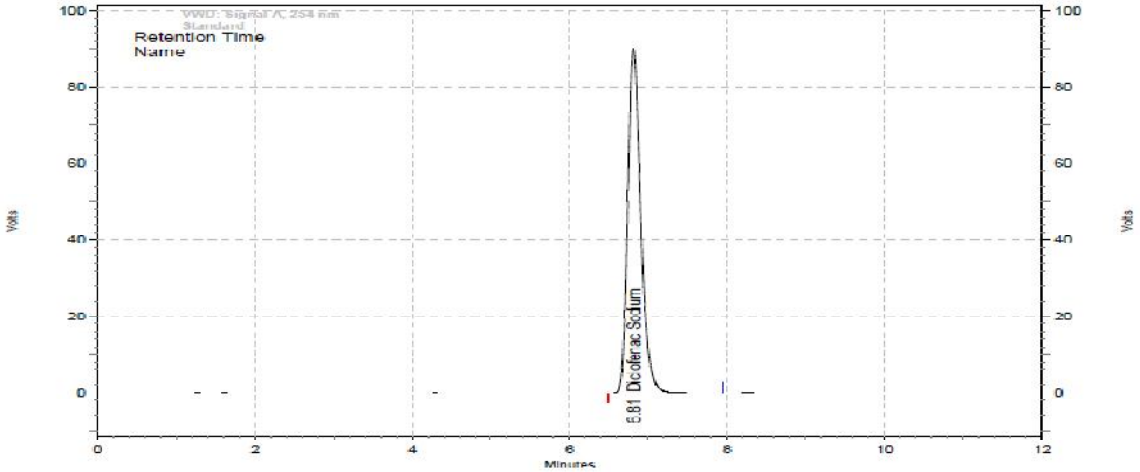
Chromatogram No – 8.11

Blank



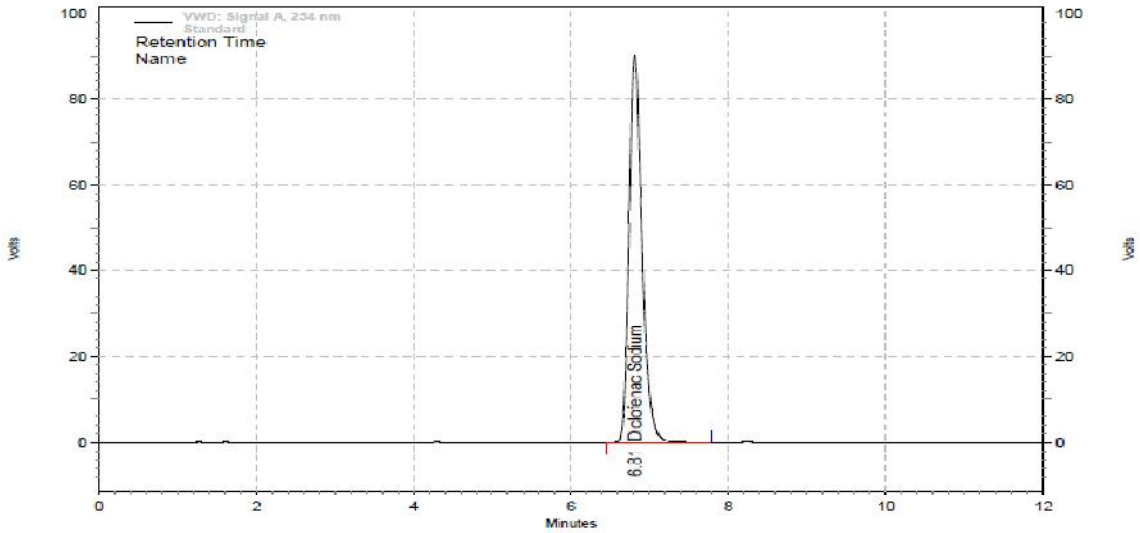
Chromatogram No – 8.12

Standard (Replicate no-1)



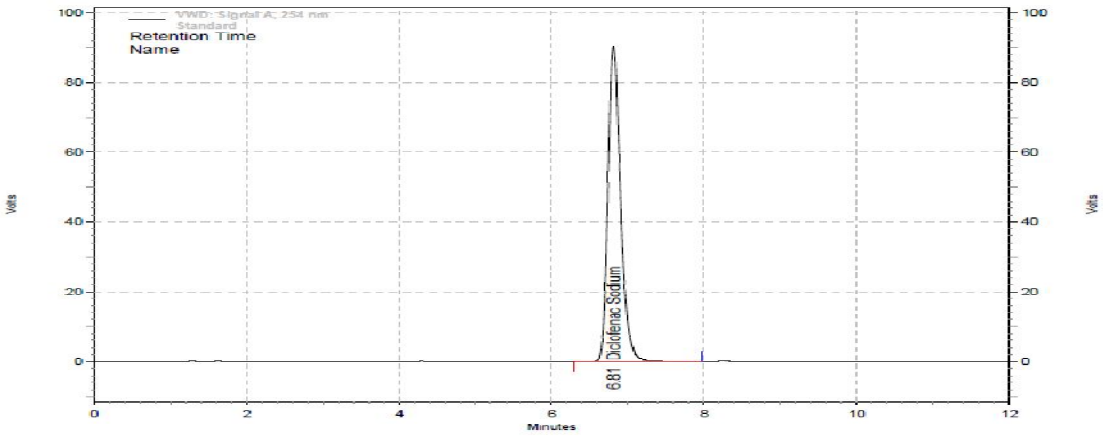
Chromatogram No – 8.13

Standard (Replicate no-2)



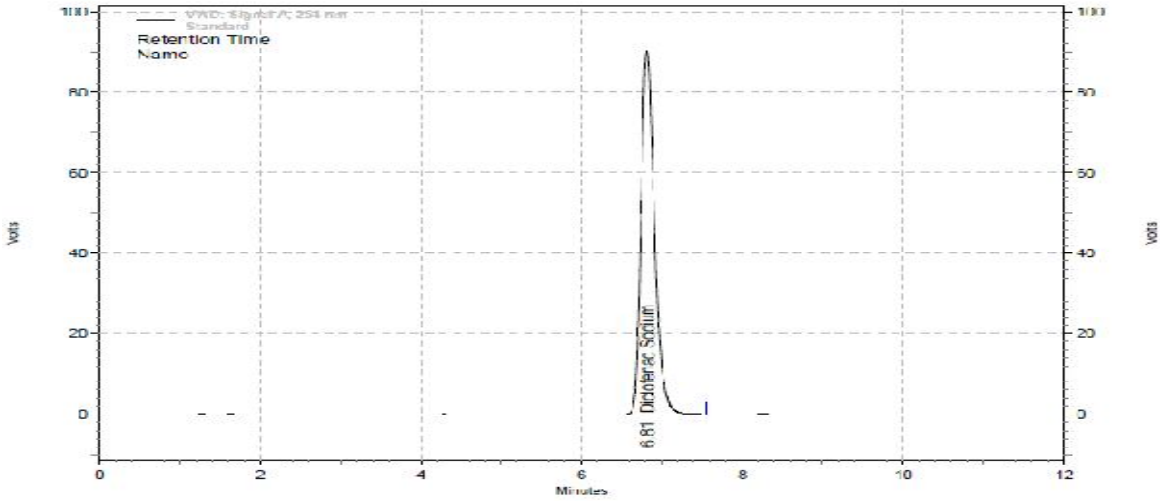
Chromatogram No – 8.14

Standard (Replicate no-3)



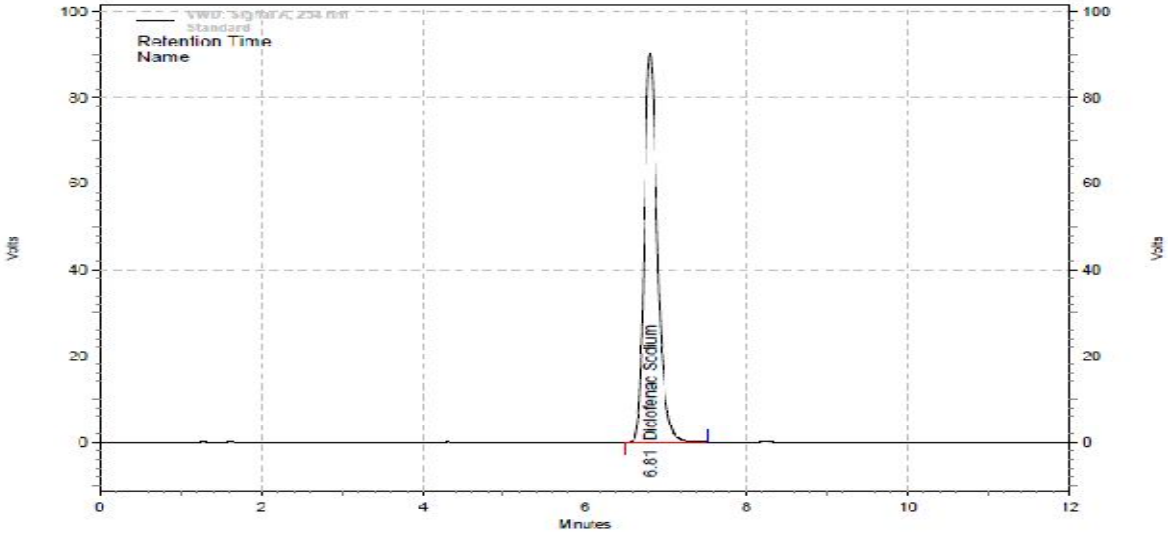
Chromatogram No – 8.15

Standard (Replicate no-4)



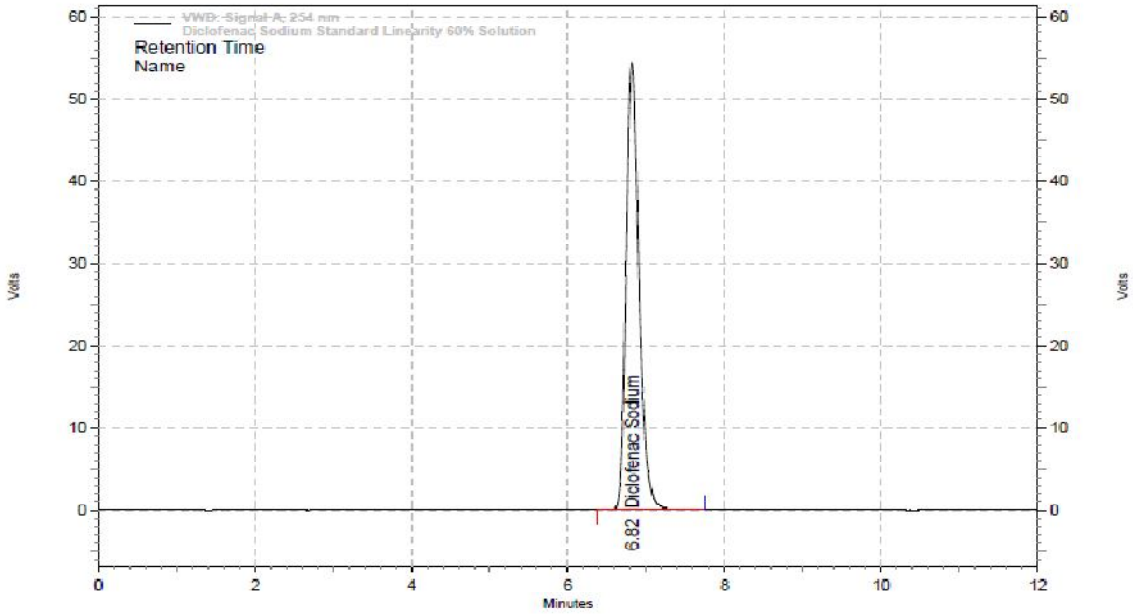
Chromatogram No – 8.16

Standard (Replicate no-5)



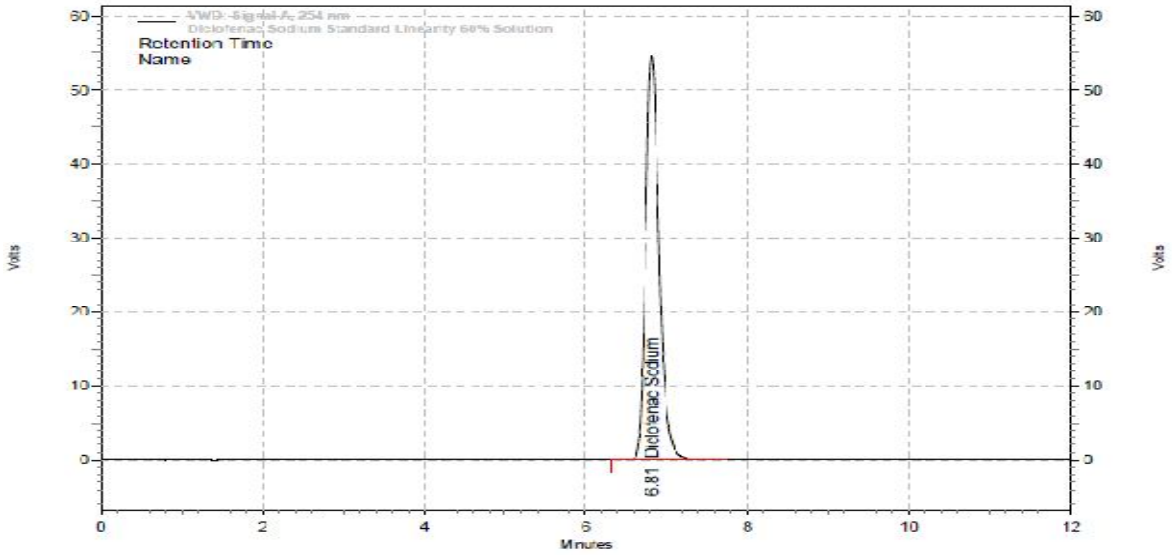
Chromatogram No – 8.17

Linearity 60%(Replicate no-1)



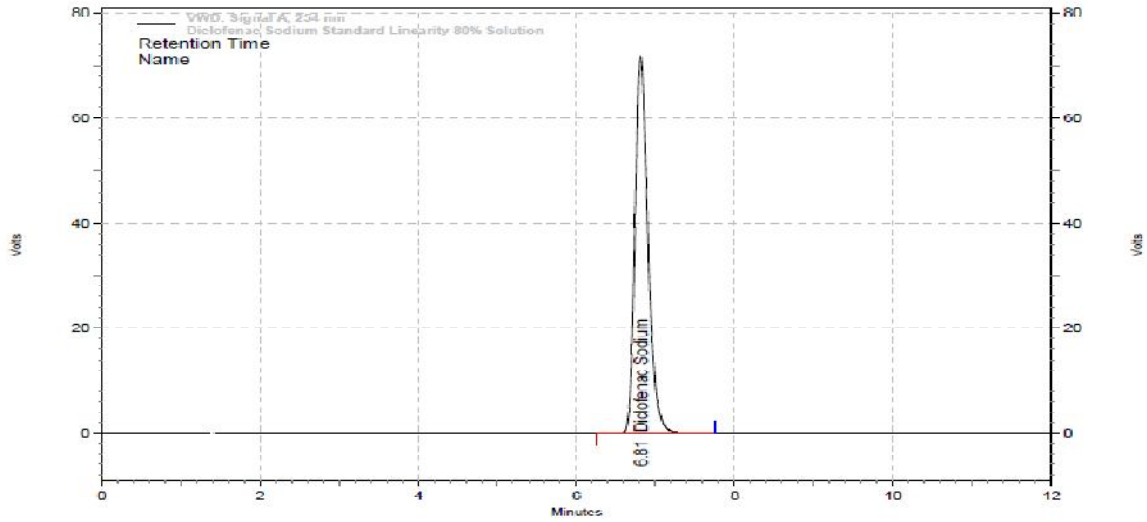
Chromatogram No – 8.18

Linearity 60%(Replicate no-2)



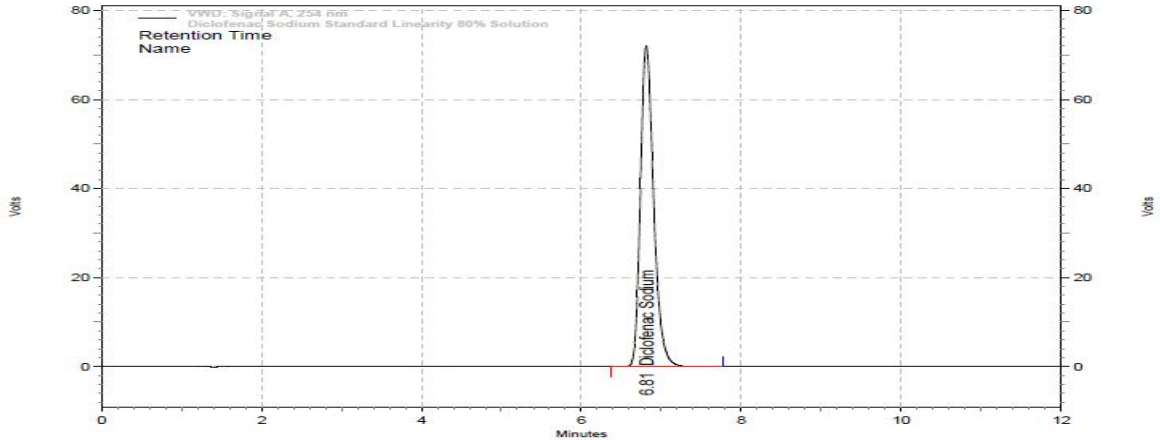
Chromatogram No – 8.19

Linearity 80%(Replicate no-1)



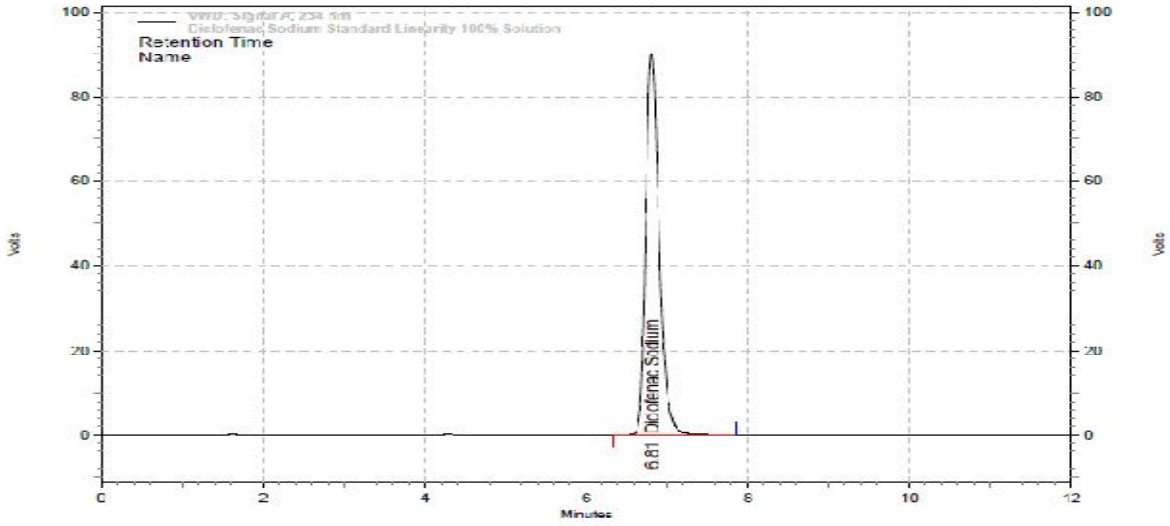
Chromatogram No – 8.20

Linearity 80%(Replicate no-2)



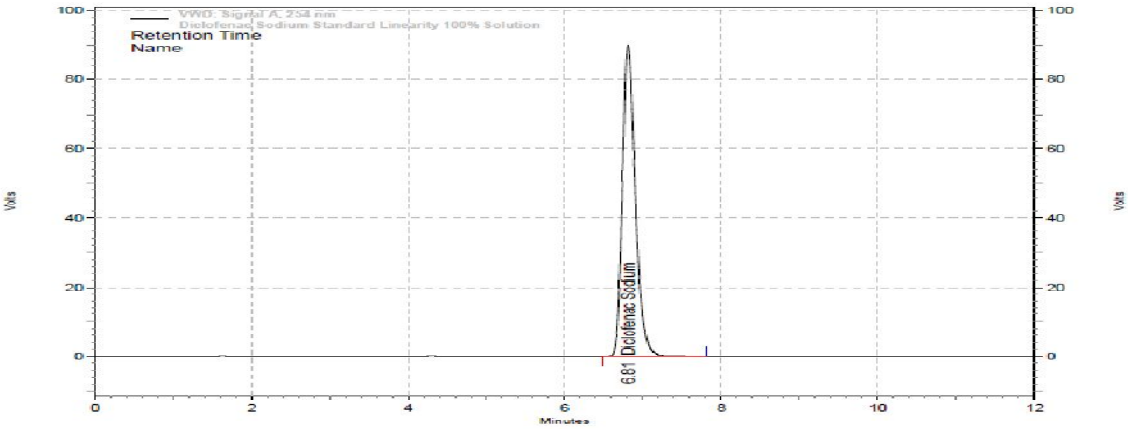
Chromatogram No – 8.21

Linearity 100%(Replicate no-1)



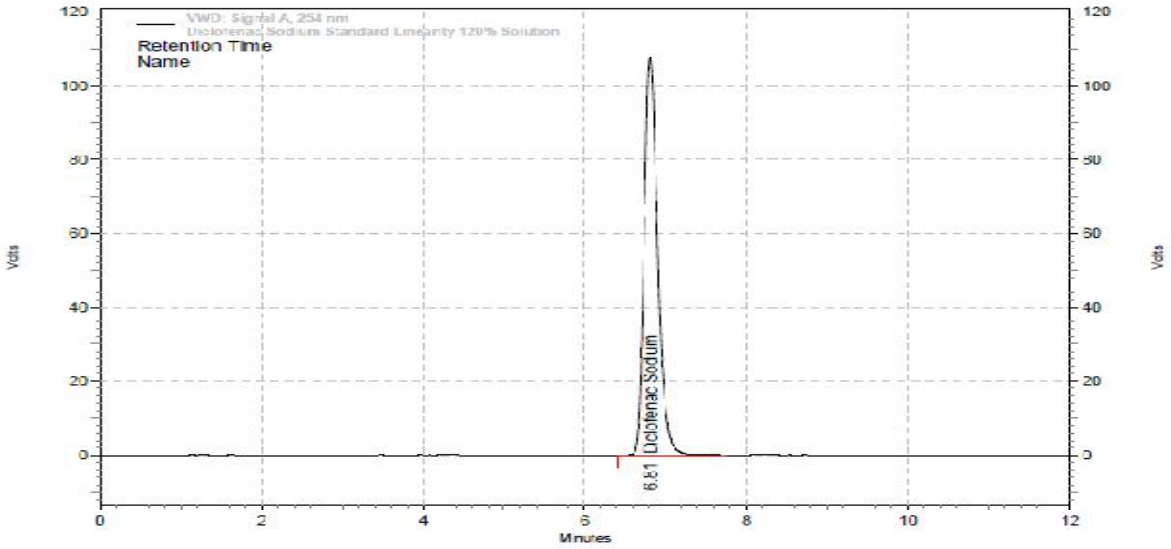
Chromatogram No – 8.22

Linearity 100%(Replicate no-2)



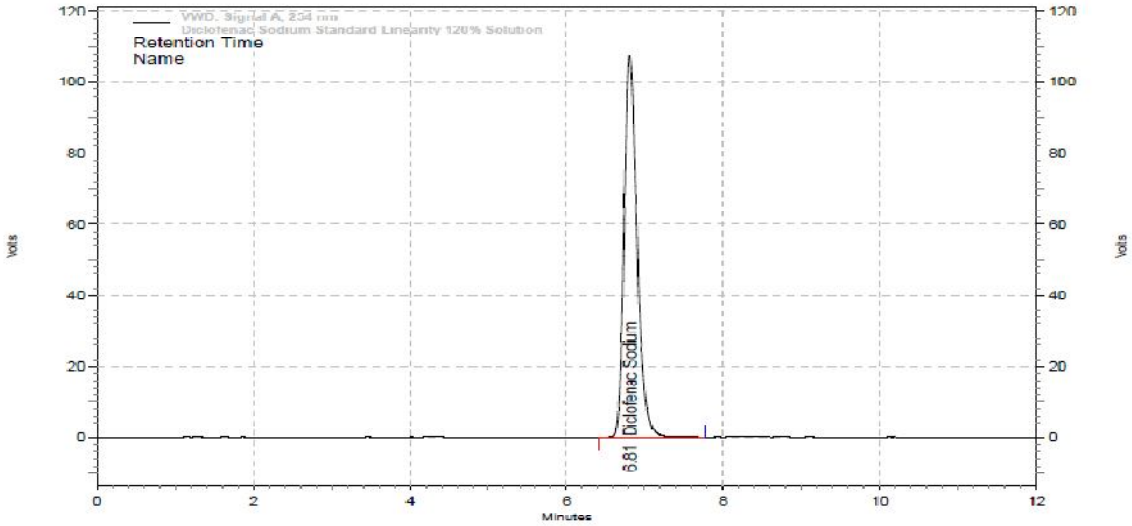
Chromatogram No – 8.23

Linearity 120%(Replicate no-1)



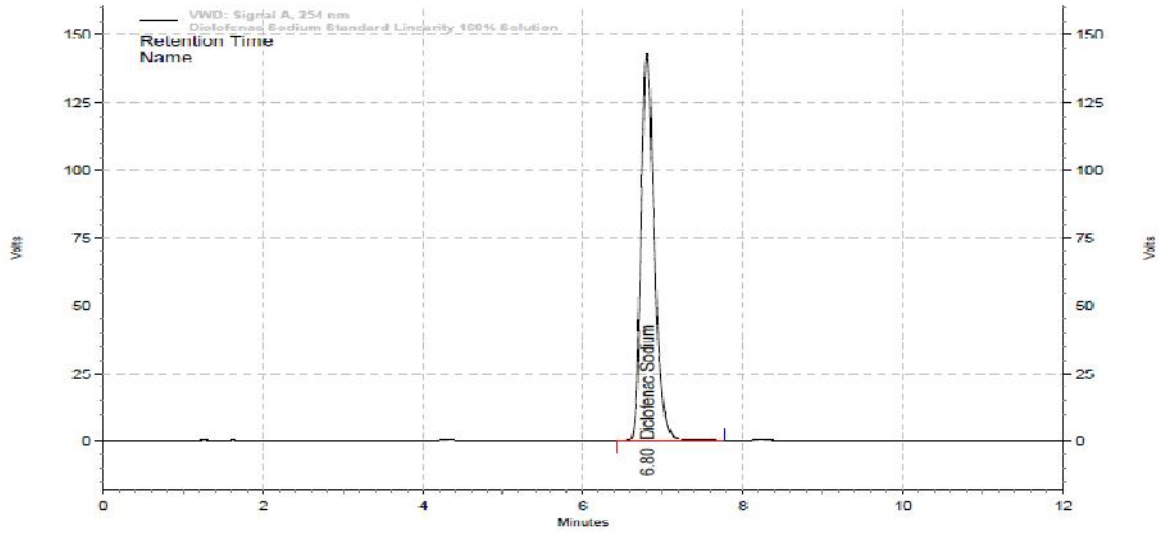
Chromatogram No – 8.24

Linearity 120%(Replicate no-2)



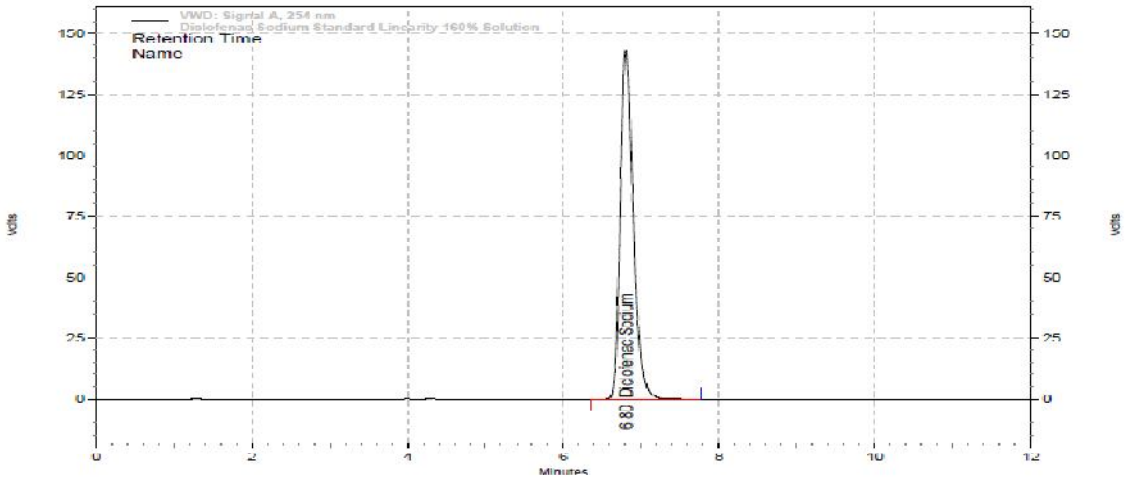
Chromatogram No – 8.25

Linearity 160%(Replicate no-1)



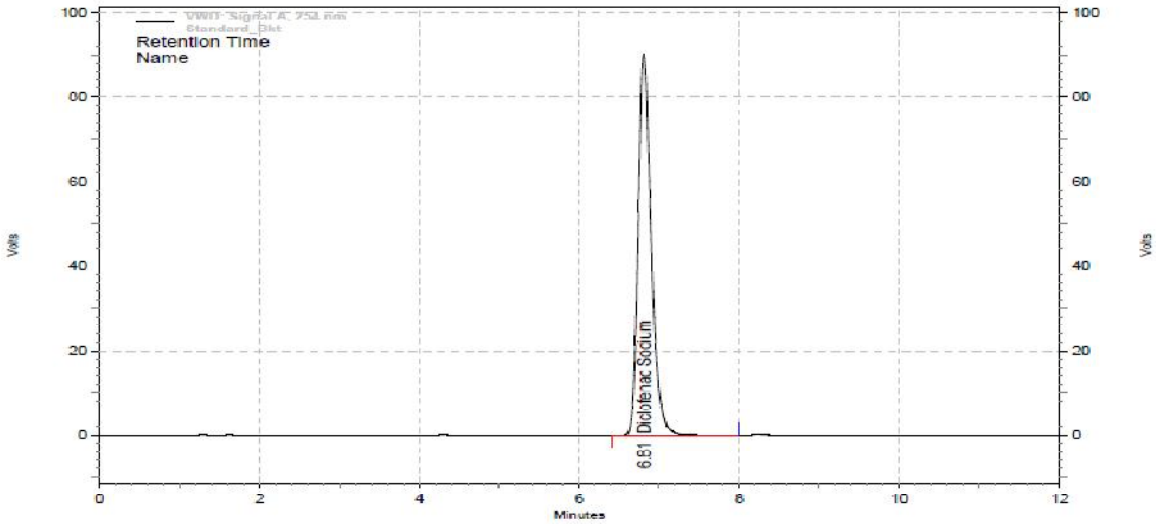
Chromatogram No – 8.26

Linearity 160%(Replicate no-2)



Chromatogram No – 8.27

Bracketing standard

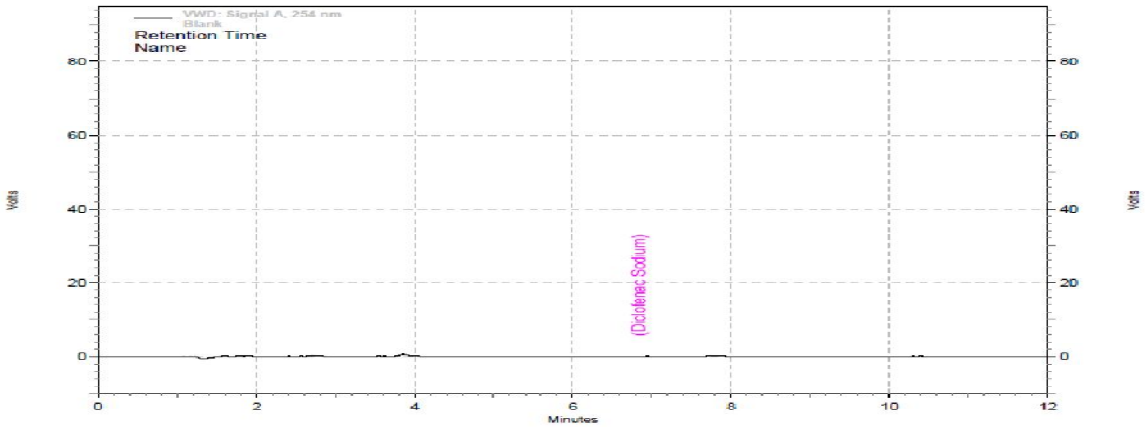


PRECISION-SYSTEM PRECISION

(Blank and standards)

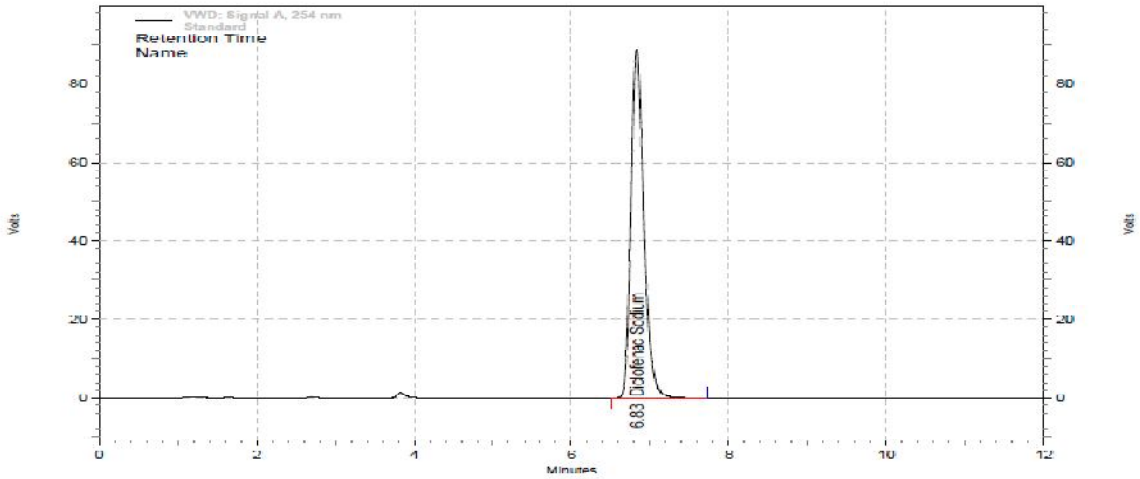
Chromatogram No – 8.28

Blank



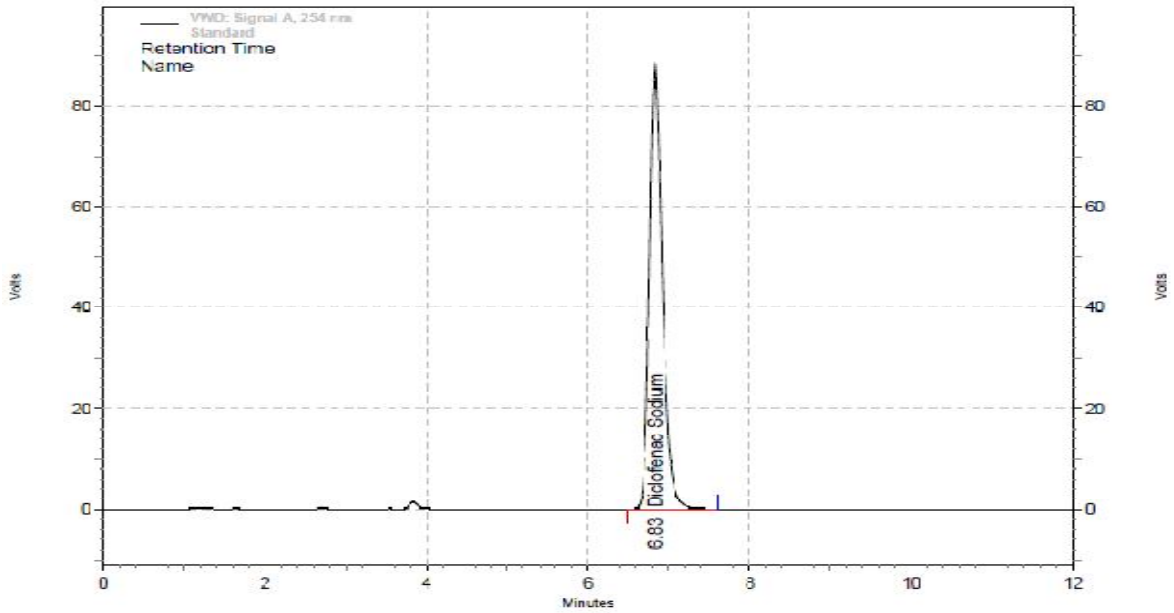
Chromatogram No – 8.29

System precision (Replicate no-1)



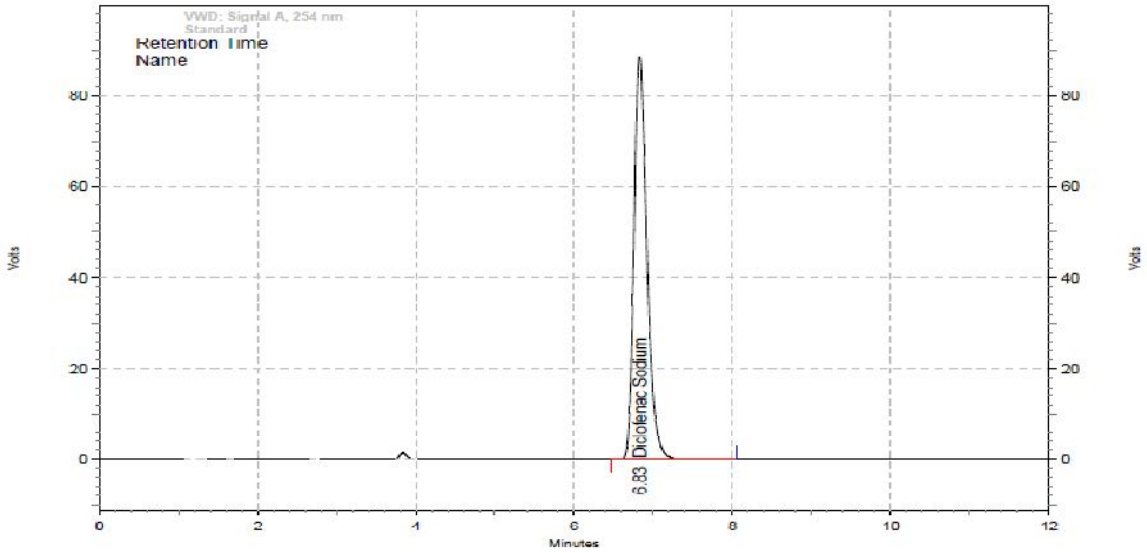
Chromatogram No – 8.30

System precision (Replicate no-2)



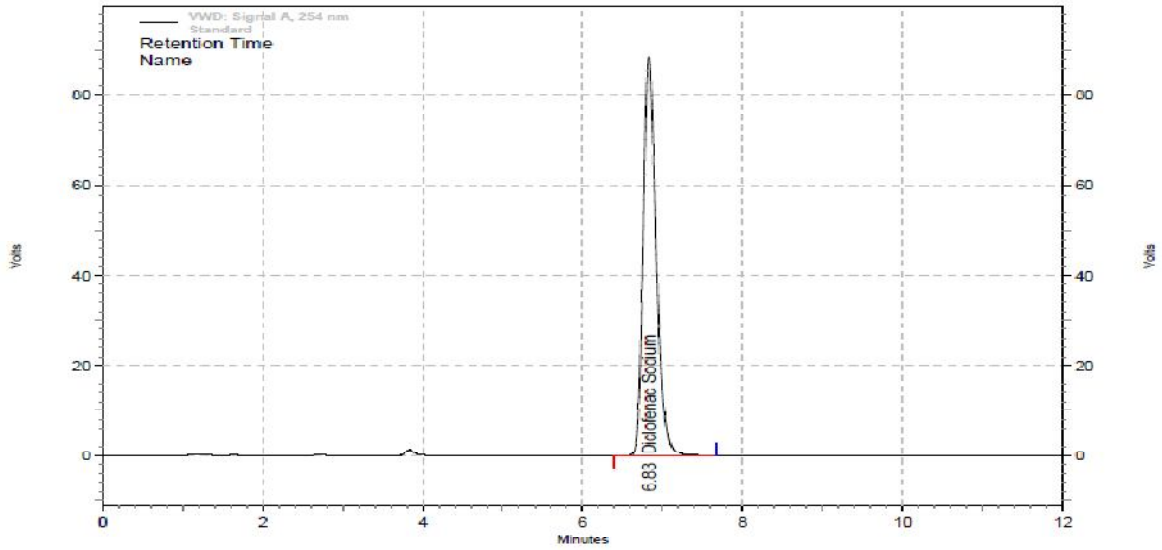
Chromatogram No – 8.31

System precision (Replicate no-3)



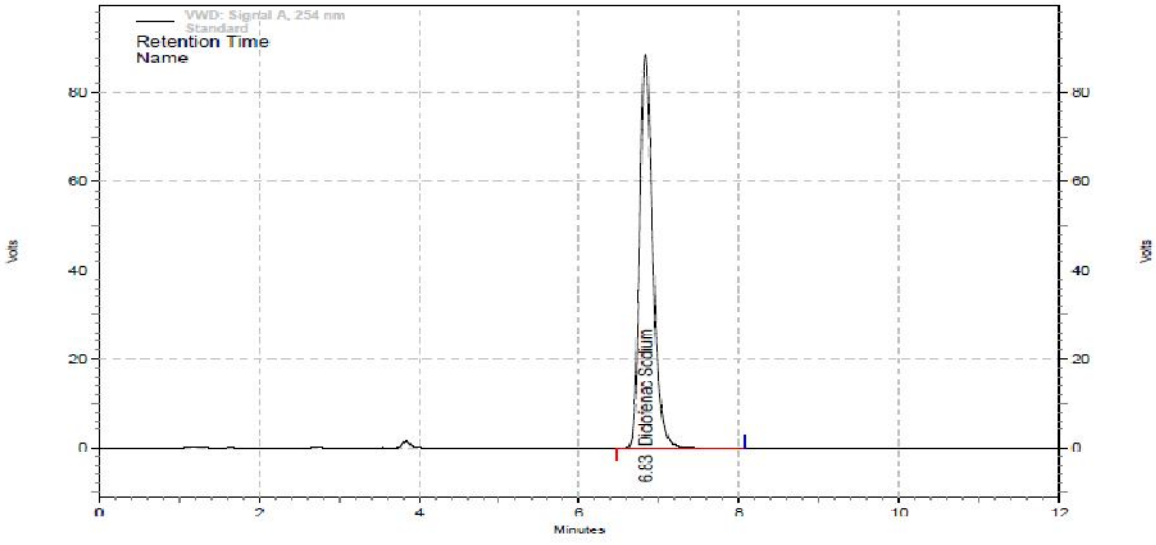
Chromatogram No – 8.32

System precision (Replicate no-4)



Chromatogram No – 8.33

System precision (Replicate no-5)

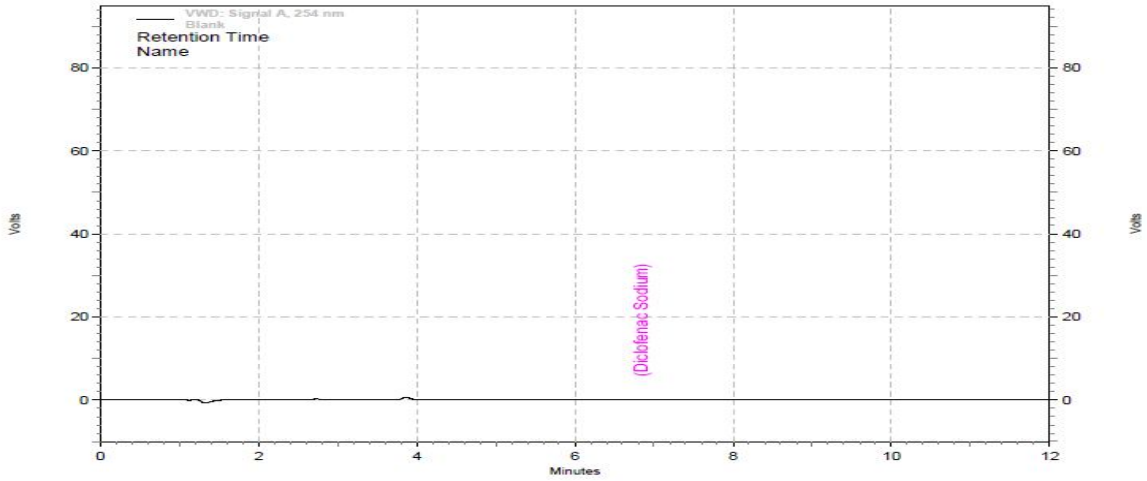


PRECISION-METHOD PRECISION

(Blank ,standards, Sample preparations 1,2,3,4,5,6)

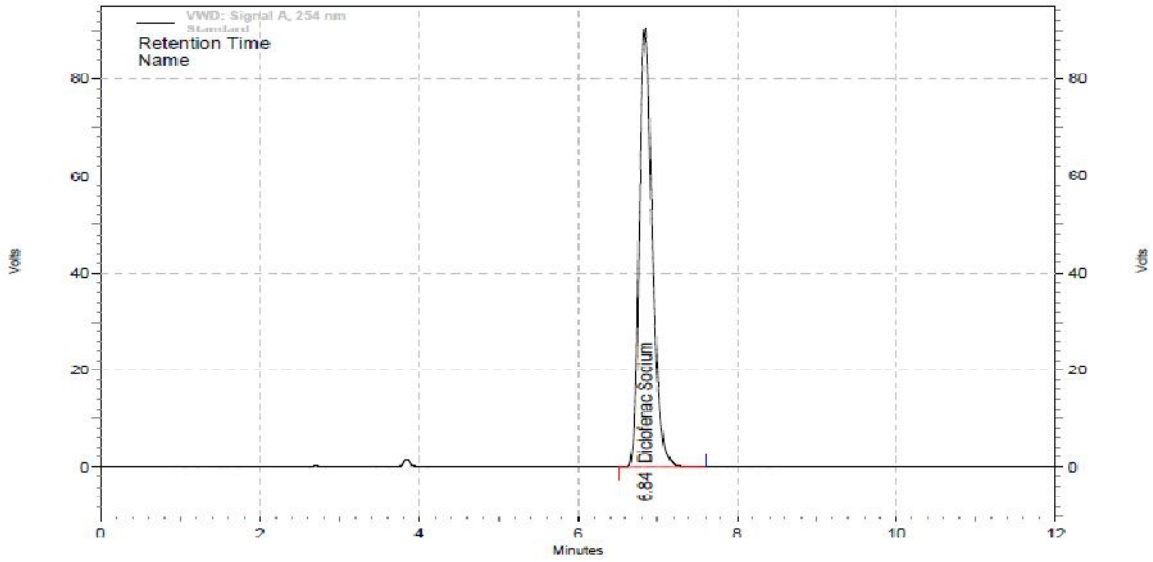
Chromatogram No – 8.34

Blank



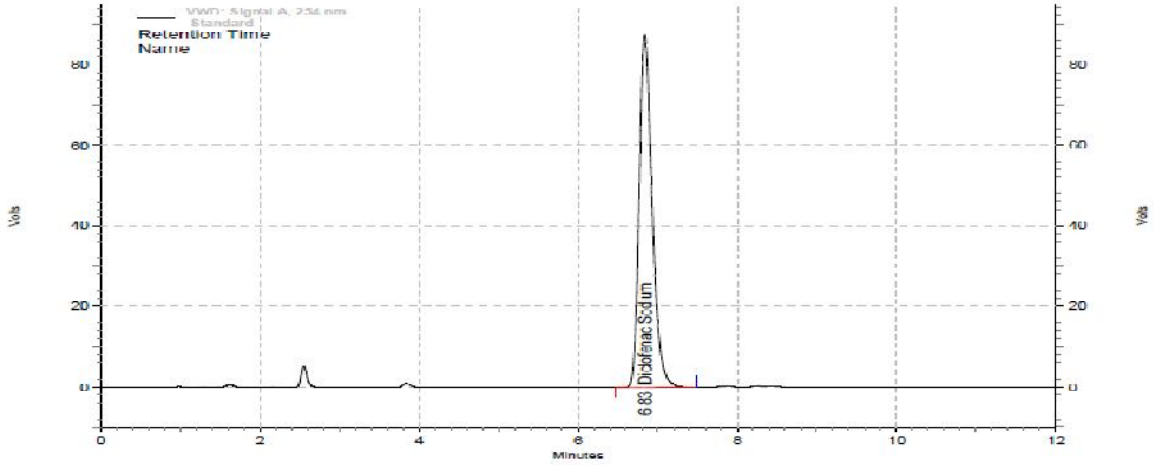
Chromatogram No – 8.35

Standard (Replicate no-1)



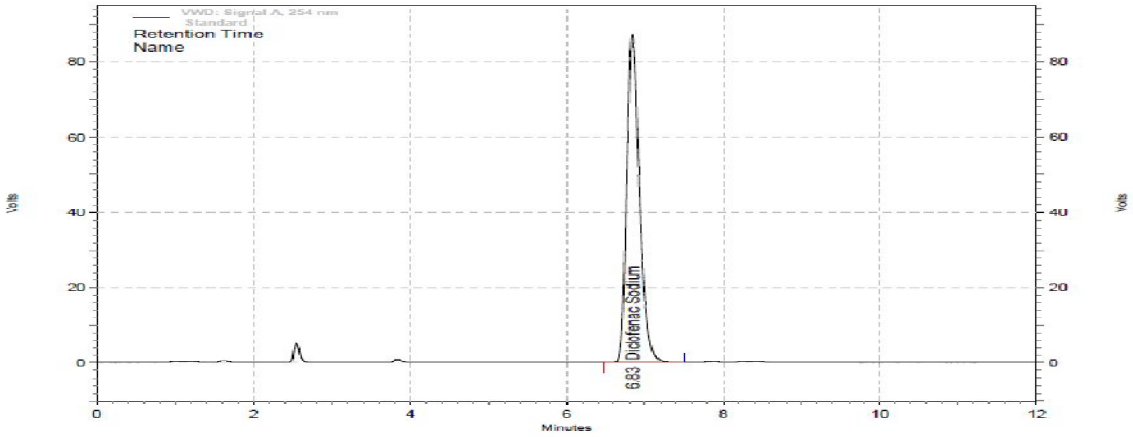
Chromatogram No – 8.36

Standard (Replicate no-2)



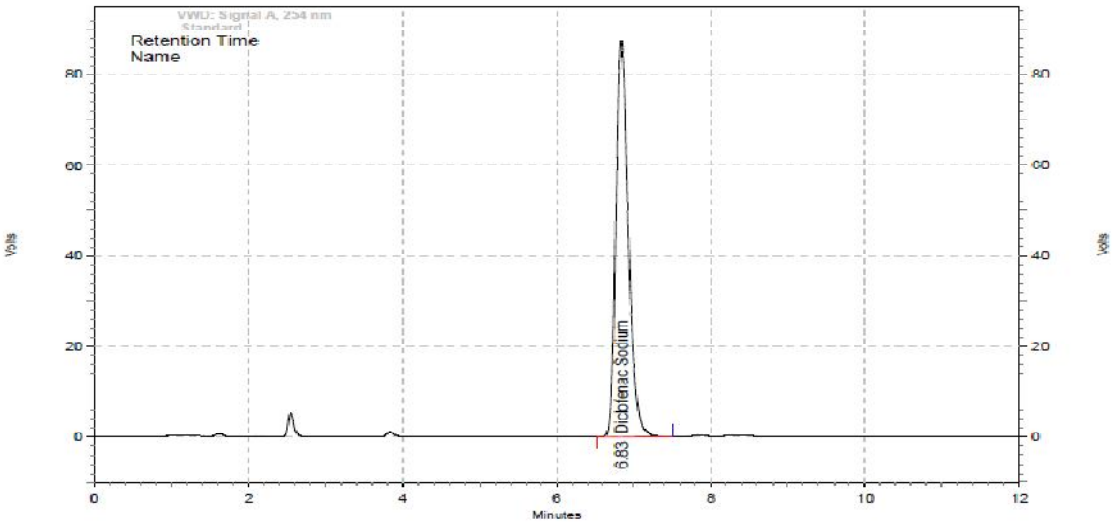
Chromatogram No – 8.37

Standard (Replicate no-3)



Chromatogram No – 8.38

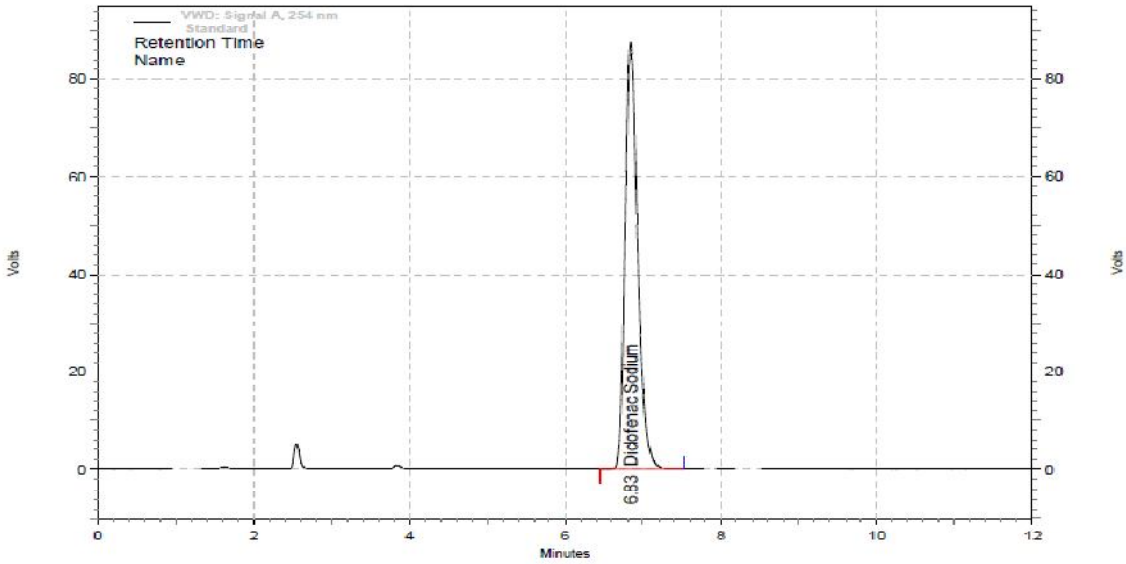
Standard (Replicate no-4)



VWD.

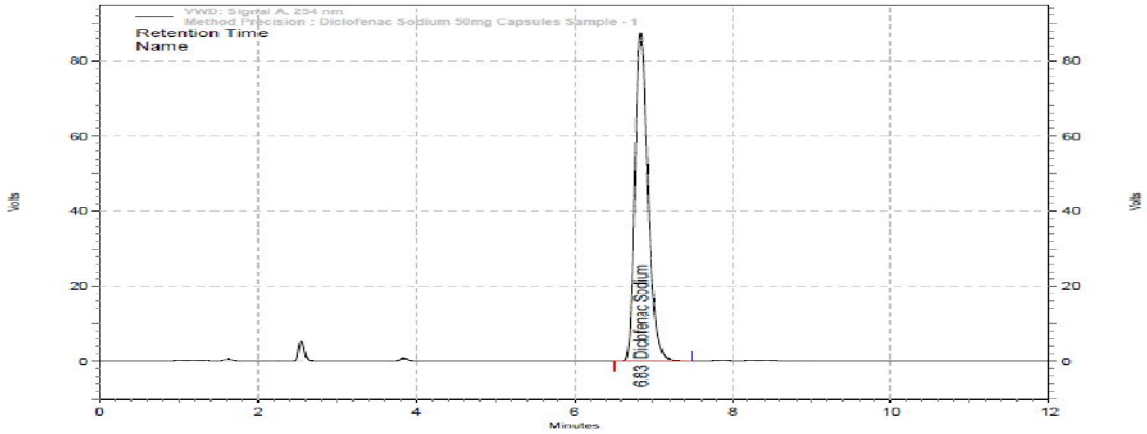
Chromatogram No – 8.39

Standard (Replicate no-5)



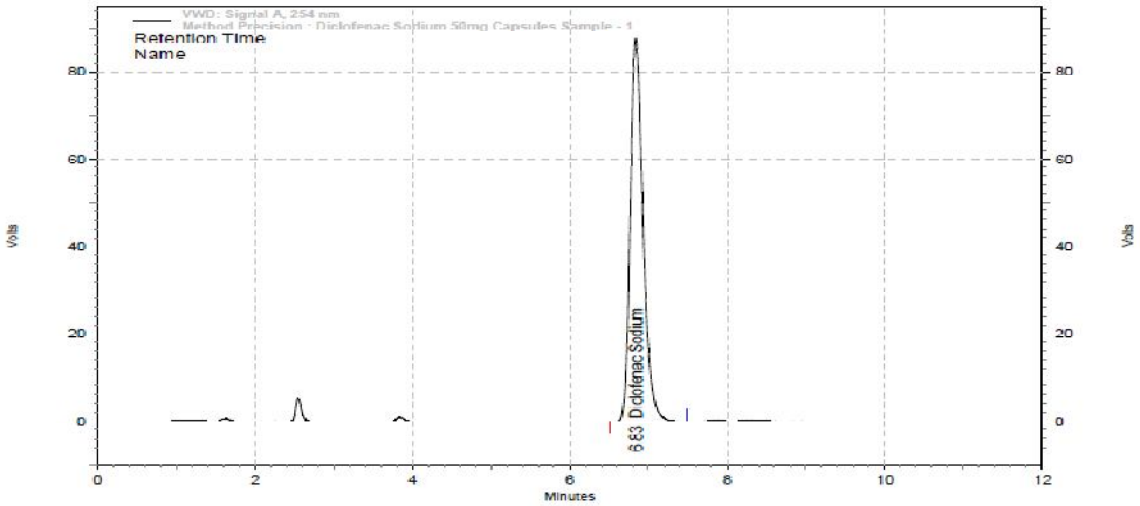
Chromatogram No – 8.40

Method precision-01 (Replicate no-1)



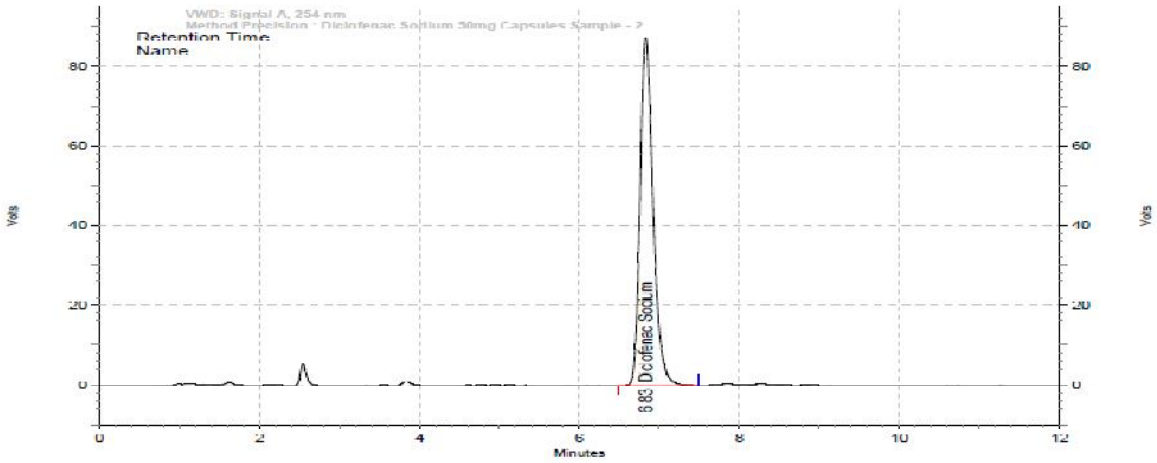
Chromatogram No – 8.41

Method precision-01 (Replicate no-2)



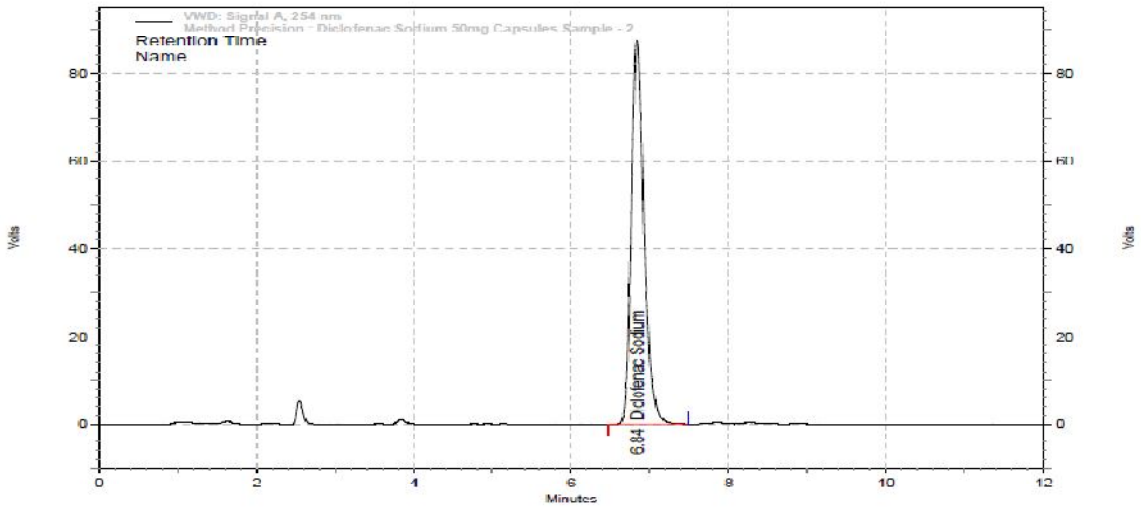
Chromatogram No – 8.42

Method precision-02 (Replicate no-1)



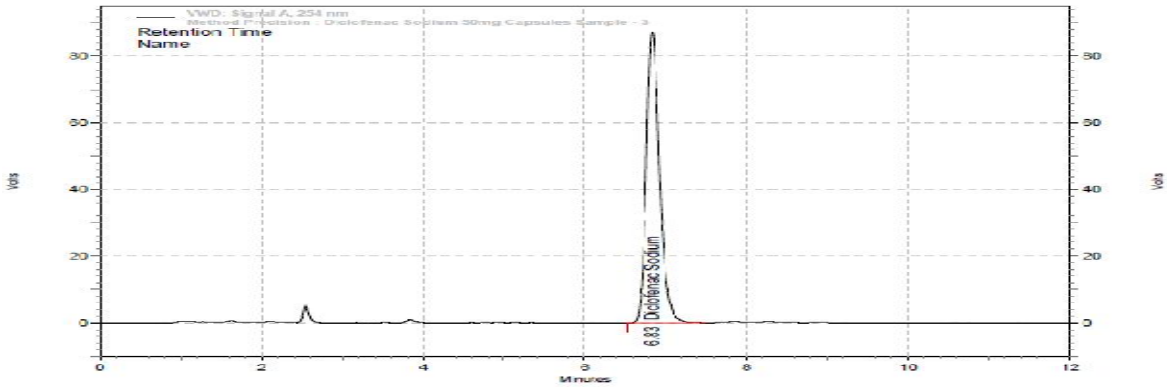
Chromatogram No – 8.43

Method precision-02 (Replicate no-2)



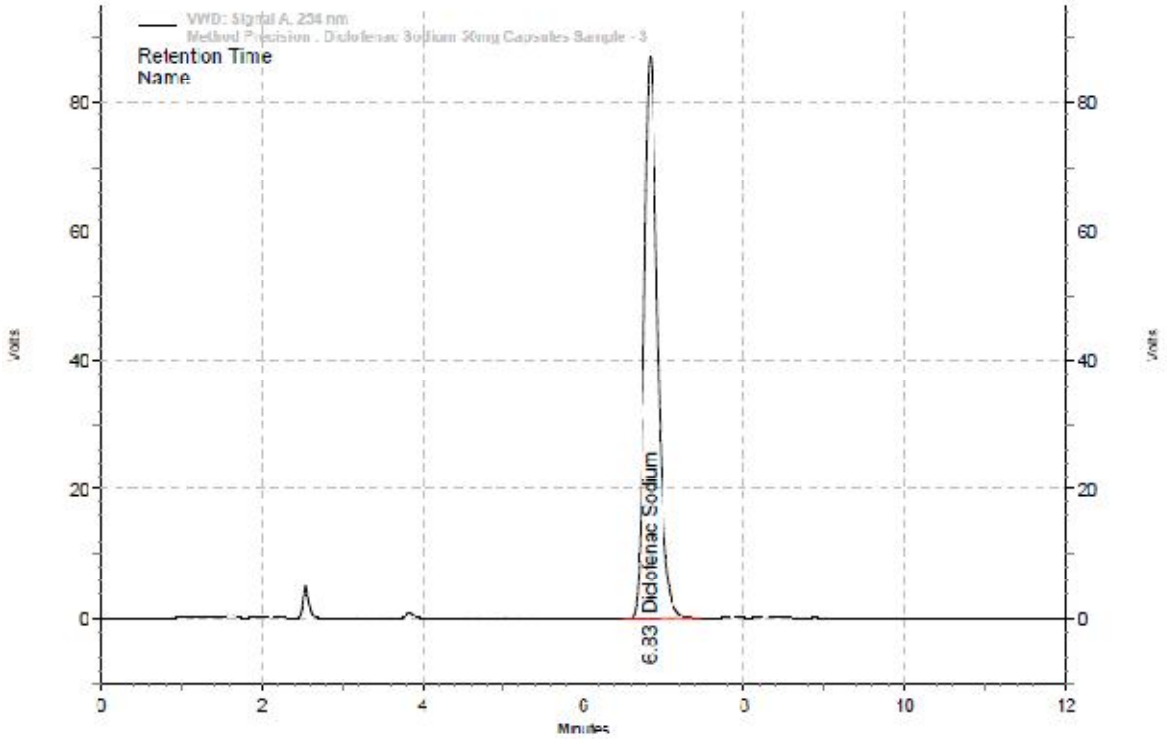
Chromatogram No – 8.43

Method precision-03 (Replicate no-1)



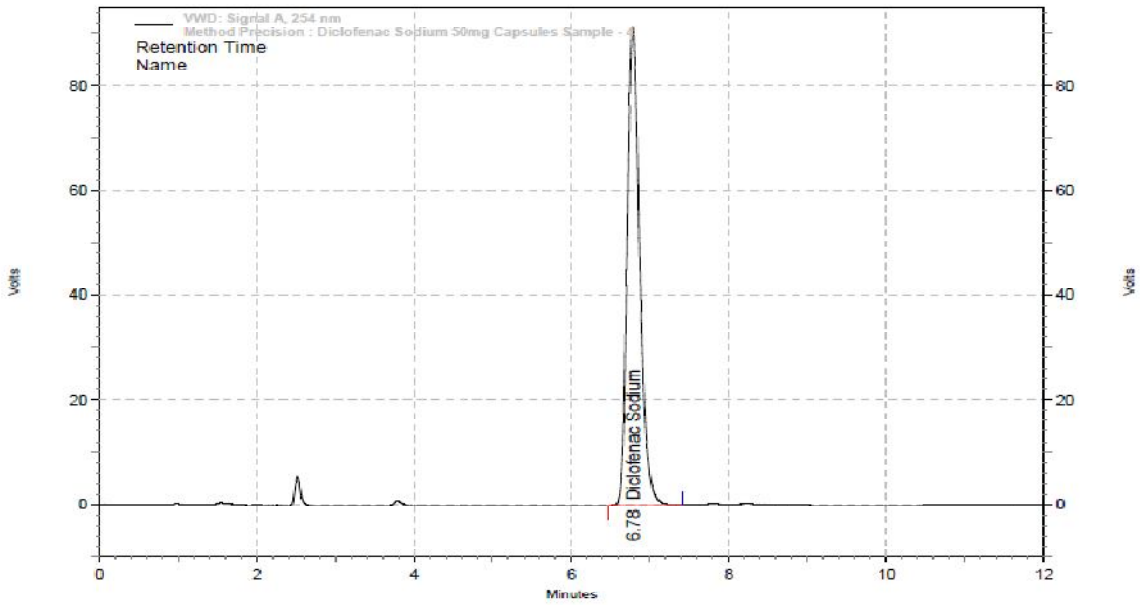
Chromatogram No – 8.44

Method precision-03 (Replicate no-2)



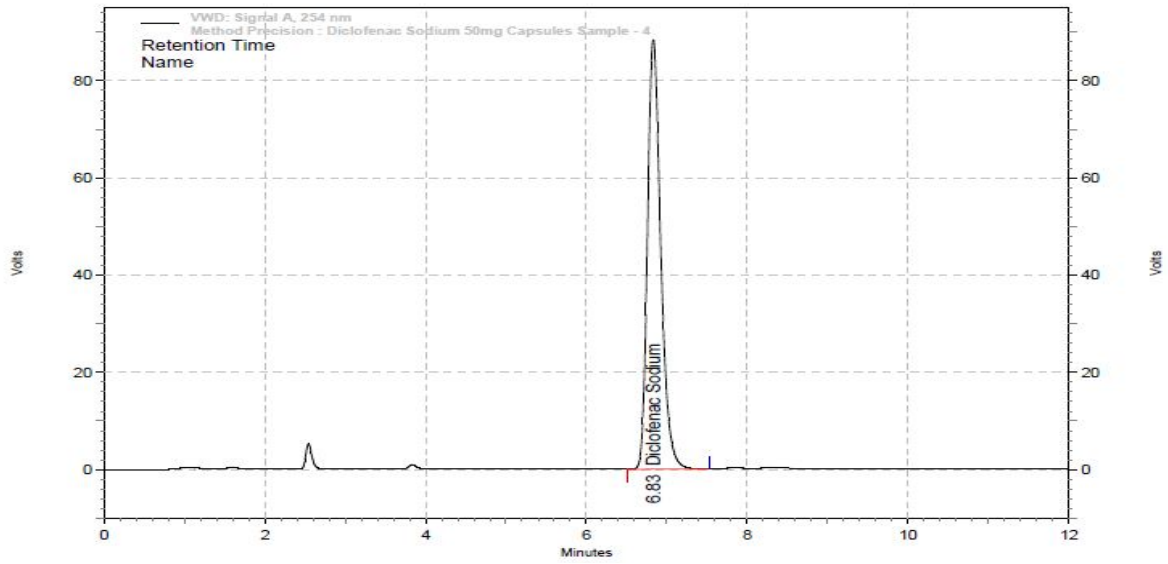
Chromatogram No – 8.45

Method precision-04 (Replicate no-1)



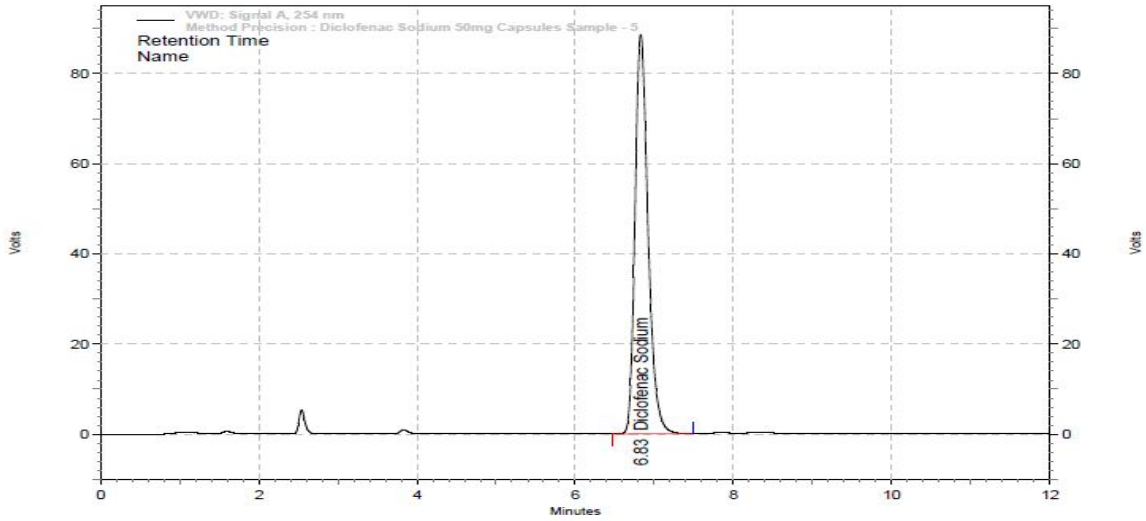
Chromatogram No – 8.46

Method precision-04 (Replicate no-2)



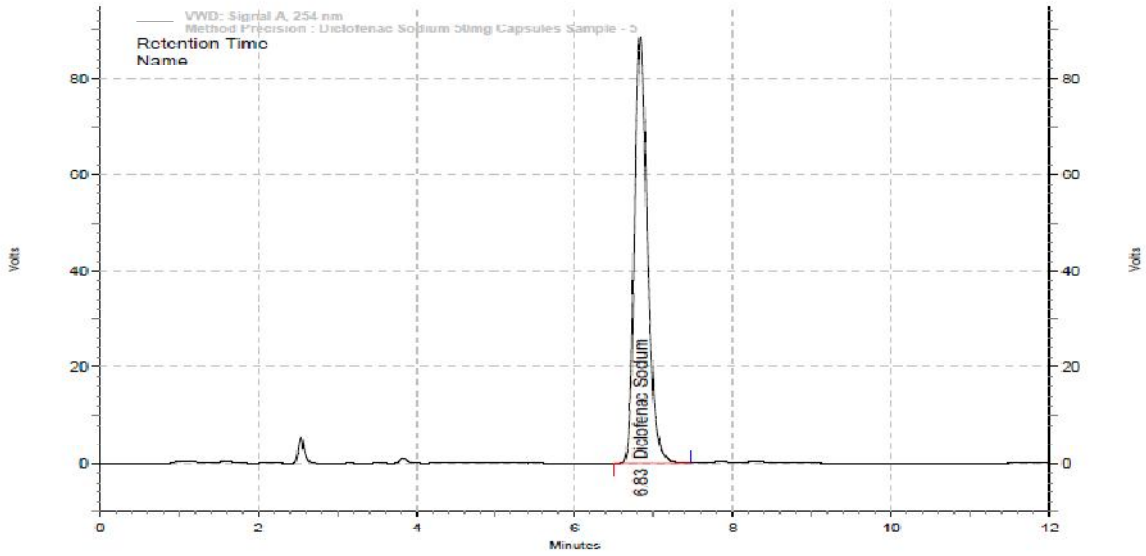
Chromatogram No – 8.47

Method precision-05 (Replicate no-1)



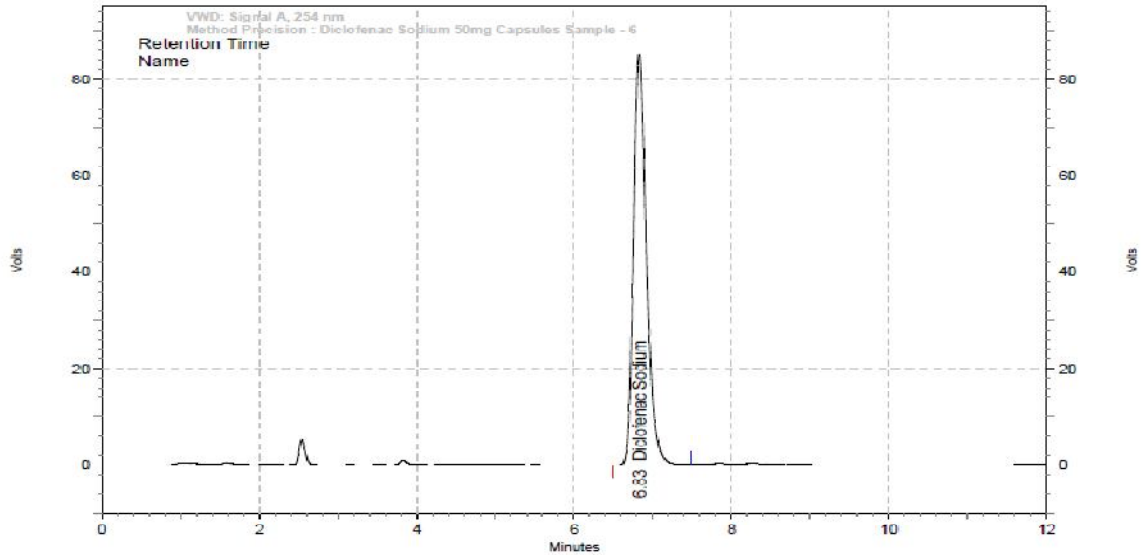
Chromatogram No – 8.48

Method precision-05 (Replicate no-2)



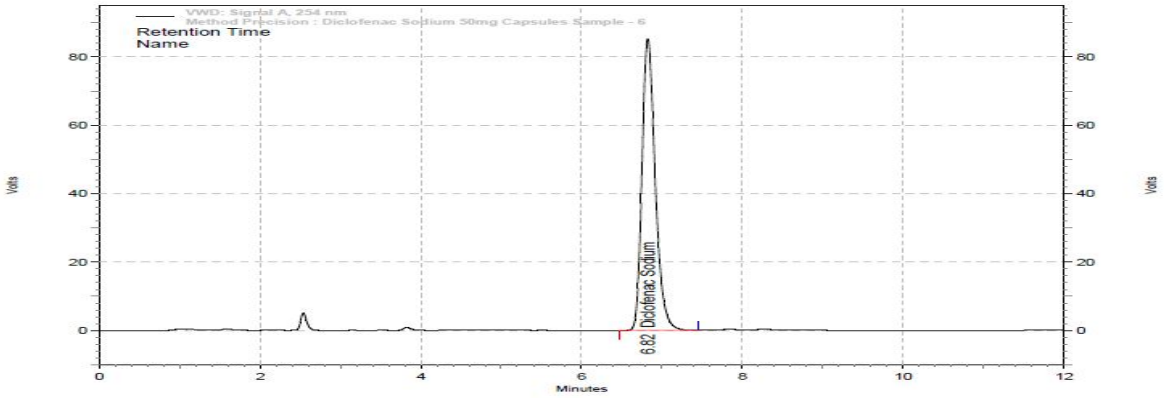
Chromatogram No – 8.49

Method precision-06 (Replicate no-1)



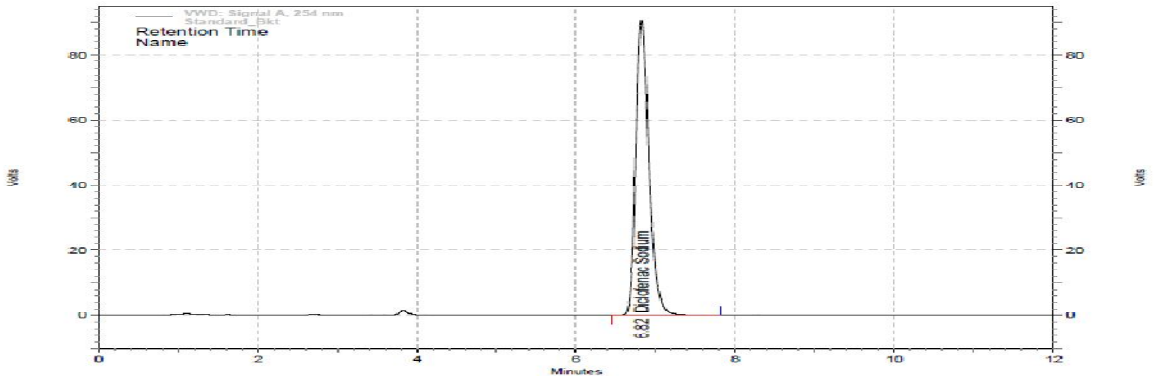
Chromatogram No – 8.50

Method precision-06 (Replicate no-2)



Chromatogram No – 8.51

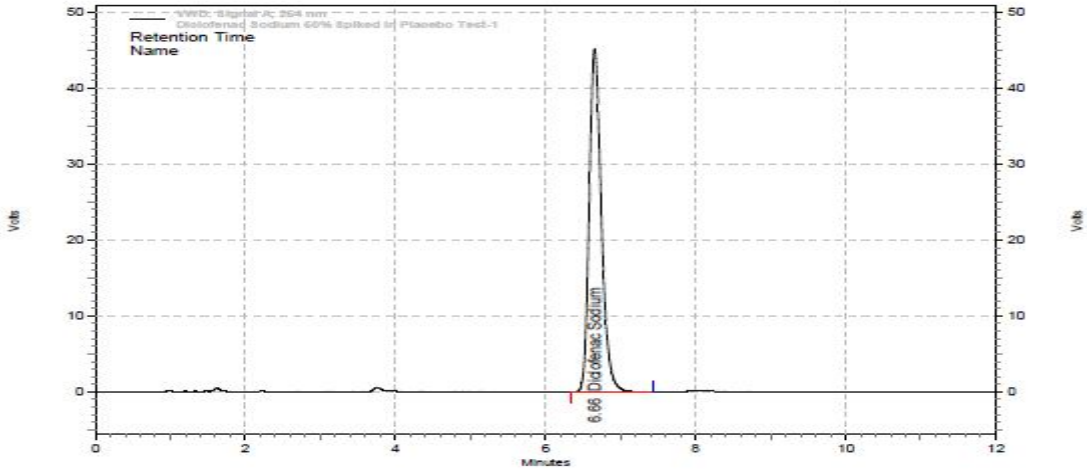
Bracketing standard



Accuracy

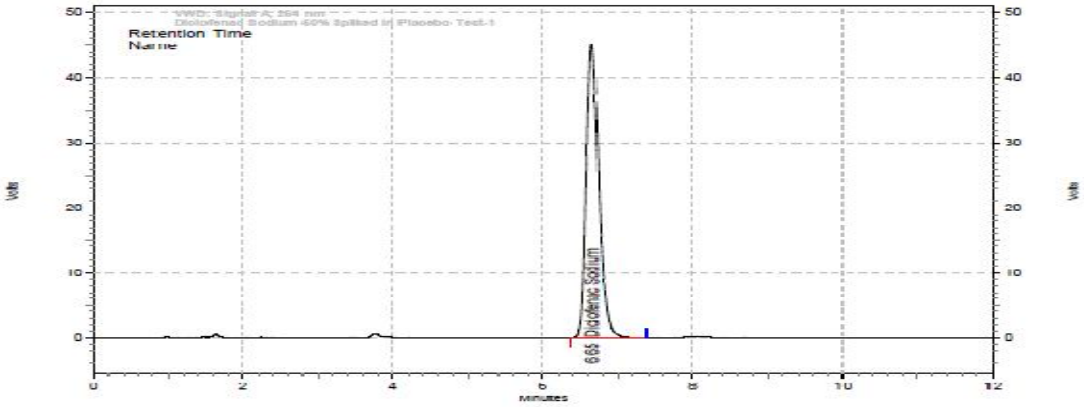
Chromatogram No – 8.52

Accuracy 50%-Preparation-1 (Replicate no-1)



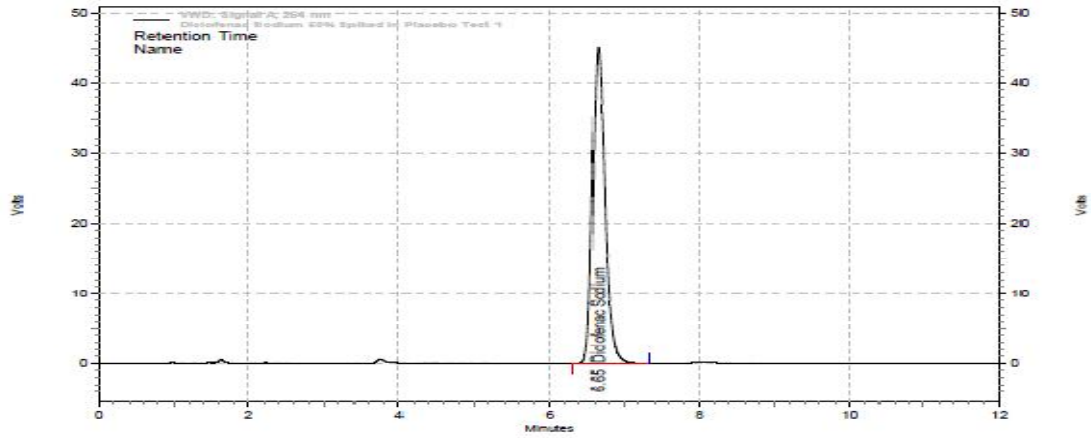
Chromatogram No – 8.53

Accuracy 50%-Preparation-1 (Replicate no-2)



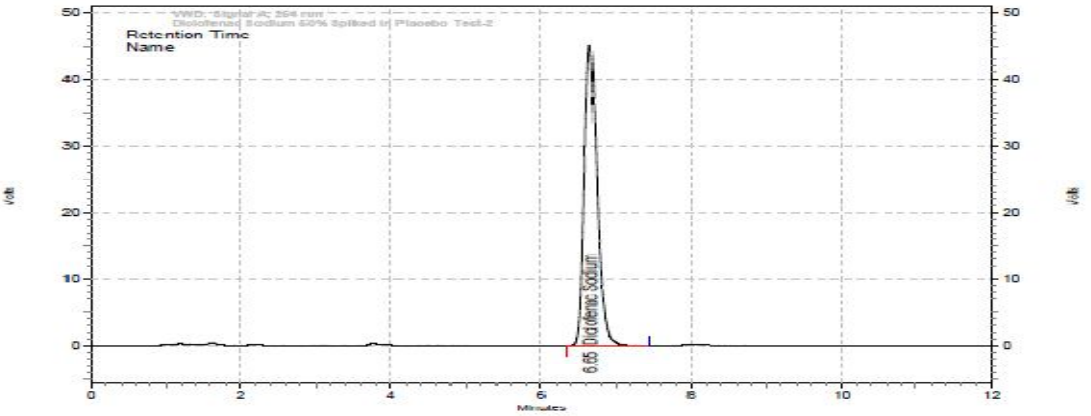
Chromatogram No – 8.54

Accuracy 50%-Preparation-1 (Replicate no-3)



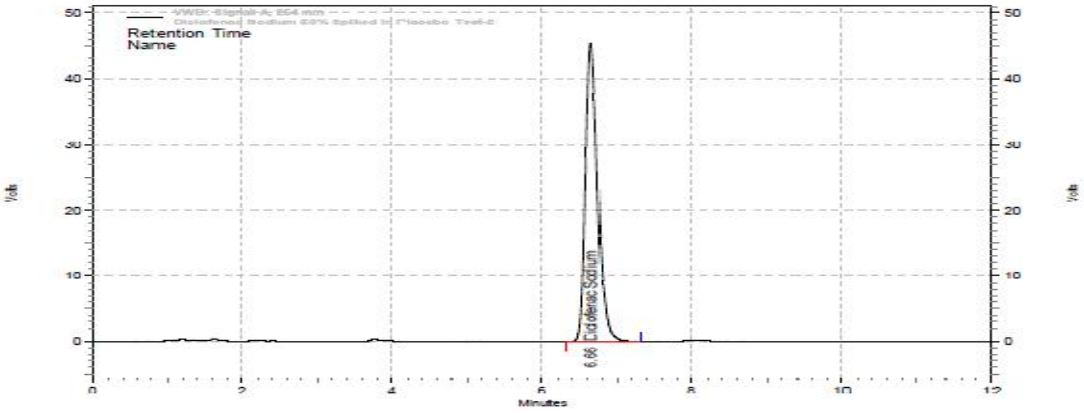
Chromatogram No – 8.55

Accuracy 50%-Preparation-2 (Replicate no-1)



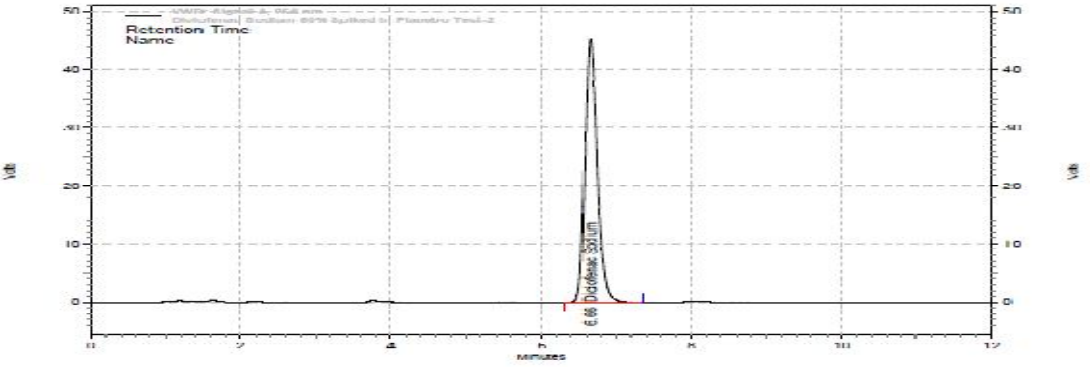
Chromatogram No – 8.56

Accuracy 50%-Preparation-2 (Replicate no-2)



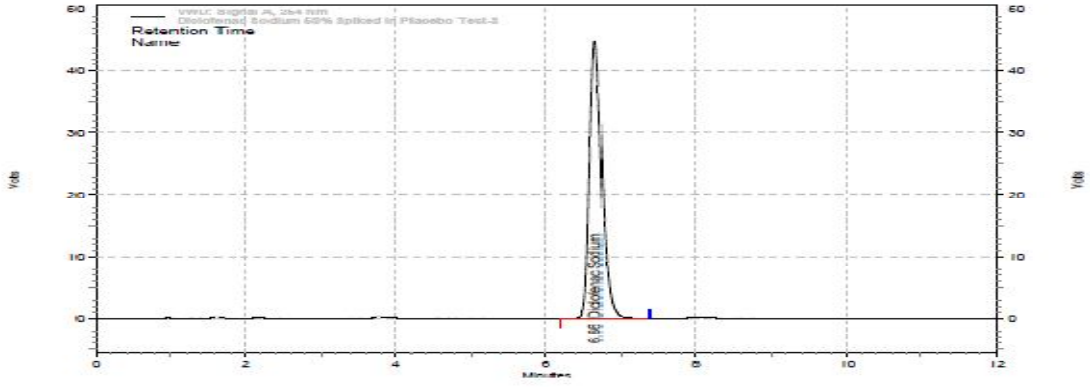
Chromatogram No – 8.57

Accuracy 50%-Preparation-2 (Replicate no-3)



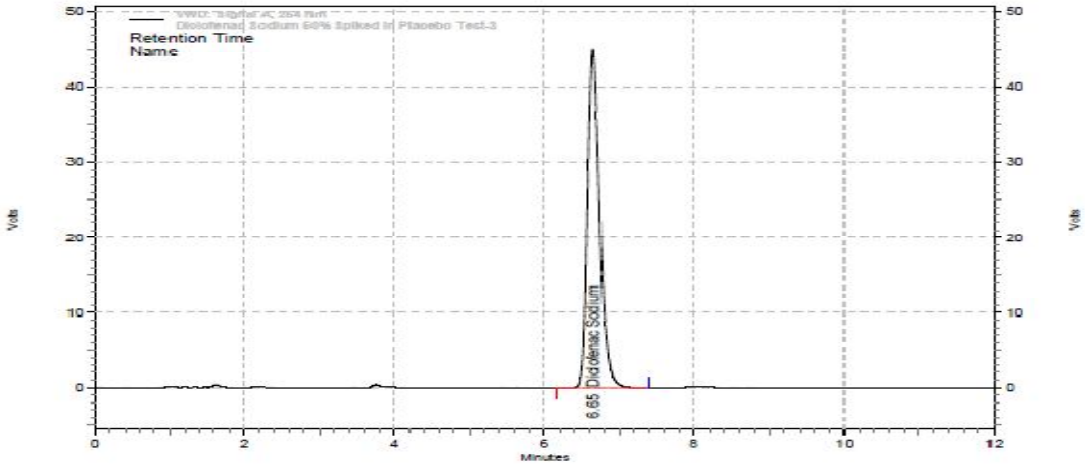
Chromatogram No – 8.58

Accuracy 50%-Preparation-3 (Replicate no-1)



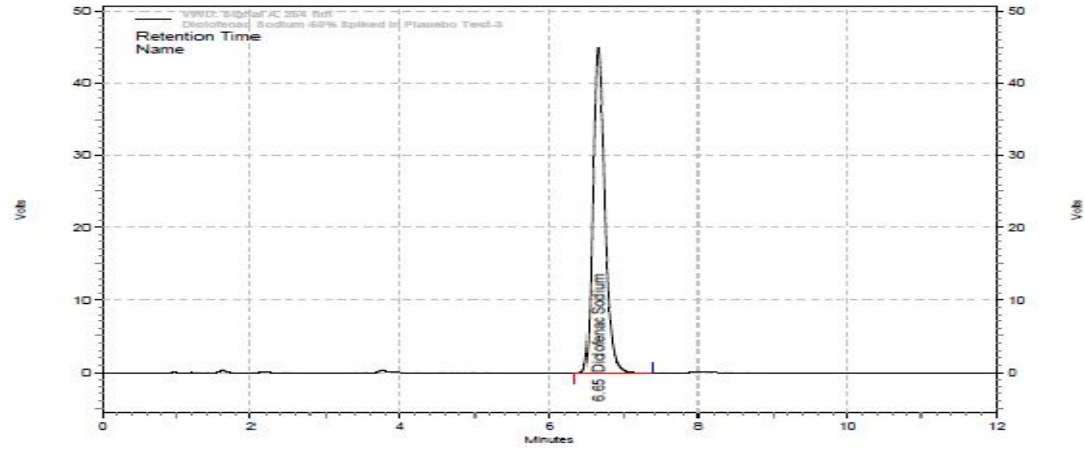
Chromatogram No – 8.59

Accuracy 50%-Preparation-3 (Replicate no-2)



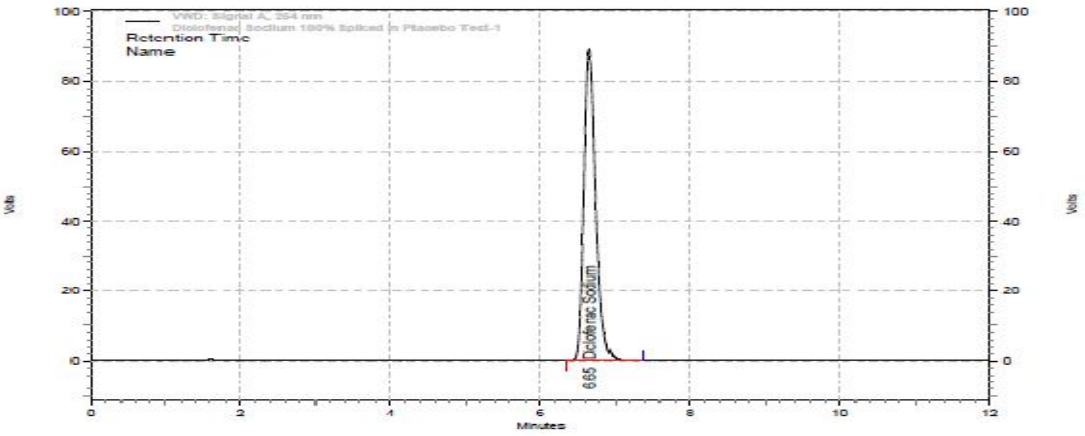
Chromatogram No – 8.60

Accuracy 50%-Preparation-3 (Replicate no-3)



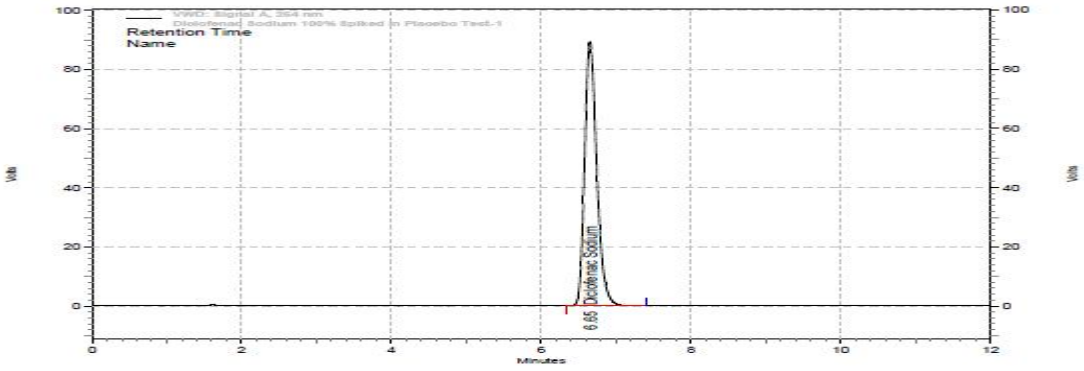
Chromatogram No – 8.61

Accuracy 100%-Preparation-1 (Replicate no-1)



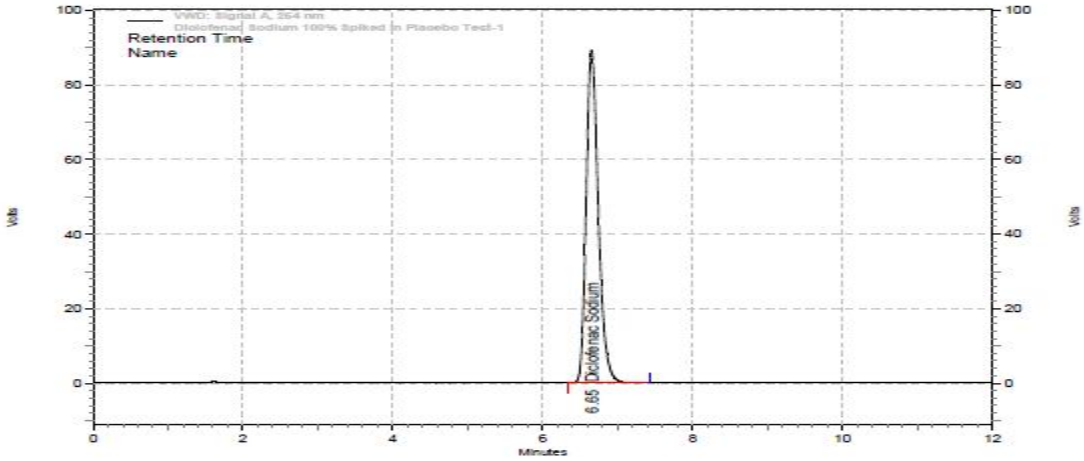
Chromatogram No – 8.62

Accuracy 100%-Preparation-1 (Replicate no-2)



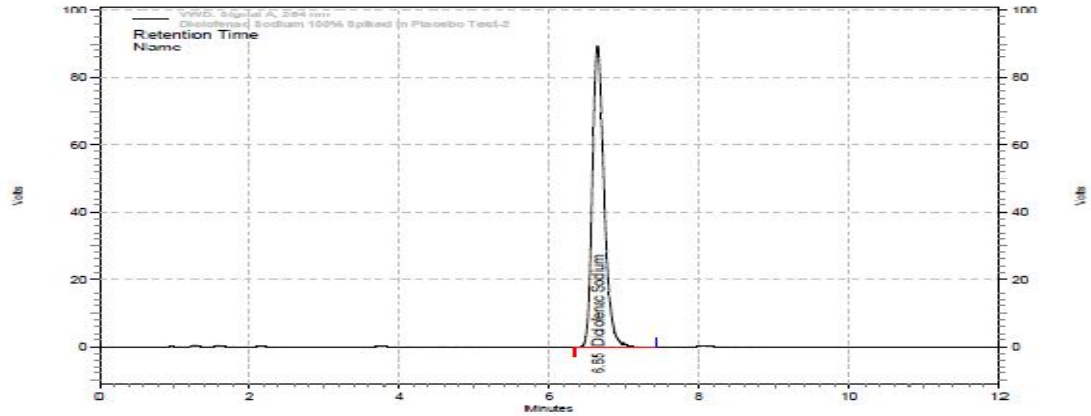
Chromatogram No – 8.63

Accuracy 100%-Preparation-1 (Replicate no-3)



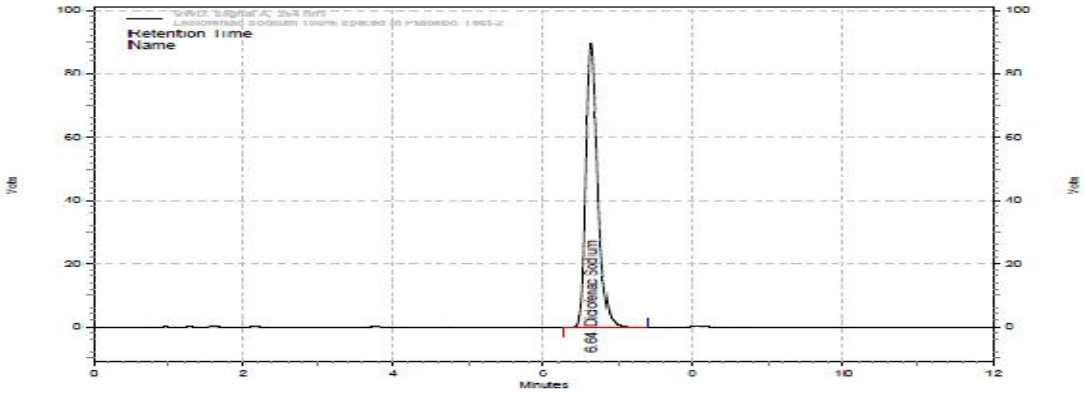
Chromatogram No – 8.64

Accuracy 100%-Preparation-2 (Replicate no-1)



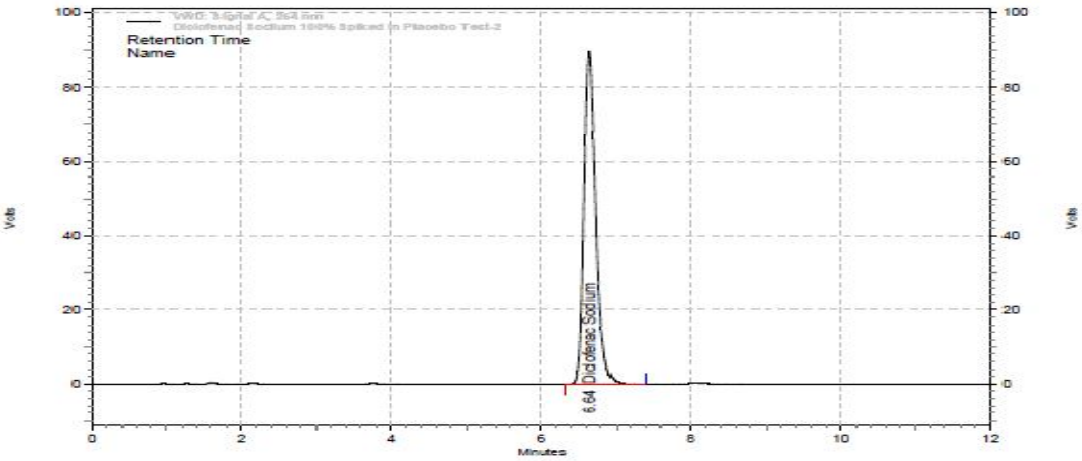
Chromatogram No – 8.65

Accuracy 100%-Preparation-2 (Replicate no-2)



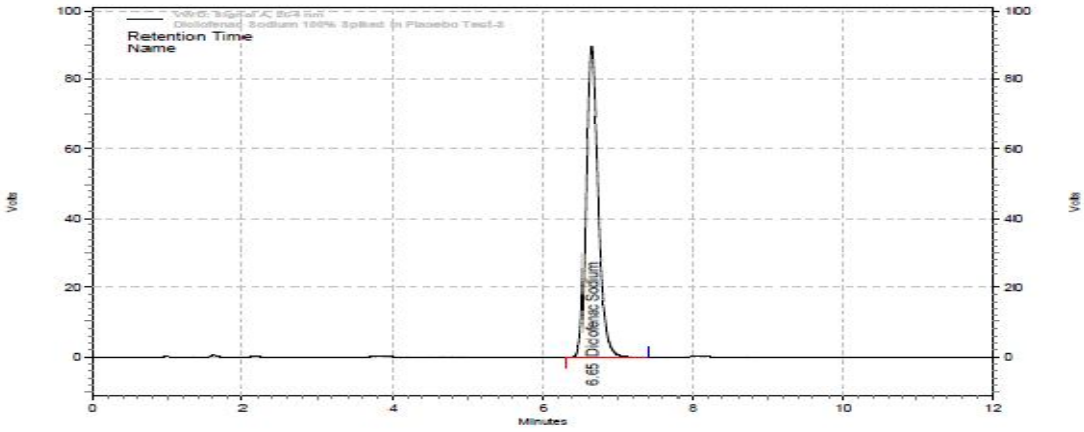
Chromatogram No – 8.66

Accuracy 100%-Preparation-2 (Replicate no-3)



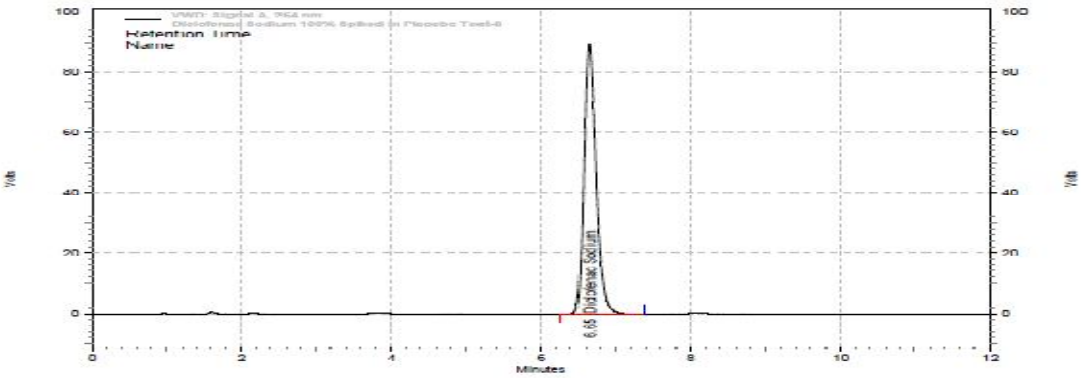
Chromatogram No – 8.67

Accuracy 100%-Preparation-3 (Replicate no-1)



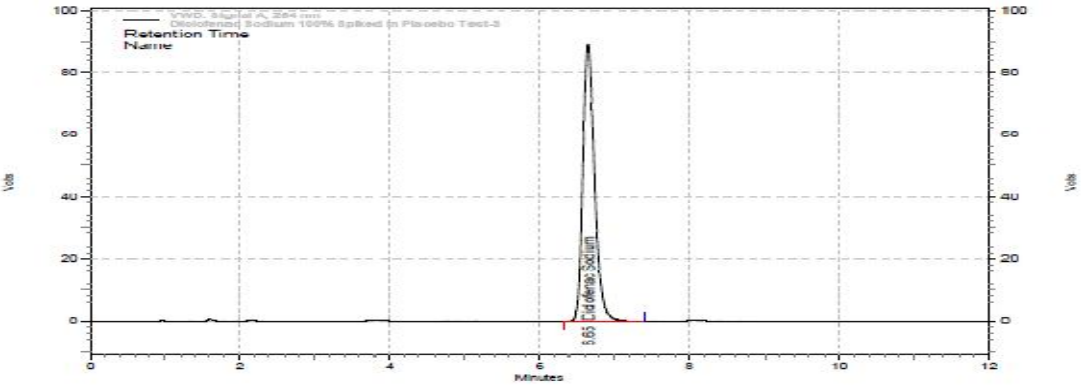
Chromatogram No – 8.68

Accuracy 100%-Preparation-3 (Replicate no-2)



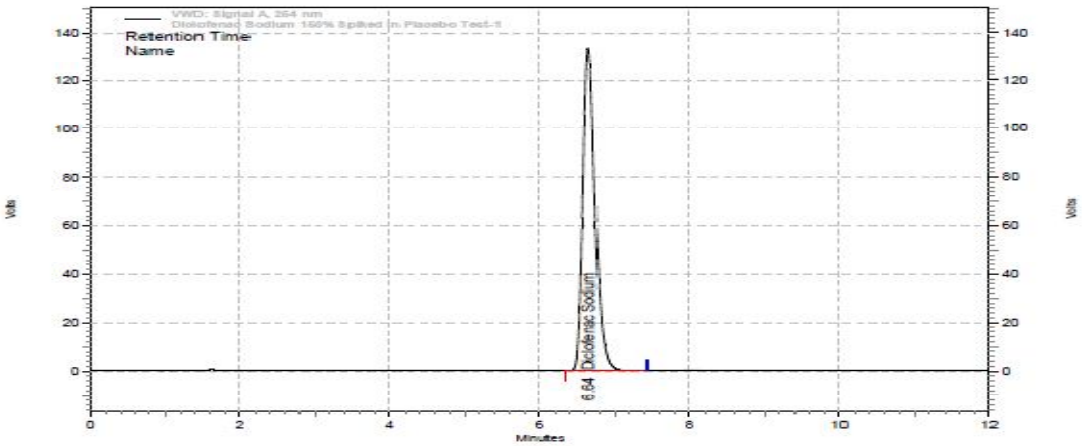
Chromatogram No – 8.69

Accuracy 100%-Preparation-3 (Replicate no-3)



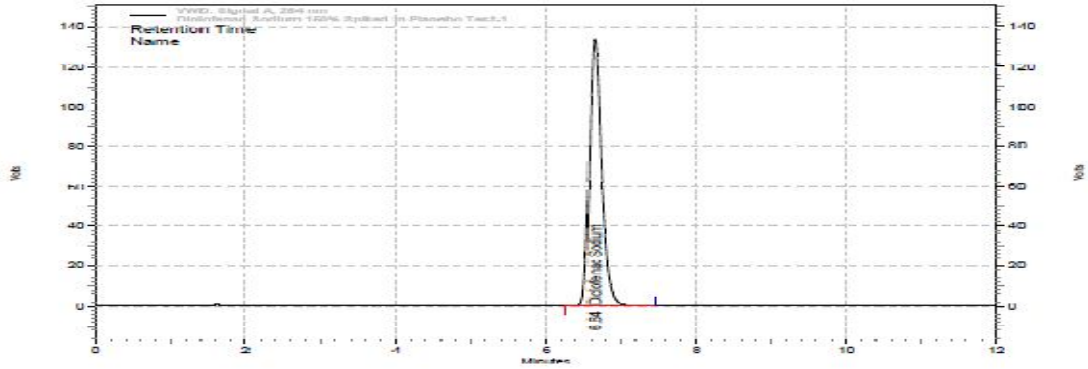
Chromatogram No – 8.70

Accuracy 150%-Preparation-1 (Replicate no-1)



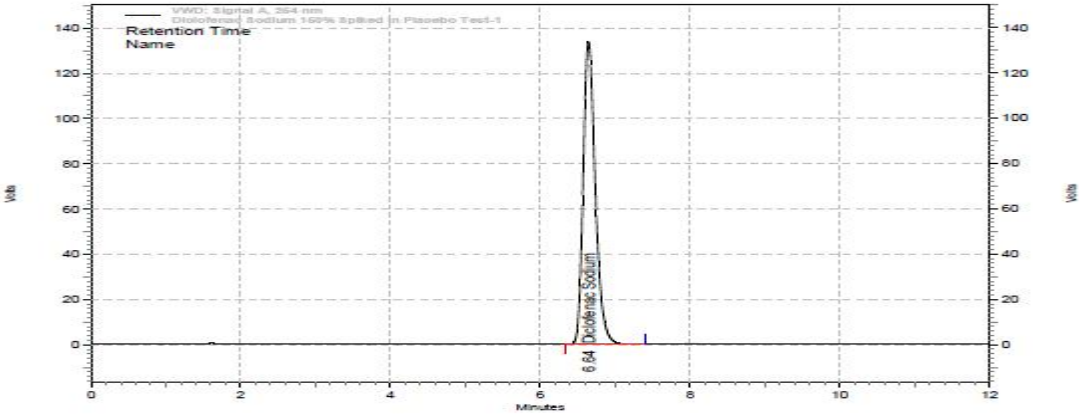
Chromatogram No – 8.71

Accuracy 150%-Preparation-1 (Replicate no-2)



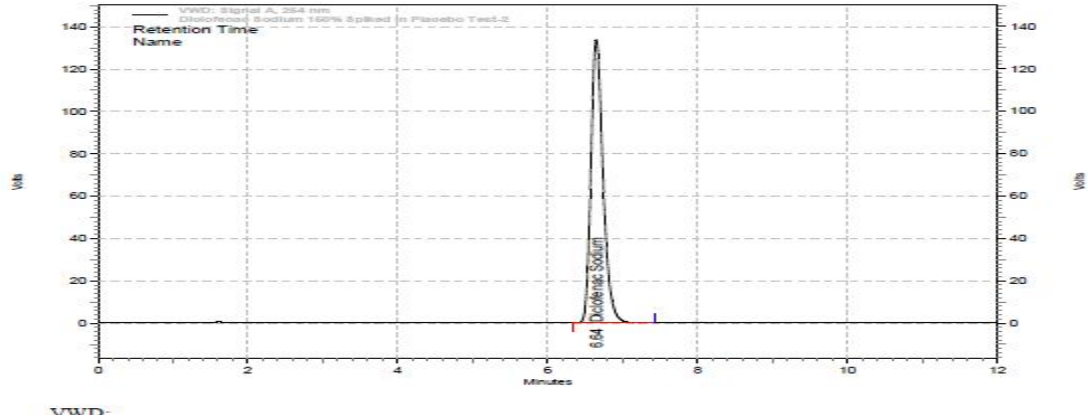
Chromatogram No – 8.72

Accuracy 150%-Preparation-1 (Replicate no-3)



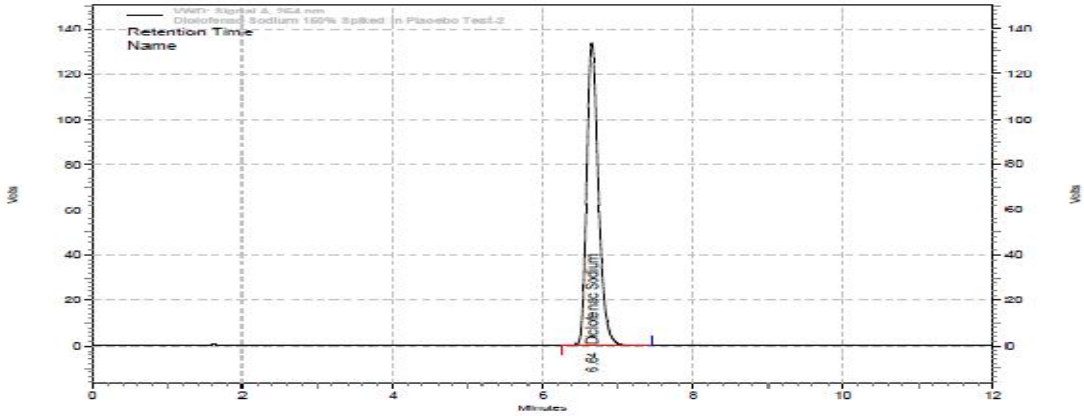
Chromatogram No – 8.73

Accuracy 150%-Preparation-2 (Replicate no-1)



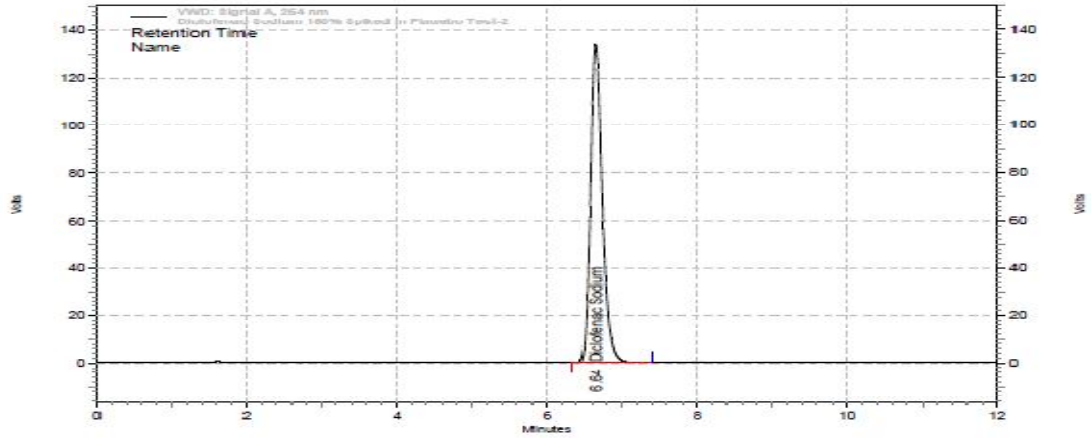
Chromatogram No – 8.74

Accuracy 150%-Preparation-2 (Replicate no-2)



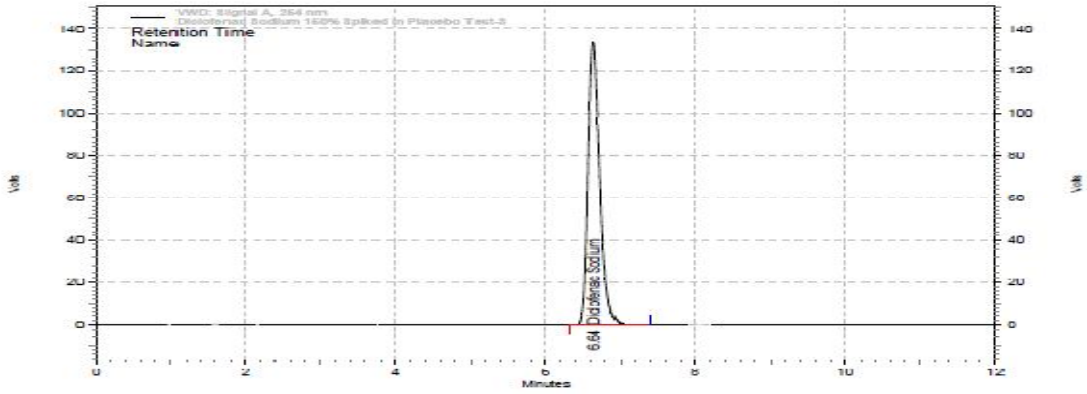
Chromatogram No – 8.75

Accuracy 150%-Preparation-2 (Replicate no-3)



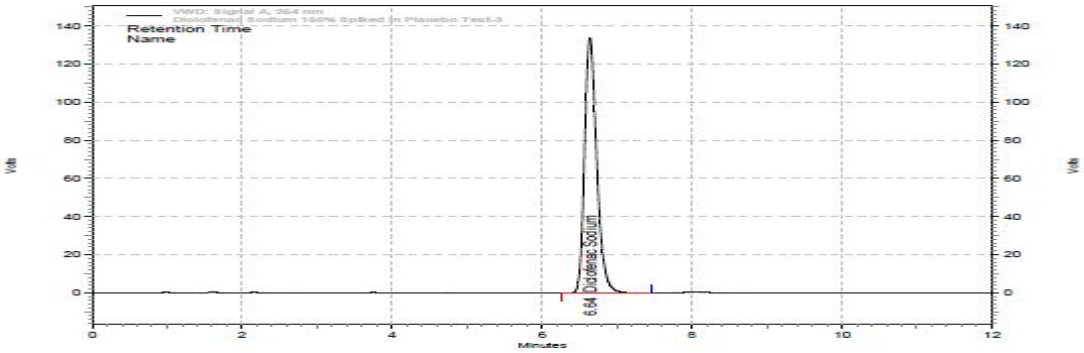
Chromatogram No – 8.76

Accuracy 150%-Preparation-3 (Replicate no-1)



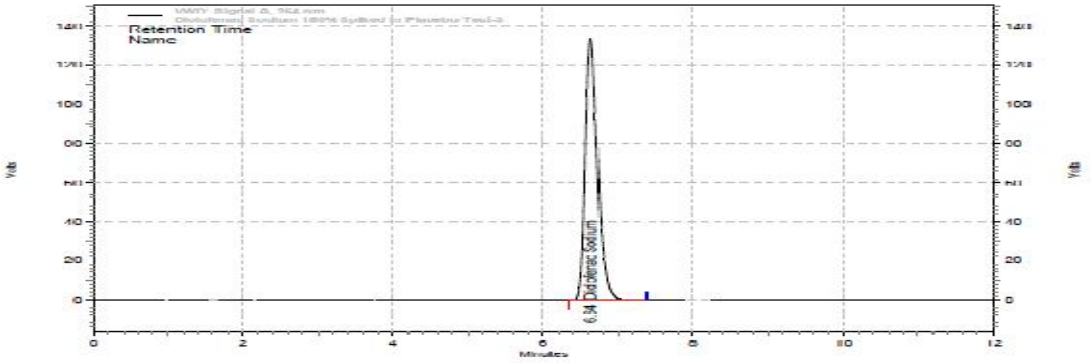
Chromatogram No – 8.77

Accuracy 150%-Preparation-3 (Replicate no-2)



Chromatogram No – 8.78

Accuracy 150%-Preparation-3 (Replicate no-3)

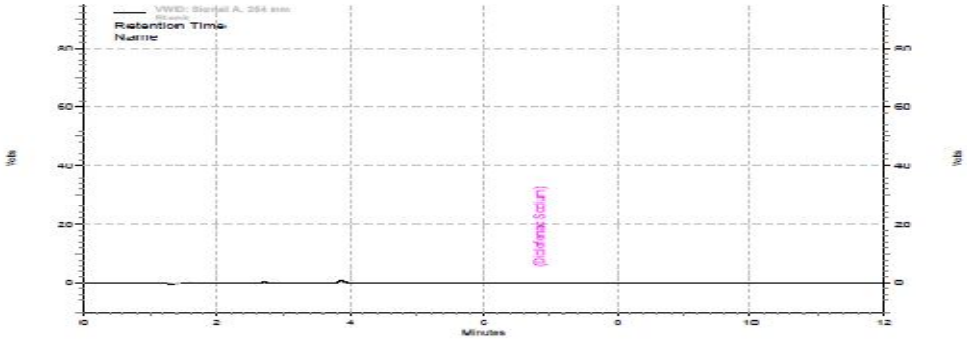


RUGGEDNESS

(Blank ,standards, Sample preparations 1,2,3,4,5,6)

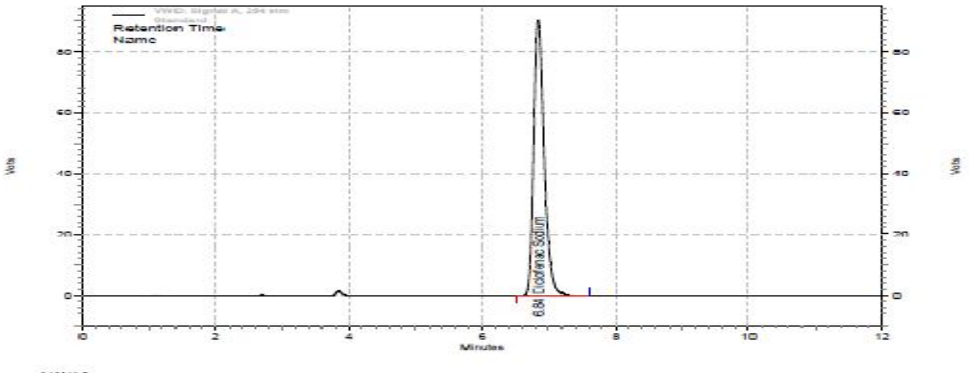
Chromatogram No – 8.78

Blank



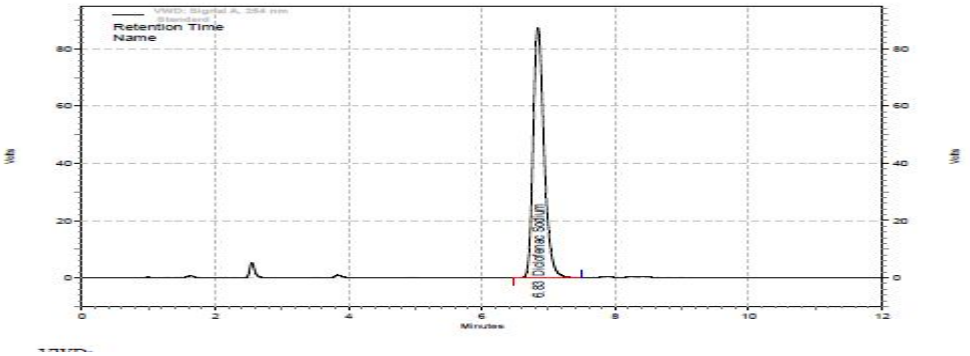
Chromatogram No – 8.79

Standard (Replicate no-1)



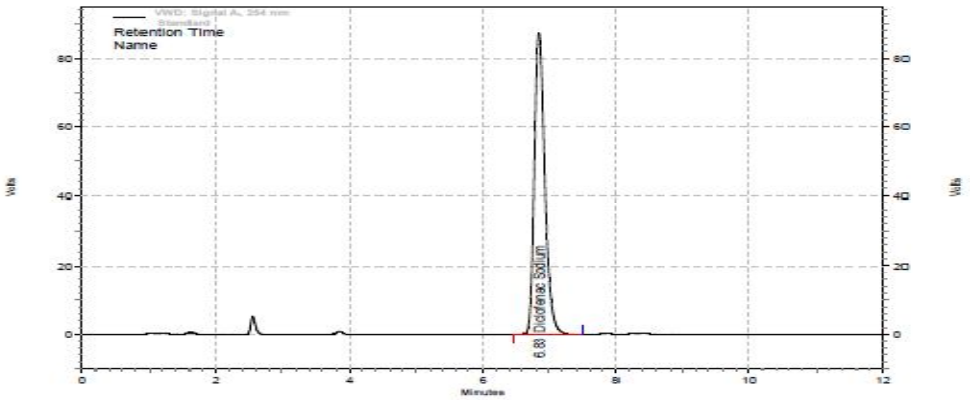
Chromatogram No – 8.80

Standard (Replicate no-2)



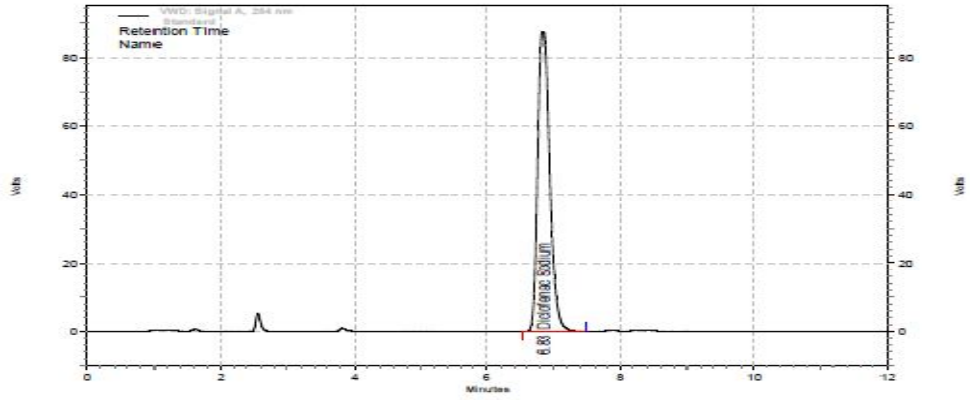
Chromatogram No – 8.81

Standard (Replicate no-3)



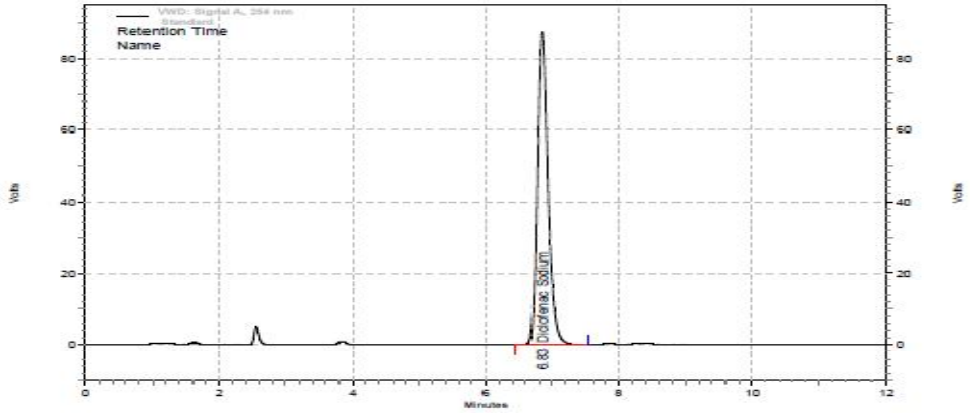
Chromatogram No – 8.82

Standard (Replicate no-4)



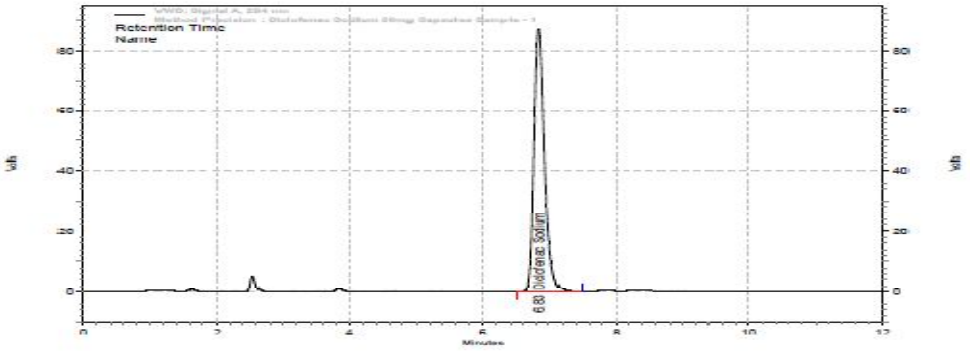
Chromatogram No – 8.83

Standard (Replicate no-5)



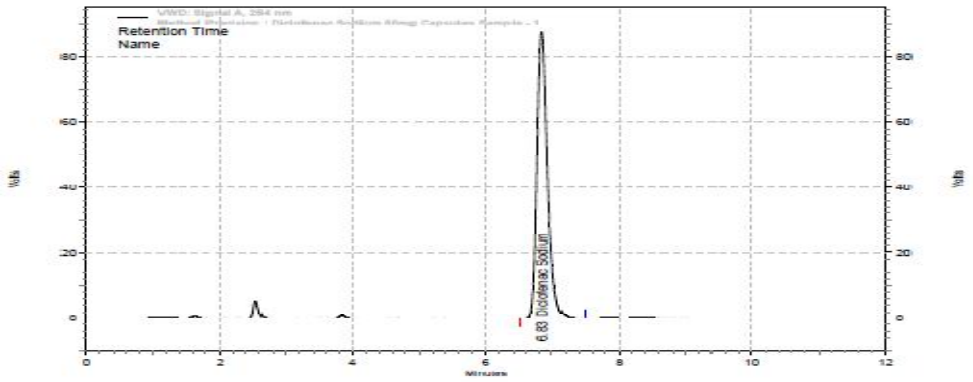
Chromatogram No – 8.84

Sample-01 (Replicate no-1)



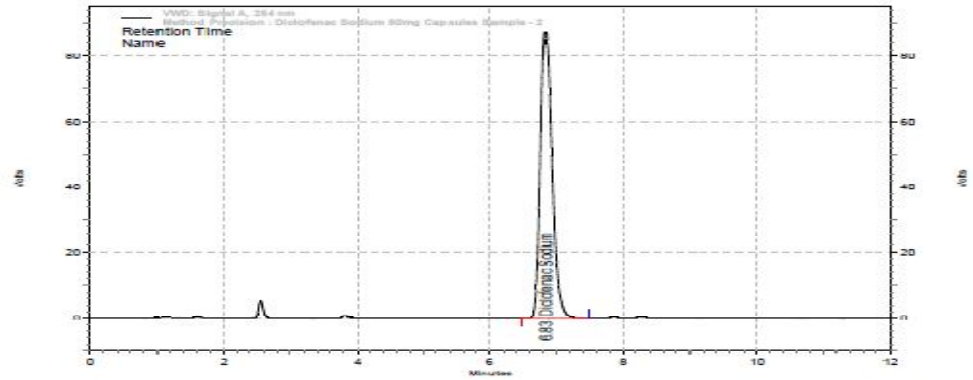
Chromatogram No – 8.85

Sample-01 (Replicate no-2)



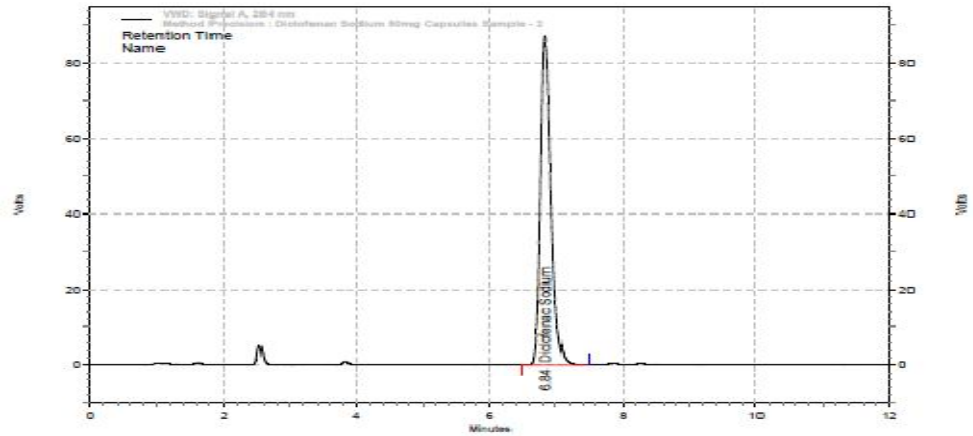
Chromatogram No – 8.86

Sample-02 (Replicate no-1)



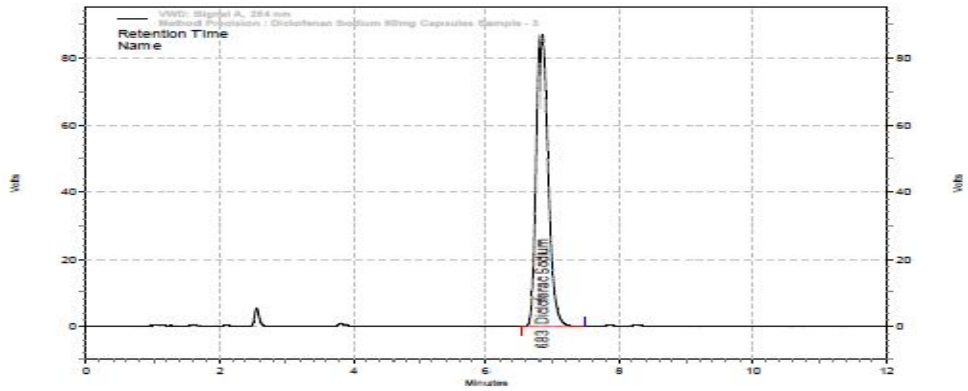
Chromatogram No – 8.87

Sample-02 (Replicate no-2)



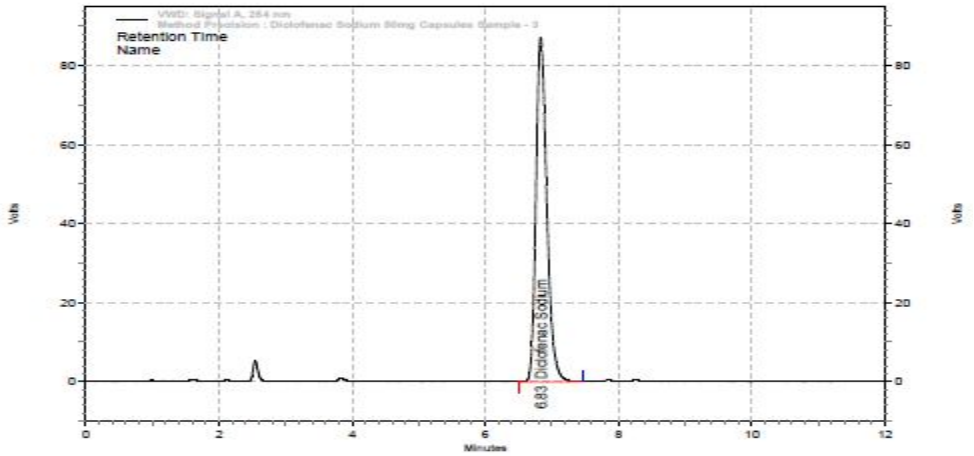
Chromatogram No – 8.88

Sample-03 (Replicate no-1)



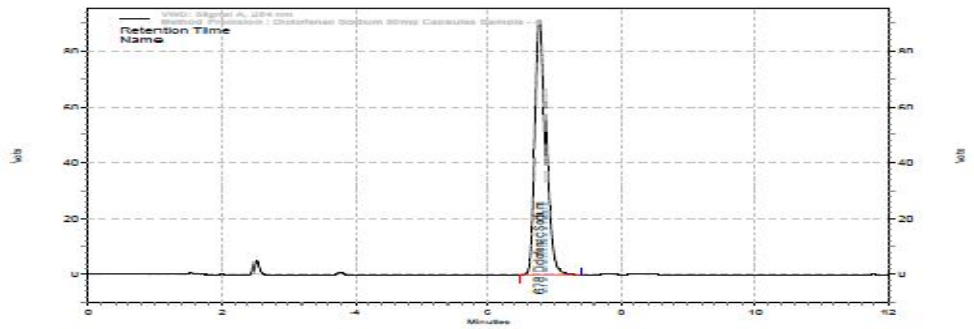
Chromatogram No – 8.89

Sample-03 (Replicate no-2)



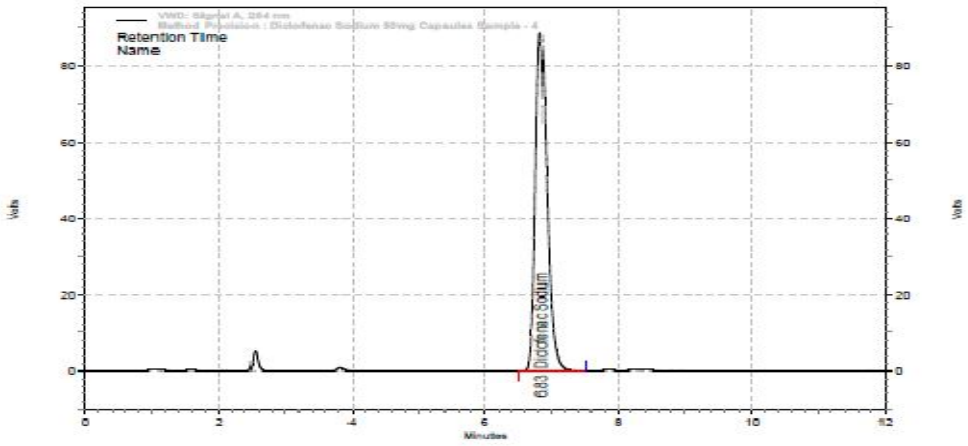
Chromatogram No – 8.90

Sample-04 (Replicate no-1)



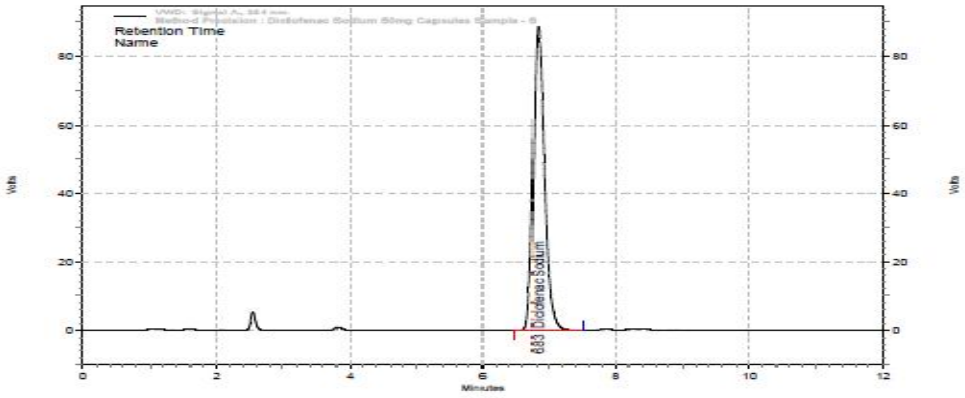
Chromatogram No – 8.91

Sample-04 (Replicate no-2)



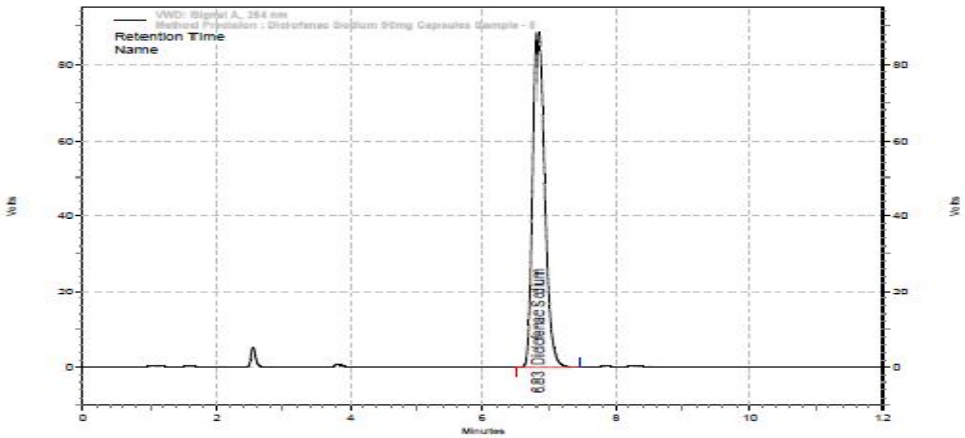
Chromatogram No – 8.92

Sample-05 (Replicate no-1)



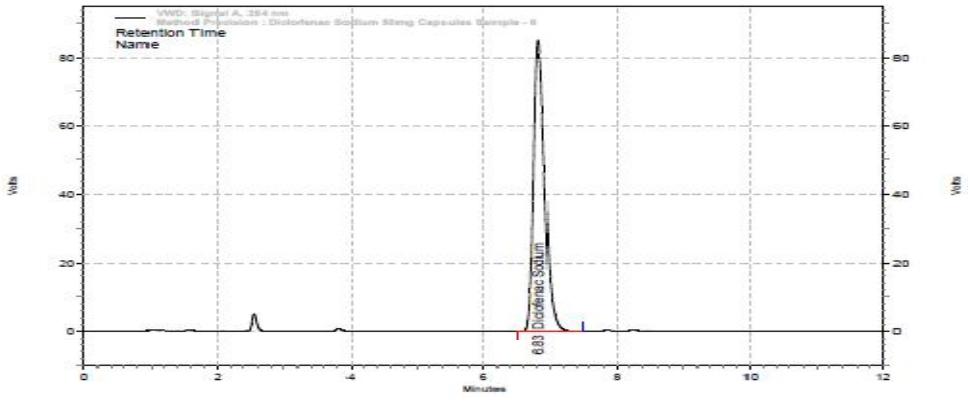
Chromatogram No – 8.93

Sample-05 (Replicate no-2)



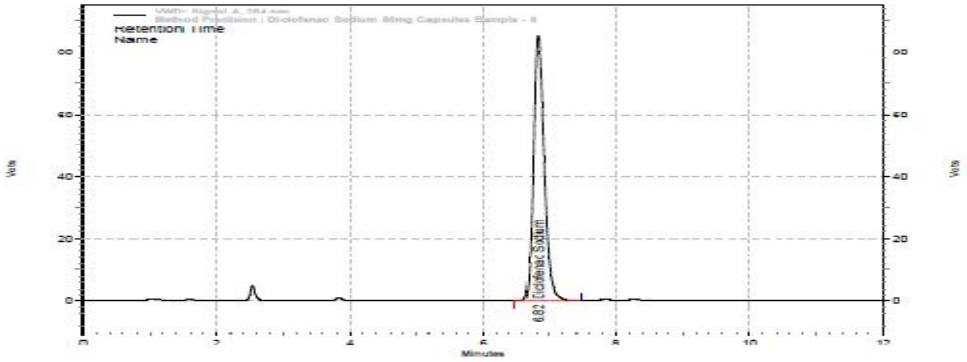
Chromatogram No – 8.94

Sample-06 (Replicate no-1)



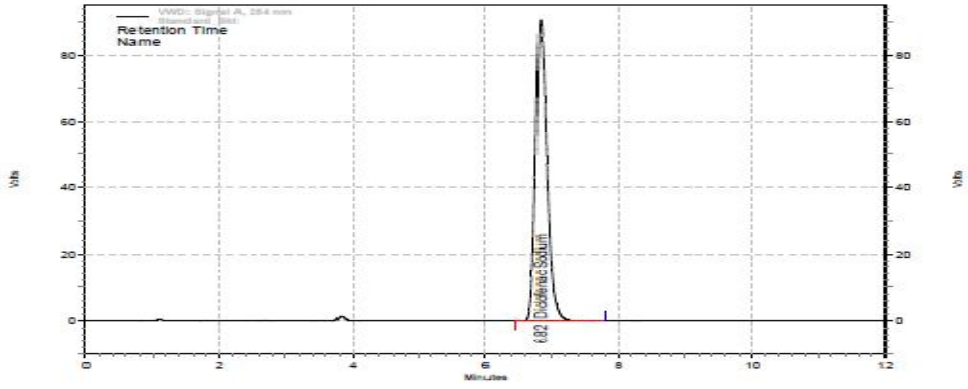
Chromatogram No – 8.95

Sample-06 (Replicate no-2)



Chromatogram No – 8.96

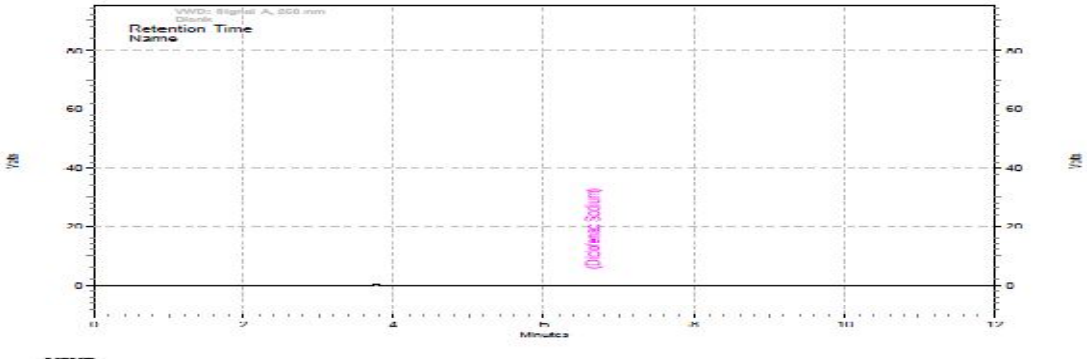
Bracketing standard



ROBUSTNESS

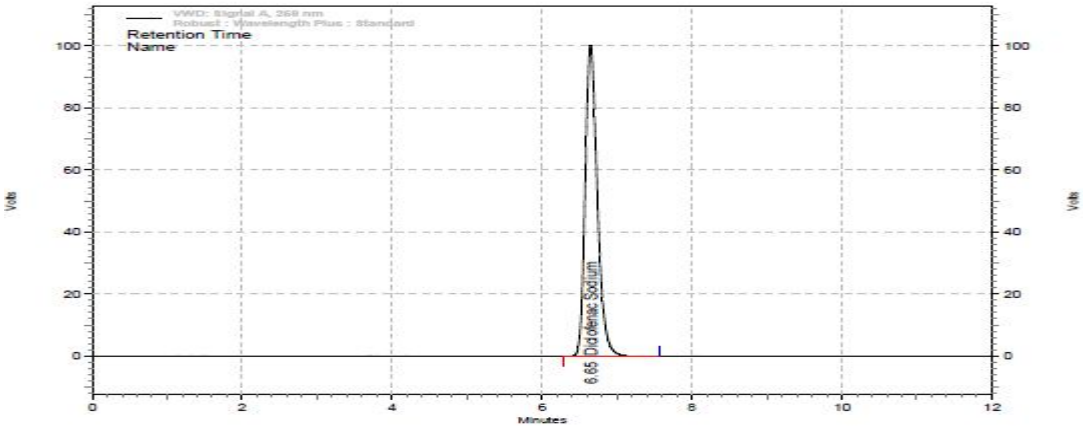
Chromatogram No – 8.97

Change in the wave length -256nm Blank



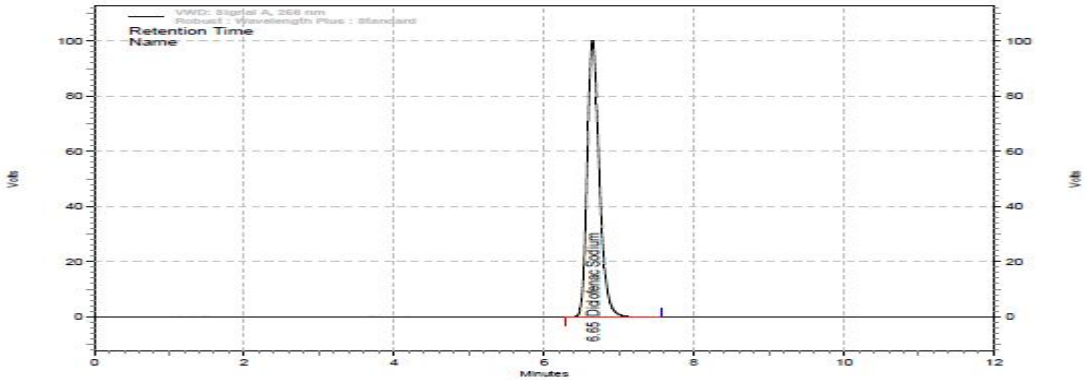
Chromatogram No – 8.98

Change in the wave length -256nm Standard
Replicate no-1



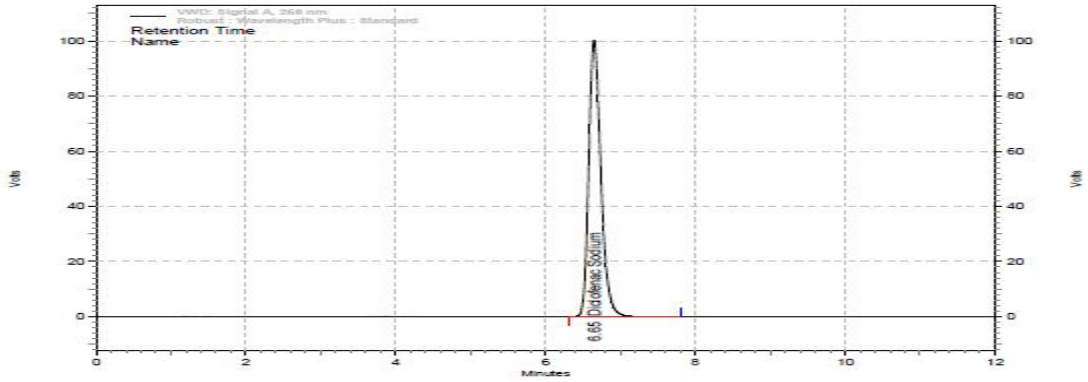
Chromatogram No – 8.99

Change in the wave length -256nm Standard
Replicate no-2



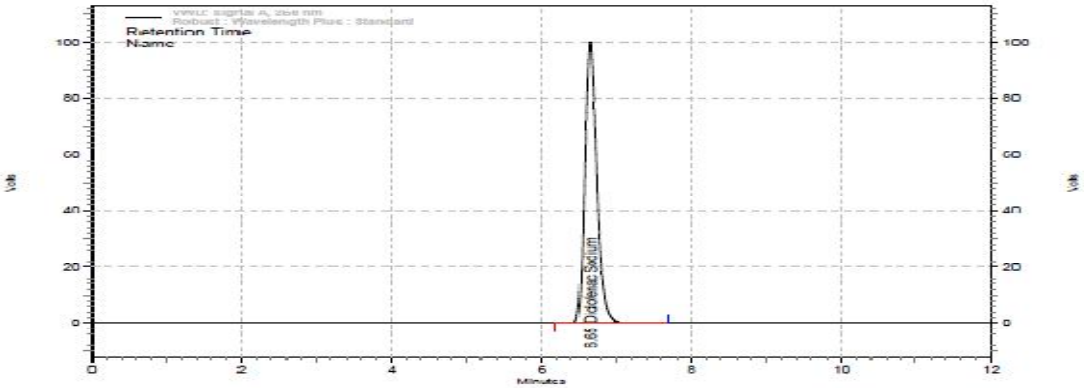
Chromatogram No – 8.100

Change in the wave length -256nm Standard
Replicate no-3



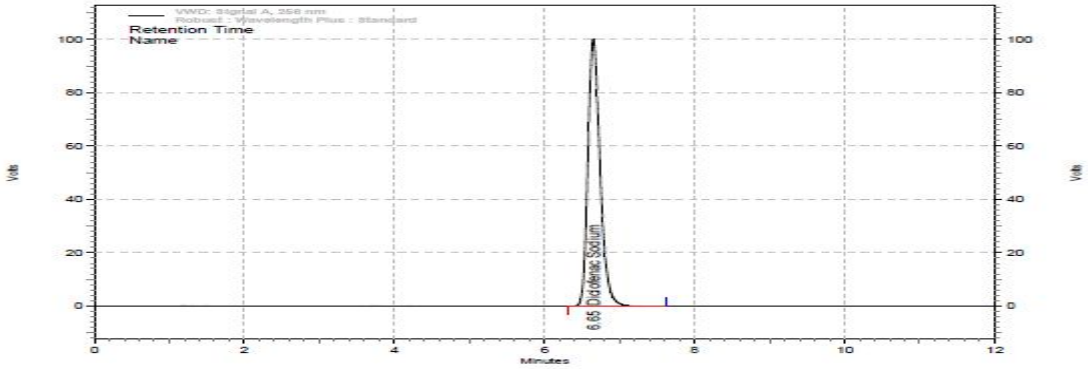
Chromatogram No – 8.101

Change in the wave length -256nm Standard
Replicate no-4



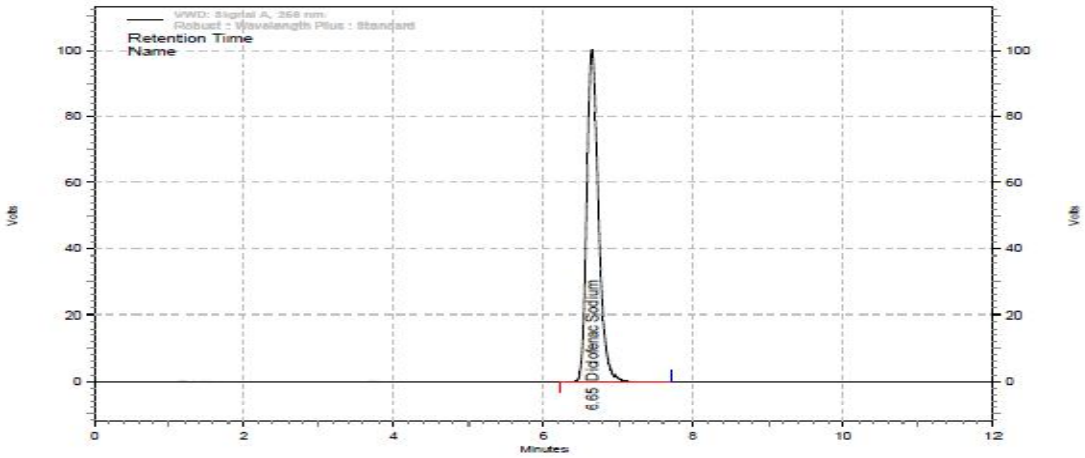
Chromatogram No – 8.102

Change in the wave length -256nm Standard
Replicate no-5



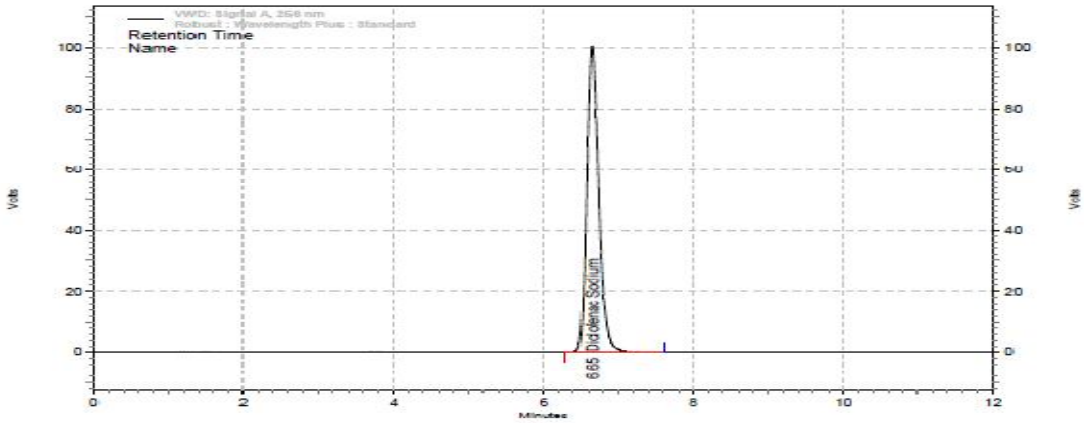
Chromatogram No – 8.103

Change in the wave length -256nm Sample Replicate no-1



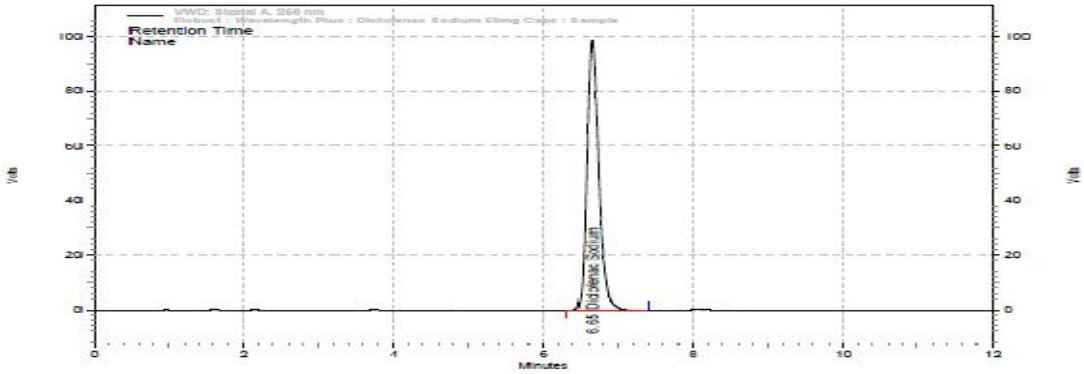
Chromatogram No – 8.104

Change in the wave length -256nm Sample Replicate no-2



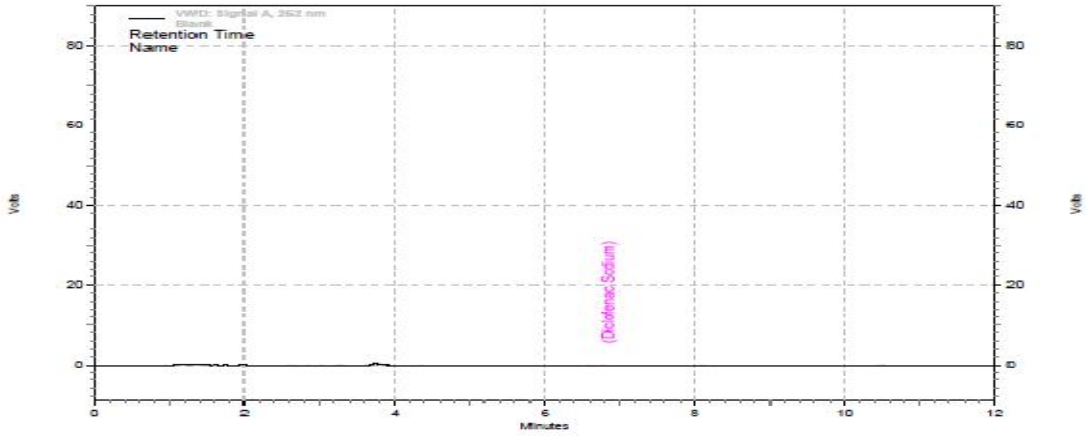
Chromatogram No – 8.105

Change in the wave length -256nm Bracketing standard



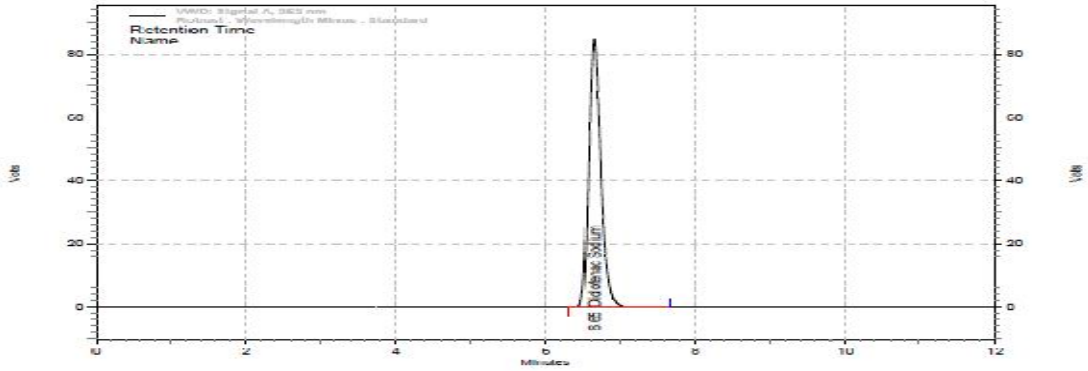
Chromatogram No – 8.106

Change in the wave length -252nm Blank



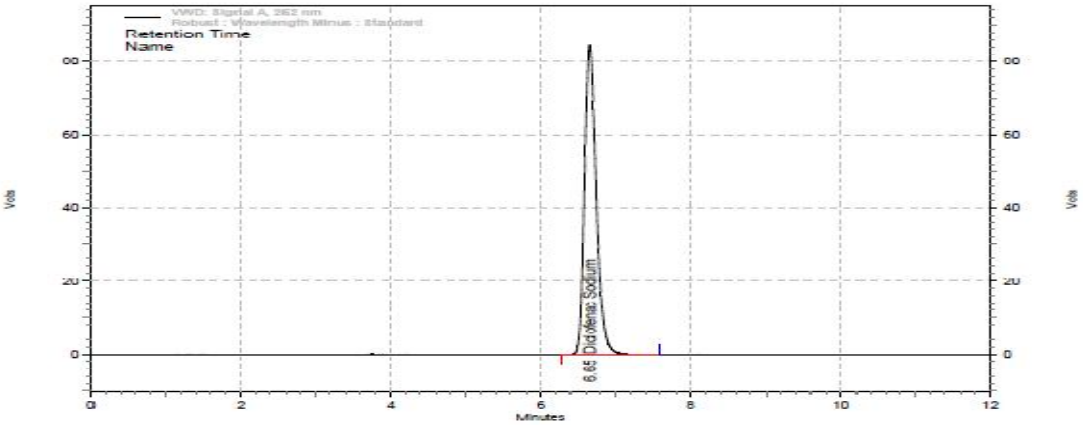
Chromatogram No – 8.107

Change in the wave length -252nm Standard
Replicate no-1



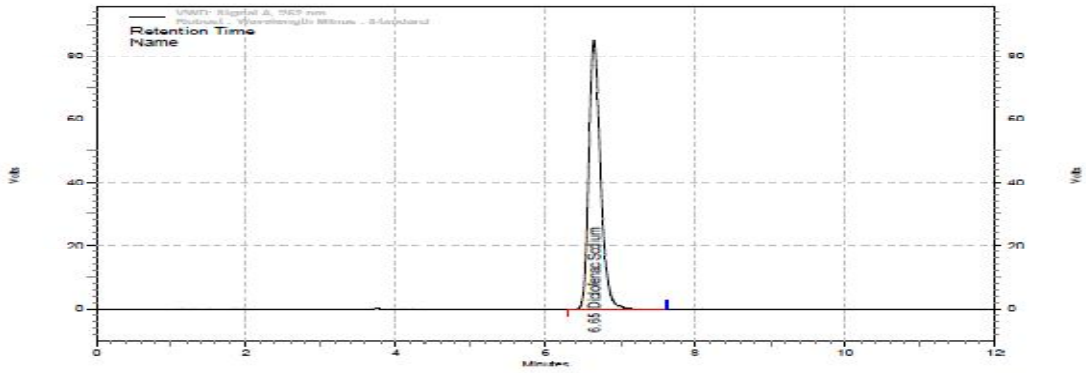
Chromatogram No – 8.108

Change in the wave length -252nm Standard
Replicate no-2



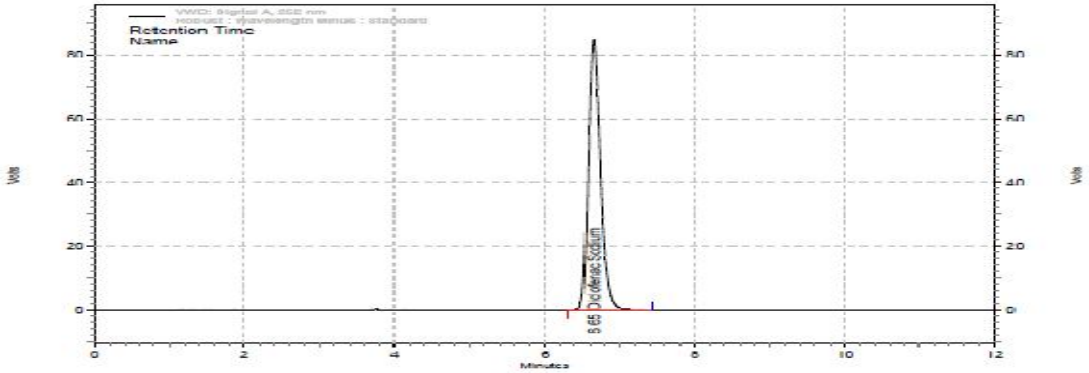
Chromatogram No – 8.109

Change in the wave length -252nm Standard
Replicate no-3



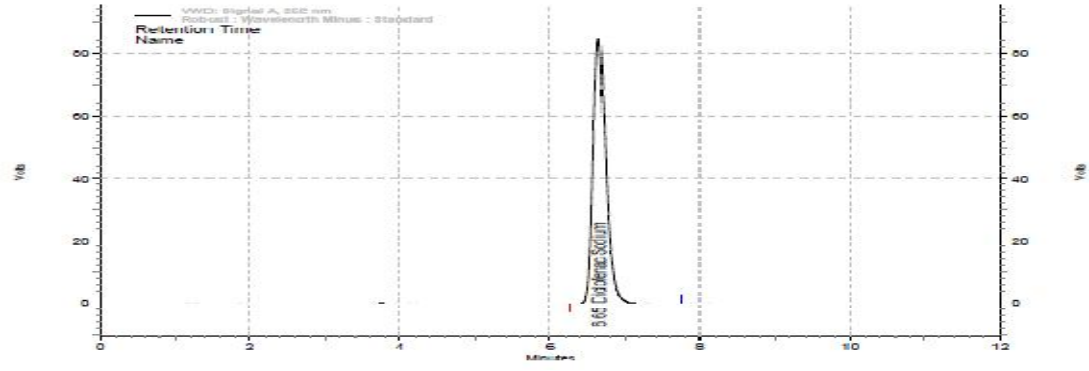
Chromatogram No – 8.110

Change in the wave length -252nm Standard
Replicate no-4



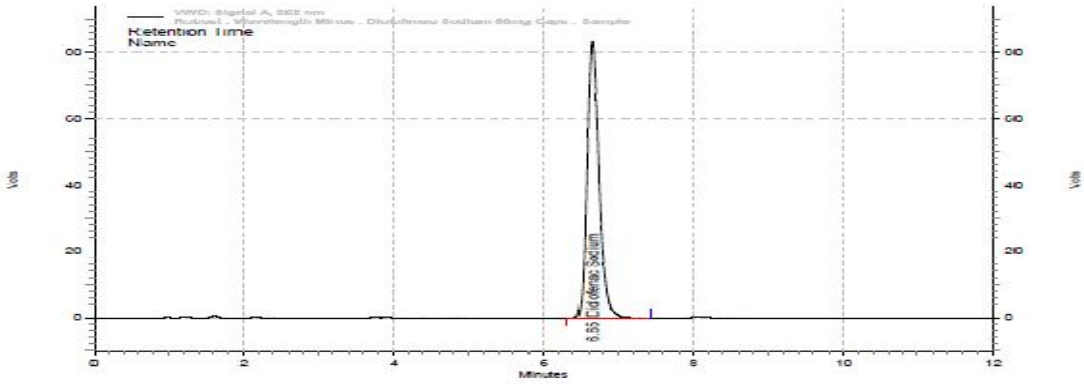
Chromatogram No – 8.111

Change in the wave length -252nm Standard
Replicate no-5



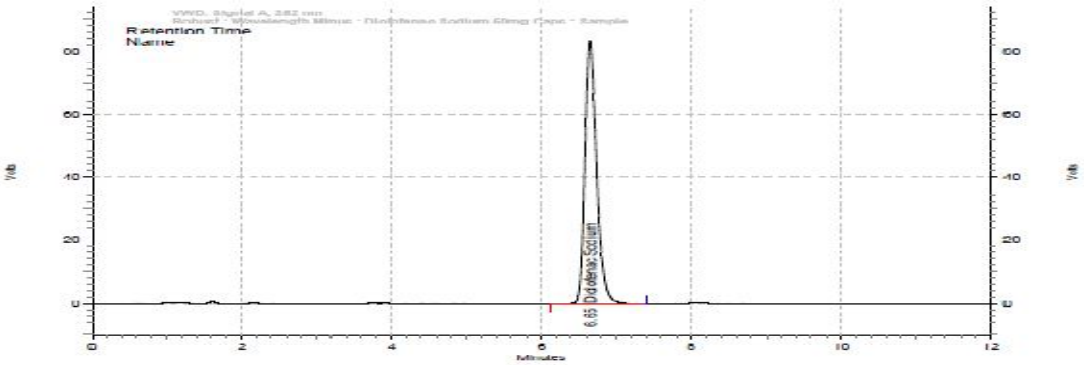
Chromatogram No – 8.112

Change in the wave length -252nm Sample Replicate no-1



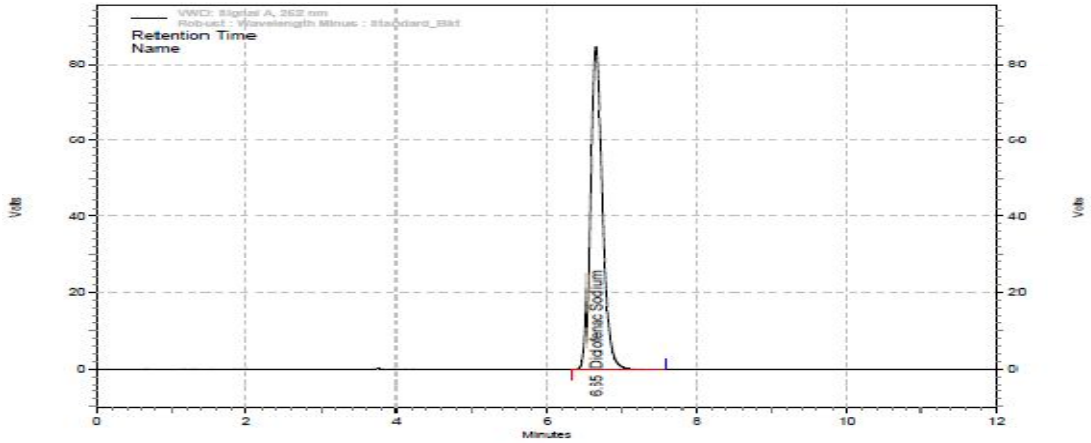
Chromatogram No – 8.113

Change in the wave length -252nm Sample Replicate no-2



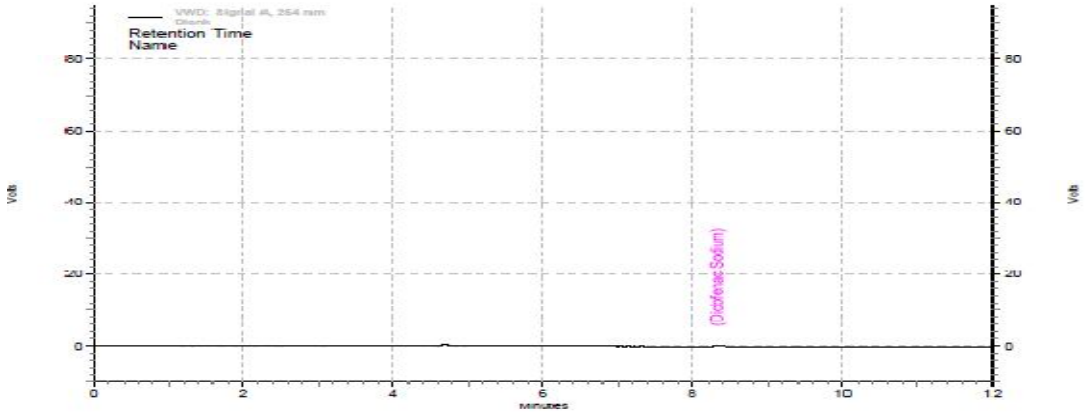
Chromatogram No – 8.114

Change in the wave length -252nm Bracketing standard



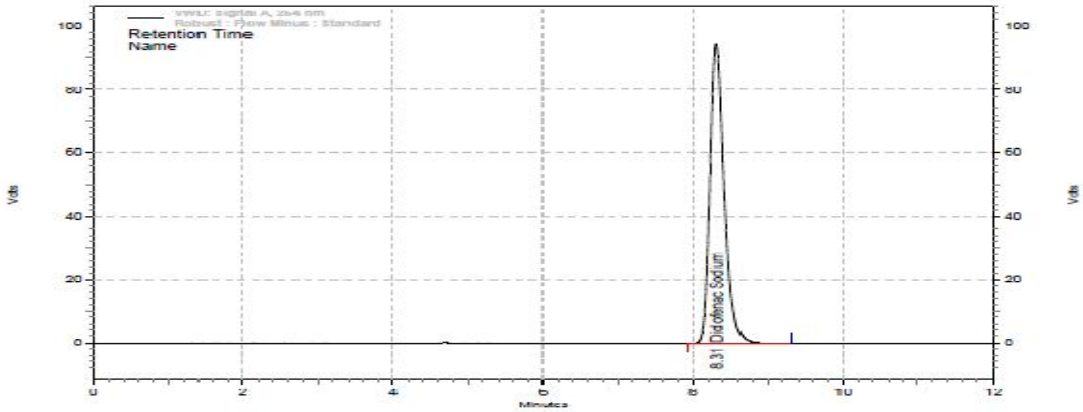
Chromatogram No – 8.115

Change in the flow rate -1.2ml Blank



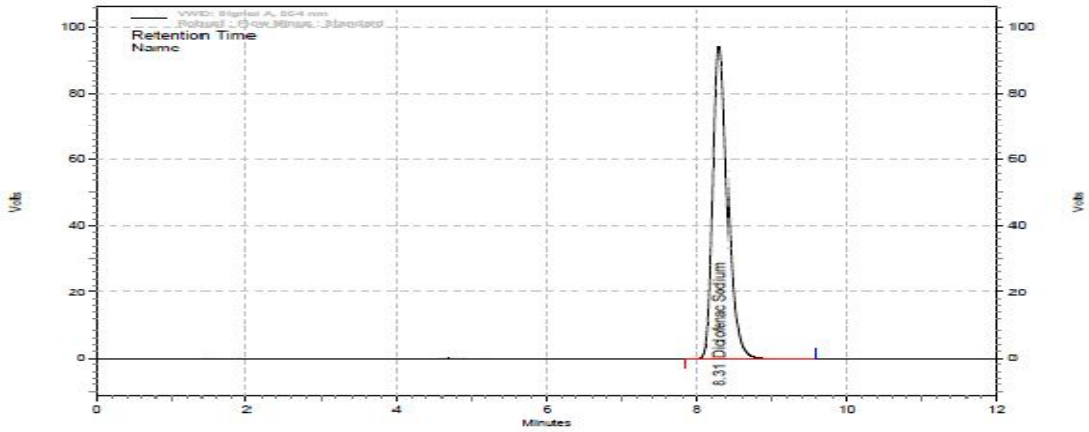
Chromatogram No – 8.116

Change in the flow rate -1.2ml Standard Replicate no-1



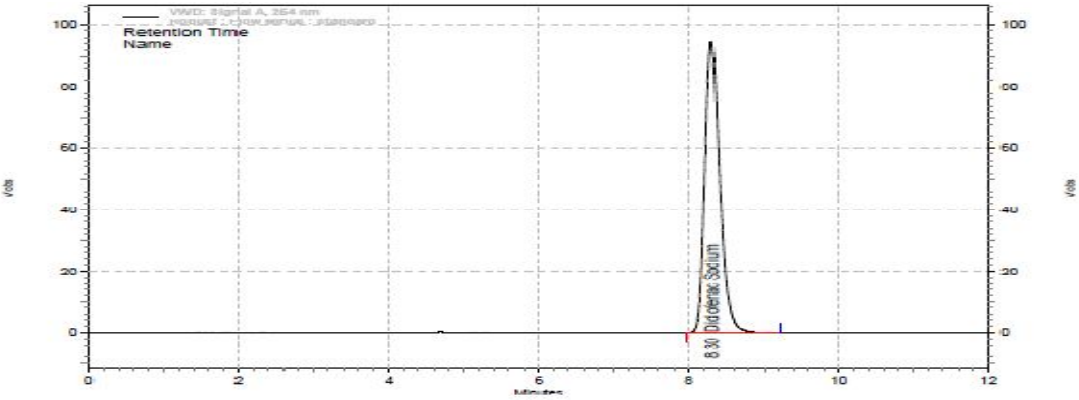
Chromatogram No – 8.117

Change in the flow rate -1.2ml Standard Replicate no-2



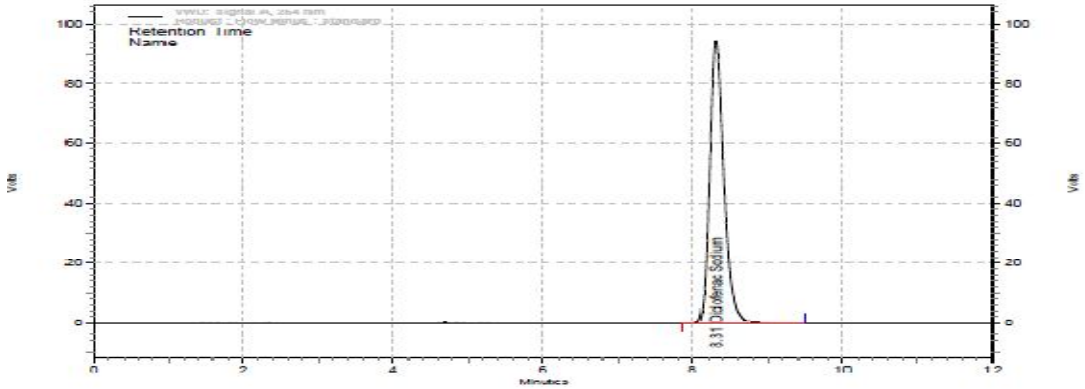
Chromatogram No – 8.118

Change in the flow rate -1.2ml Standard Replicate no-3



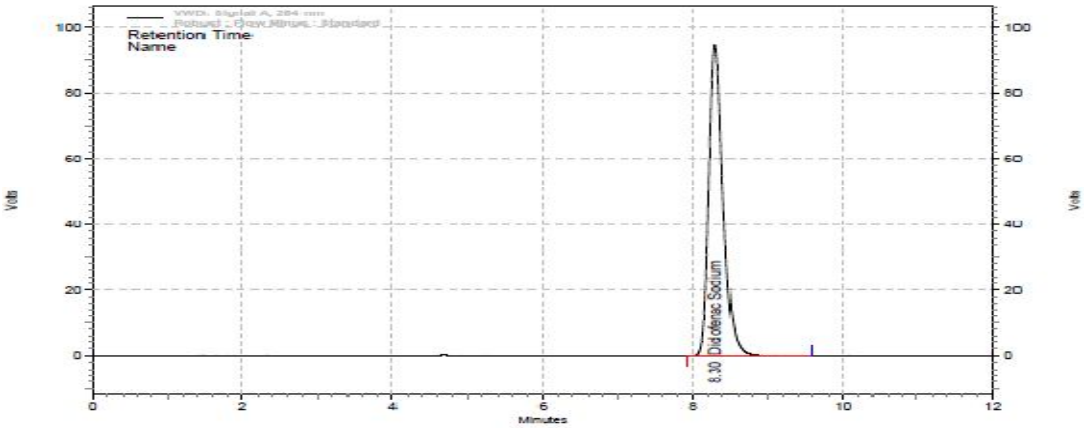
Chromatogram No – 8.119

Change in the flow rate -1.2ml Standard Replicate no-4



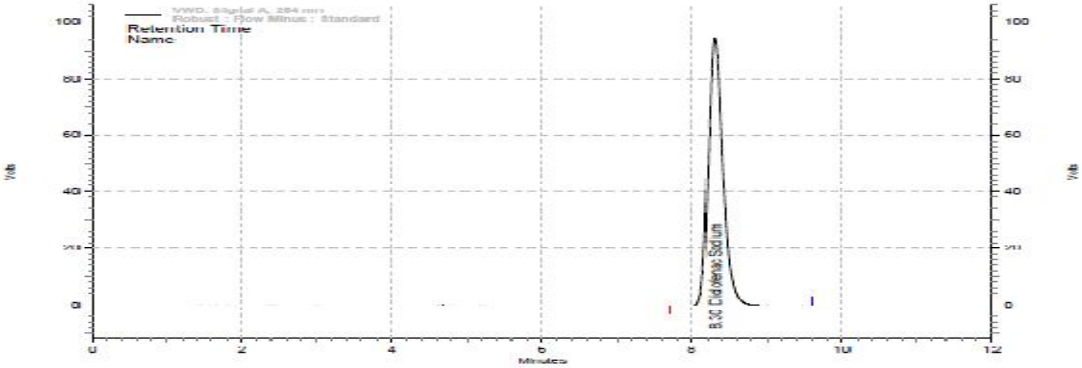
Chromatogram No – 8.120

Change in the flow rate -1.2ml Standard Replicate no-5



Chromatogram No – 8.121

Change in the flow rate -1.2ml Sample Replicate no-1



Chromatogram No – 8.122

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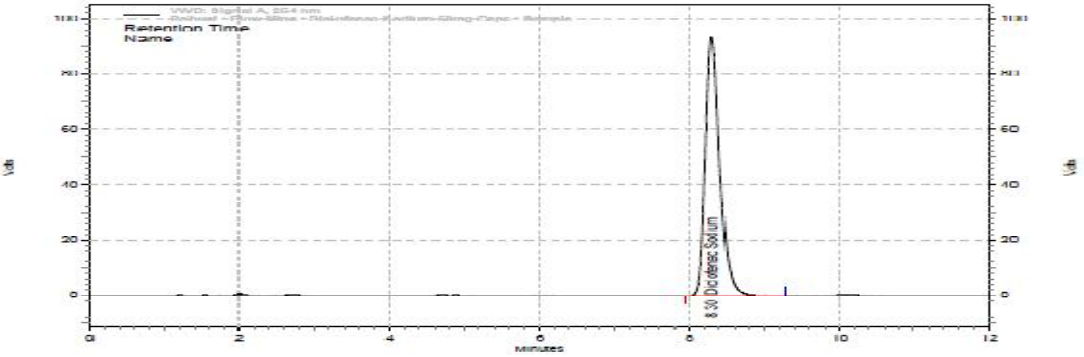

Chromatogram No – 8.123

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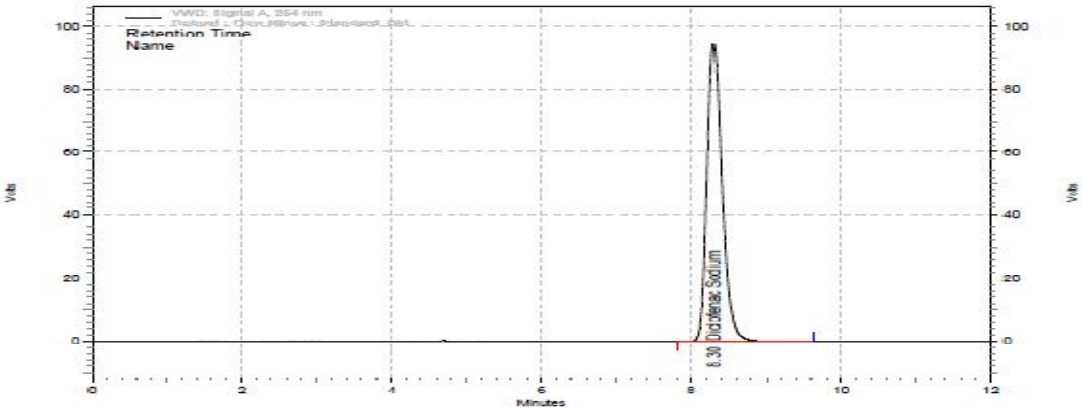

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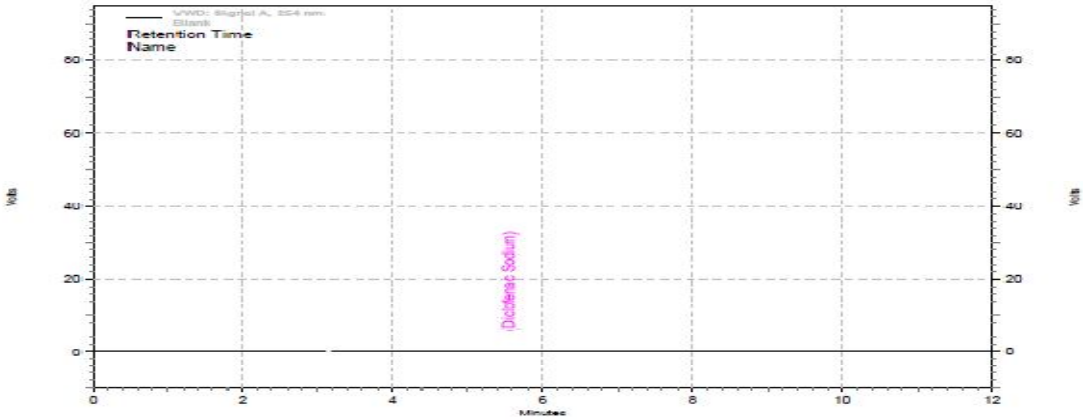

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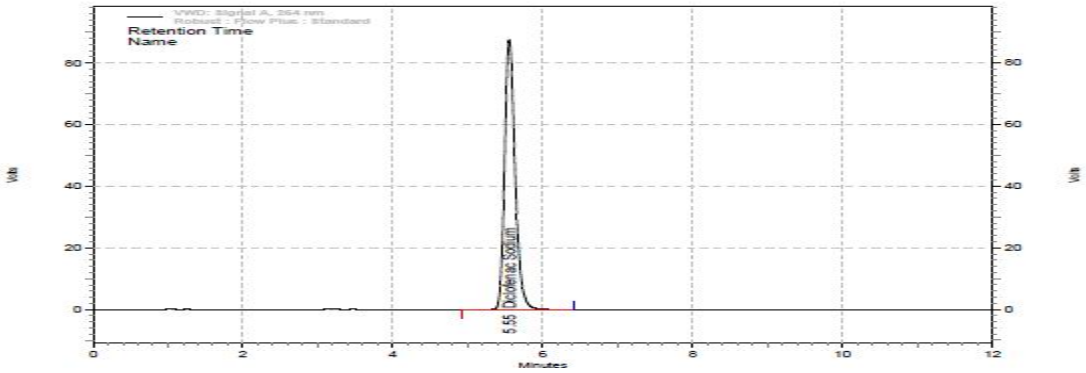

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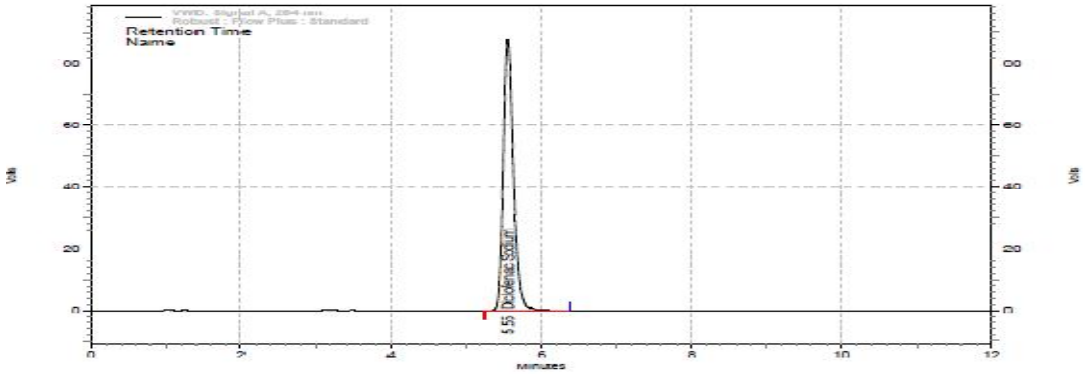

Chromatogram No – 8.127

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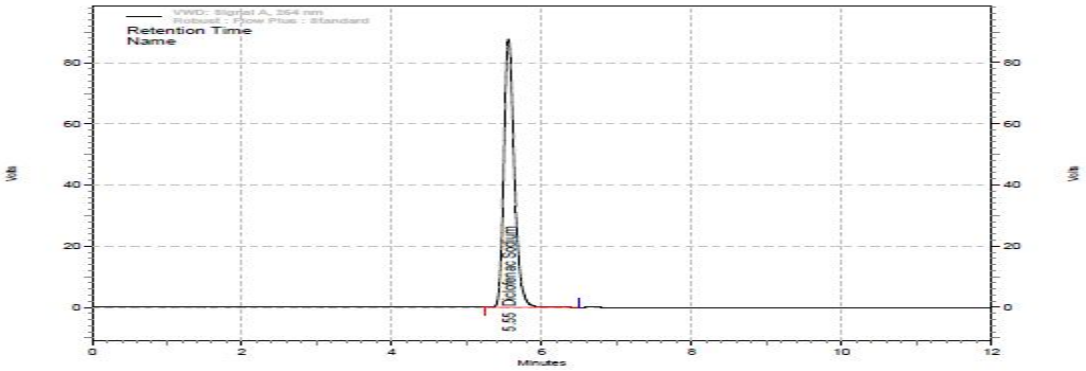

Chromatogram No – 8.128

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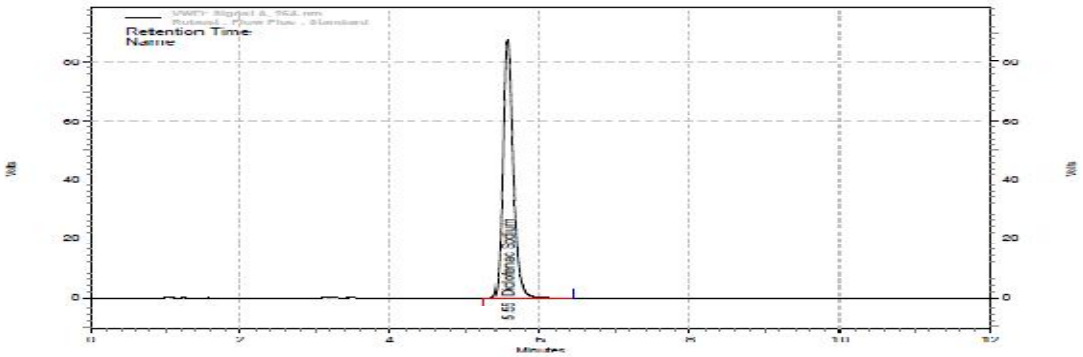

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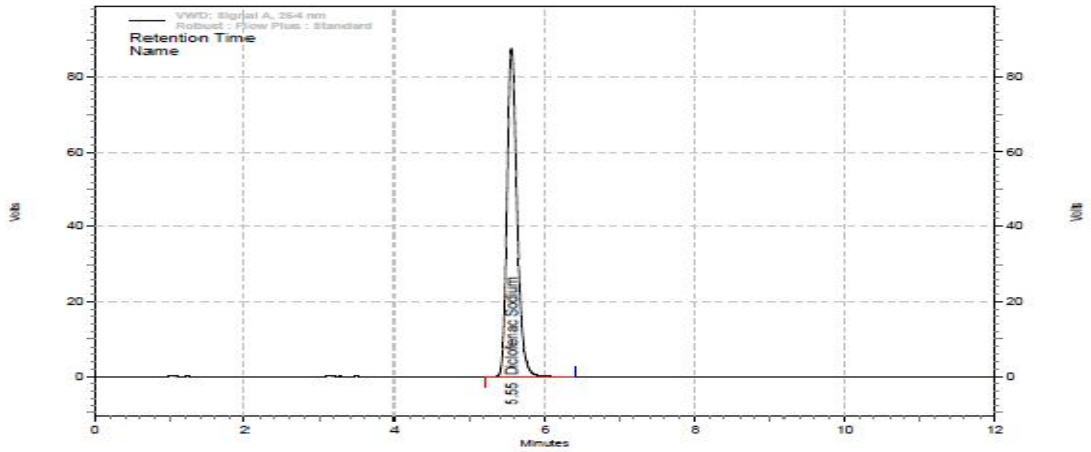

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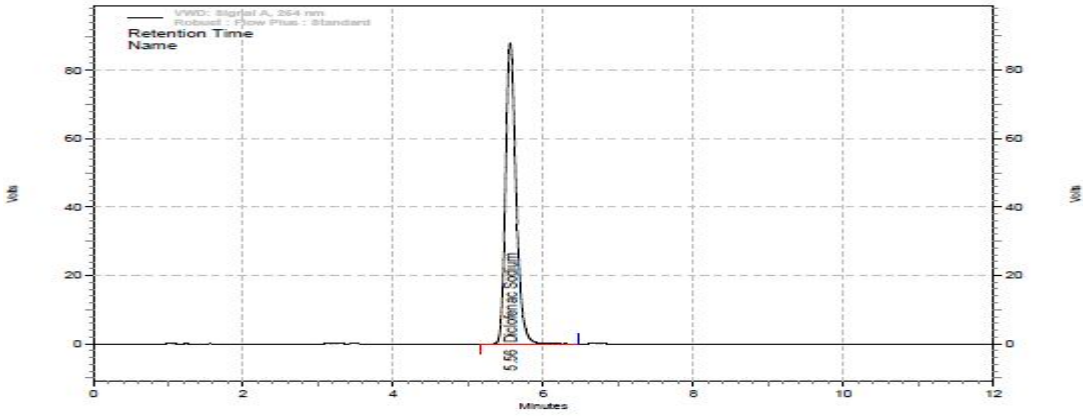

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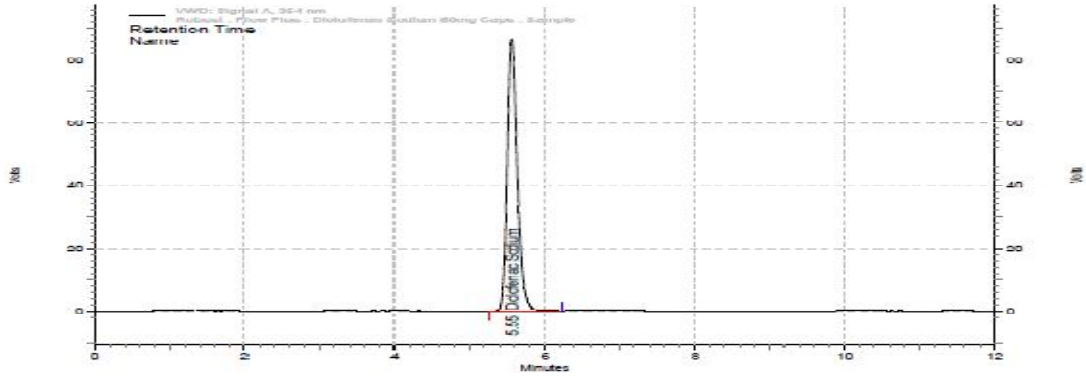
Sample Replicate-02



Chromatogram No – 8.132

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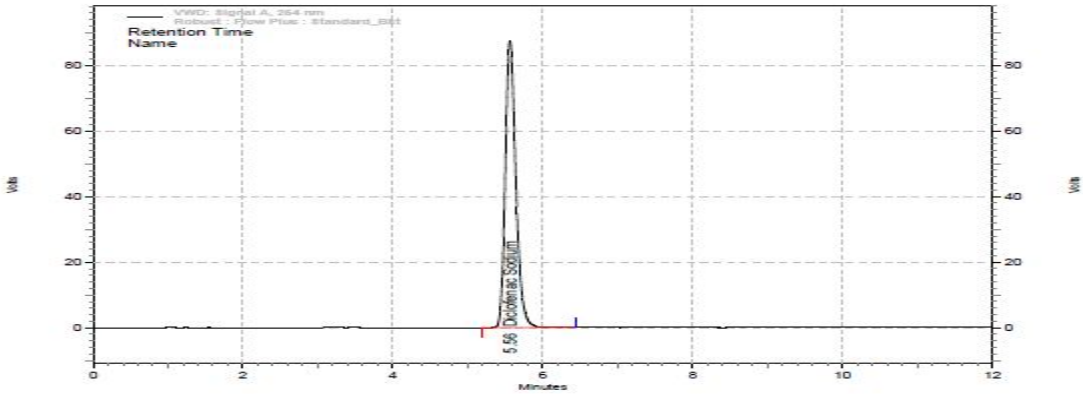
Bracketing standard



Stability studies

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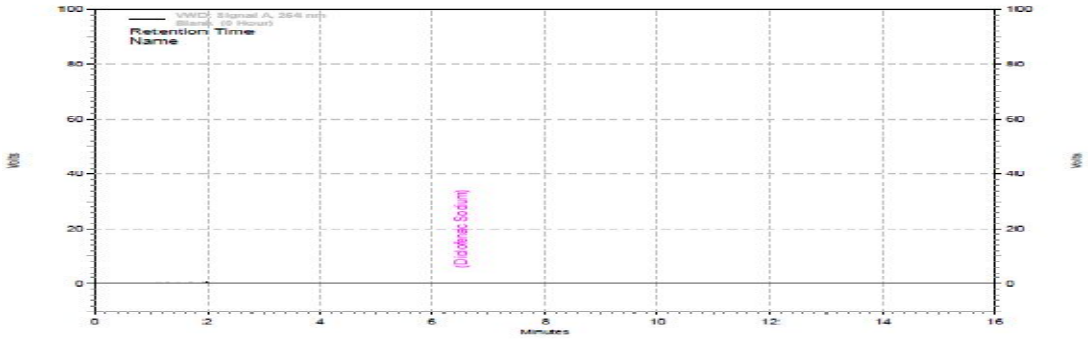
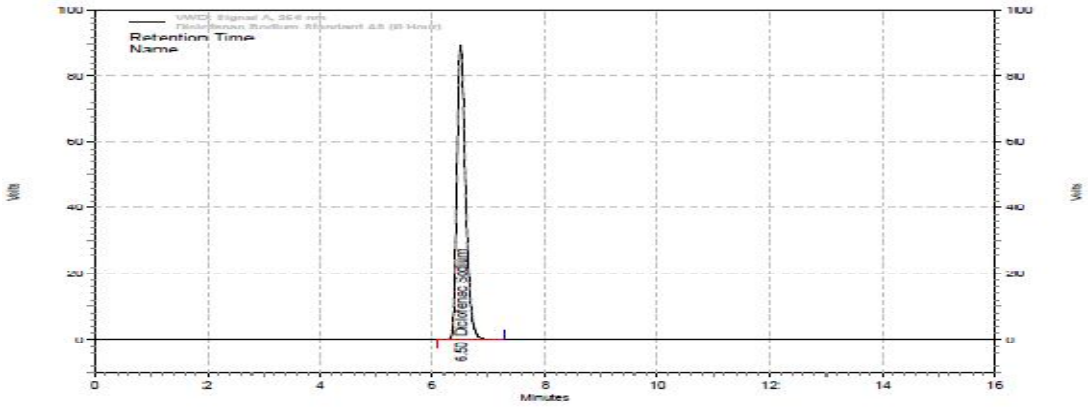


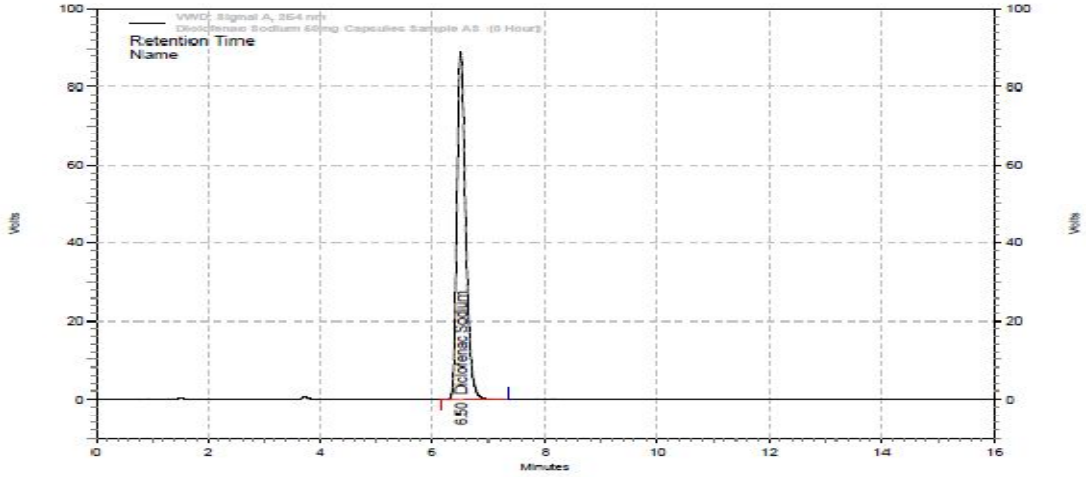
Chromatogram No – 8.134

0 hour Standard



Chromatogram No – 8.135

0 hour Sample



Chromatogram No – 8.136

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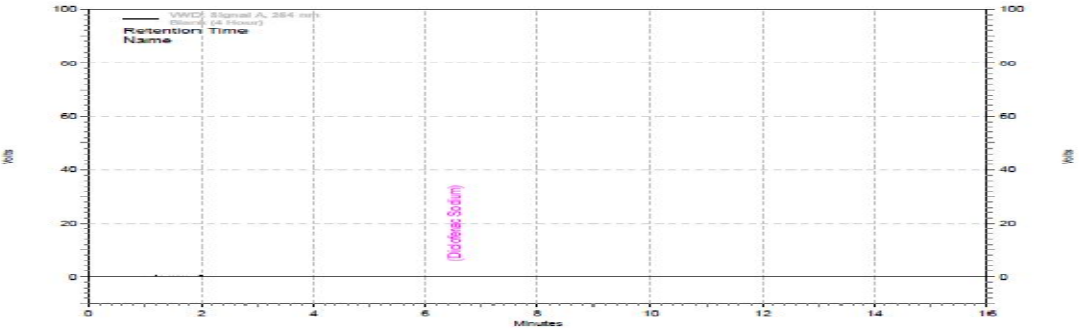

Chromatogram No – 8.137

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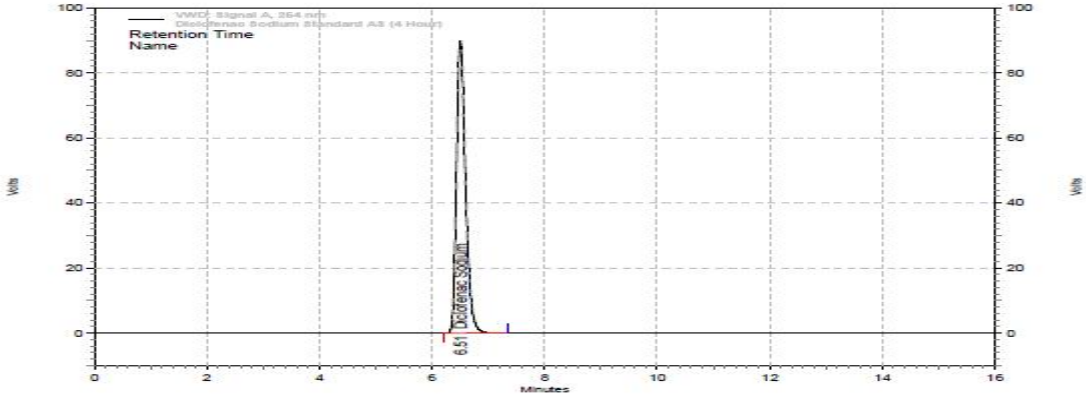

Chromatogram No – 8.138

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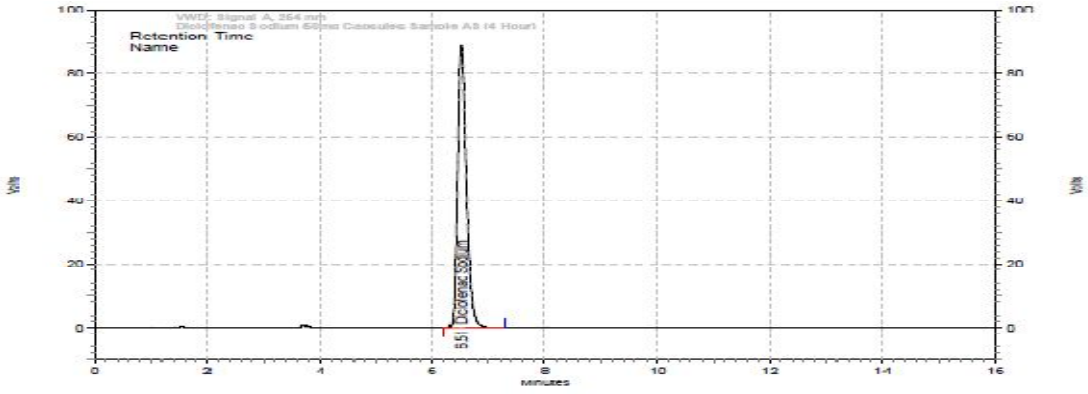

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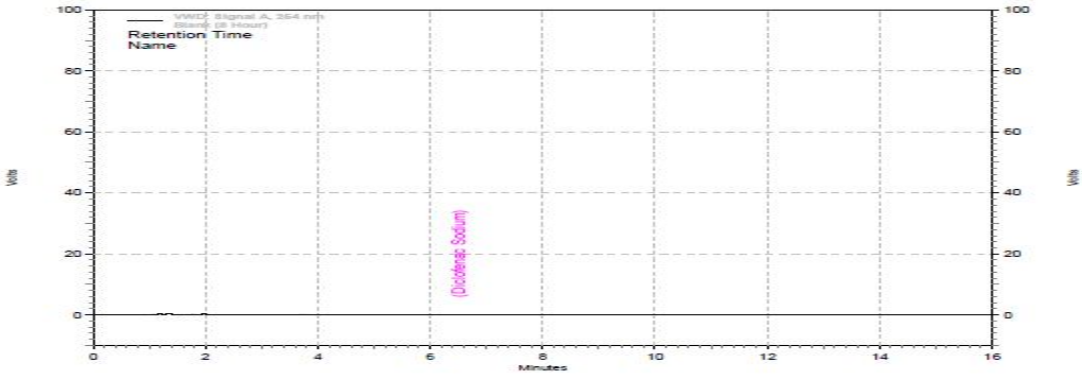

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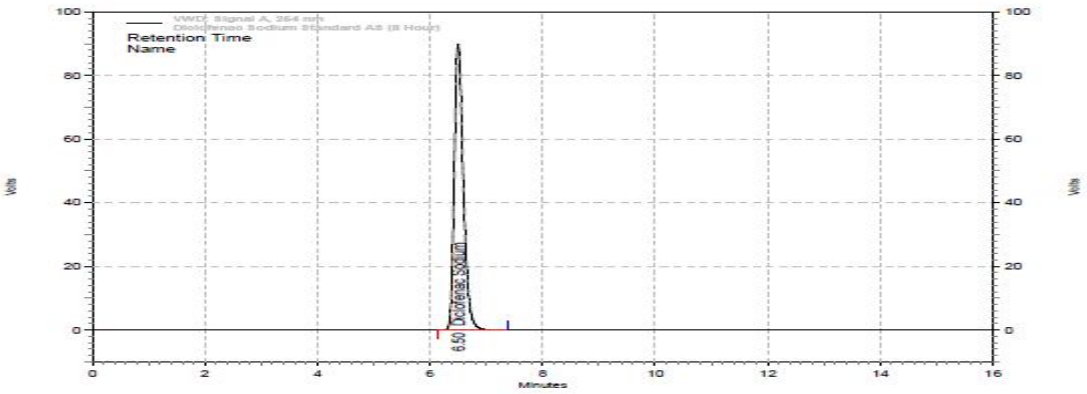
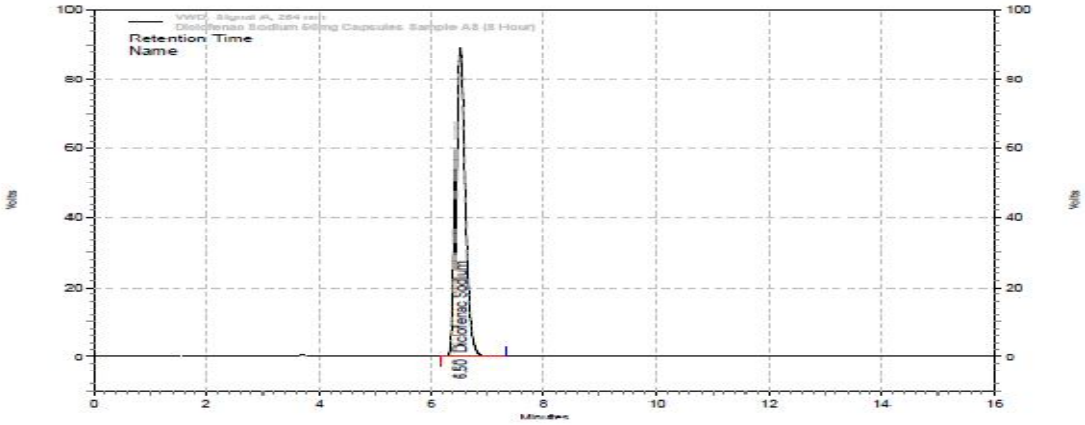
8th hour Standard



Chromatogram No – 8.141

8th hour Sample



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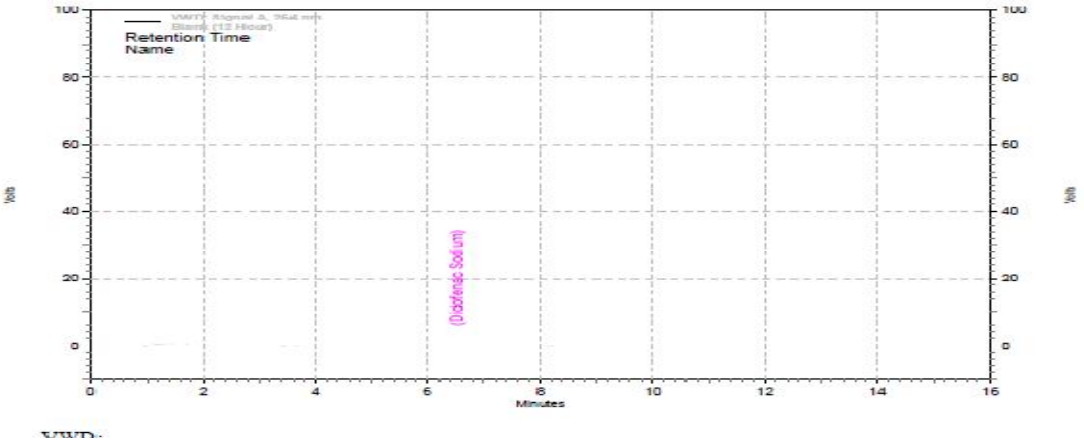

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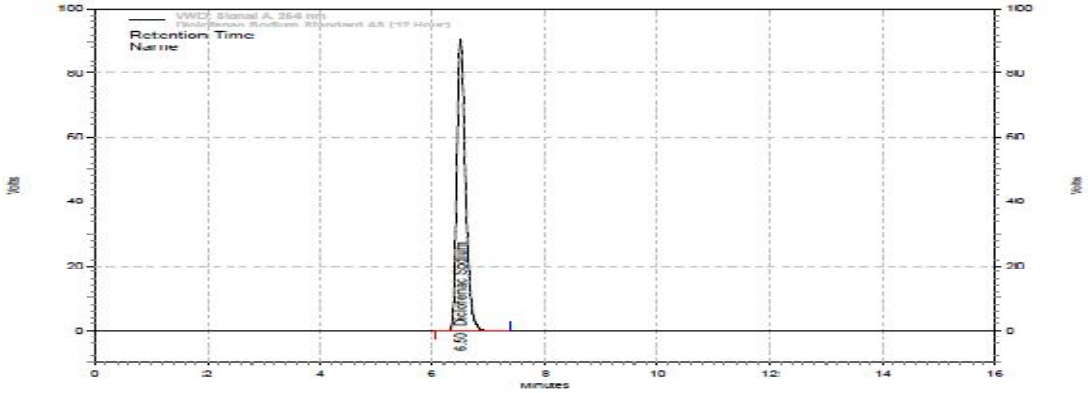

Chromatogram No – 8.144

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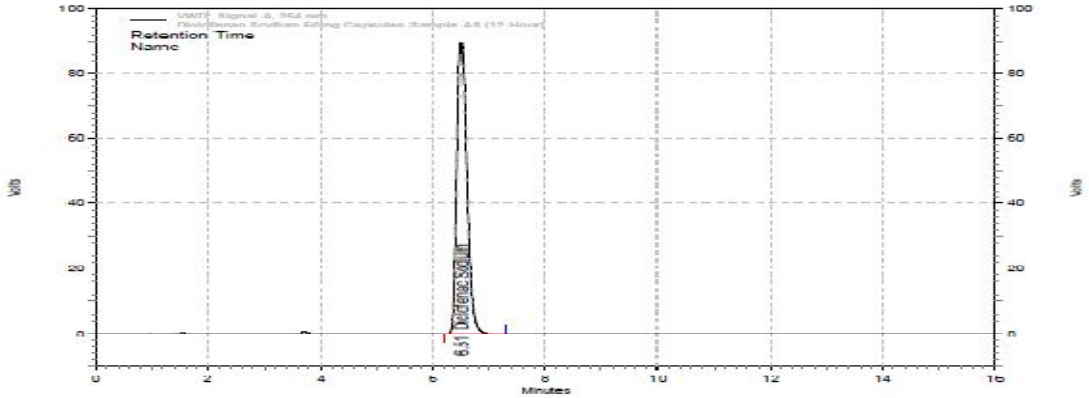

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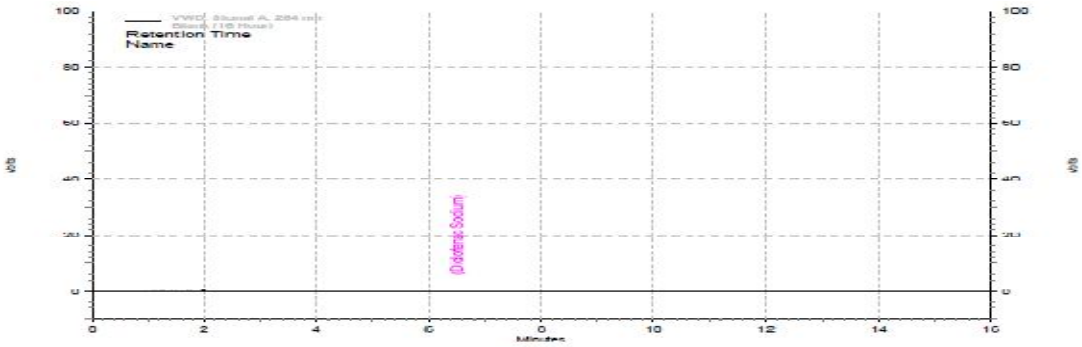

Chromatogram No – 8.146

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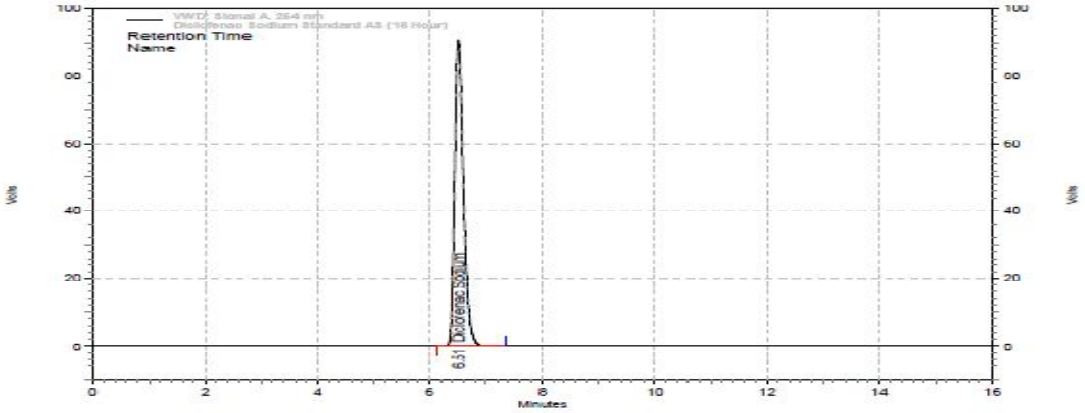

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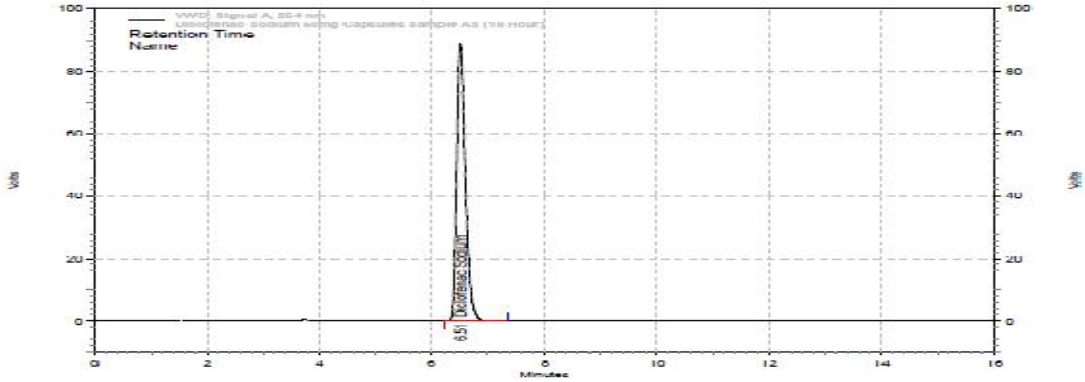

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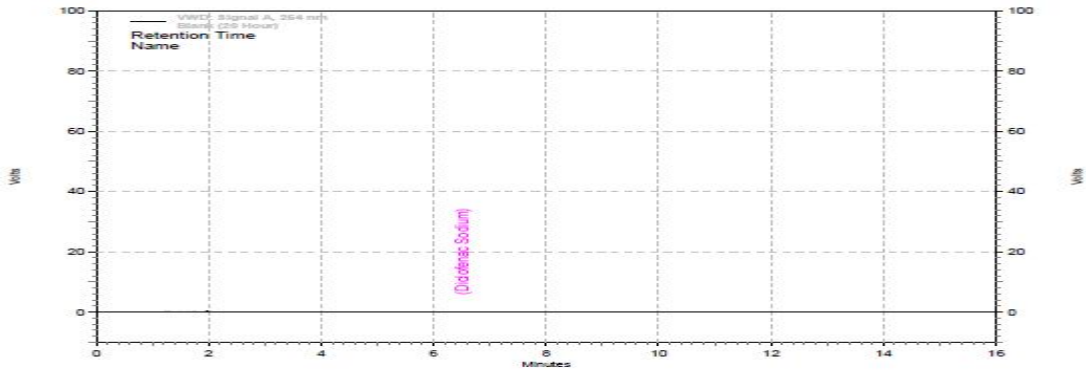

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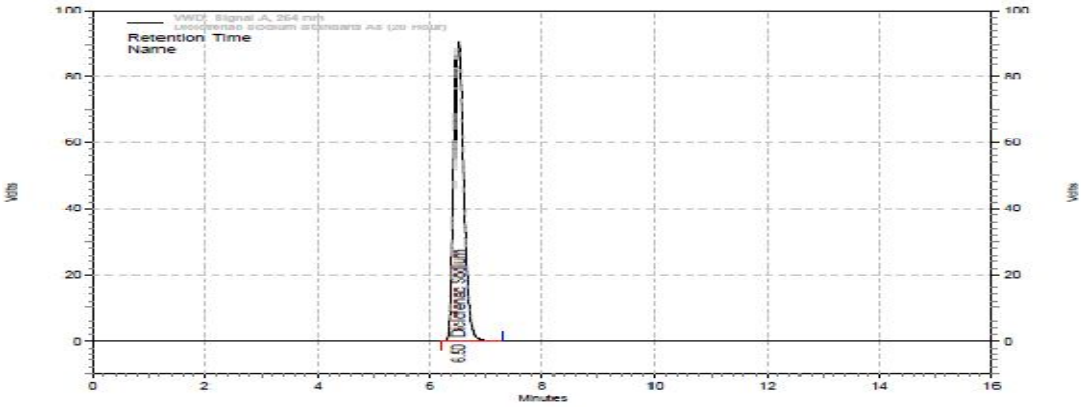

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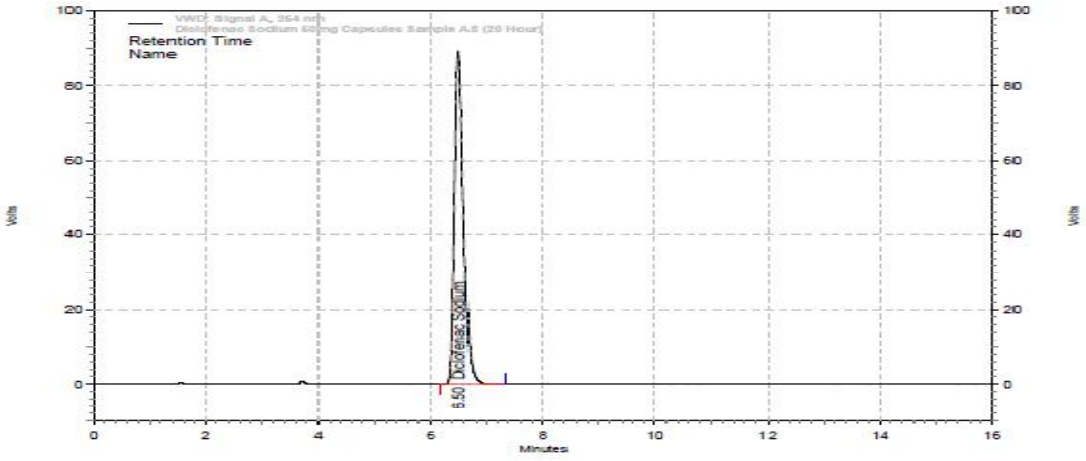

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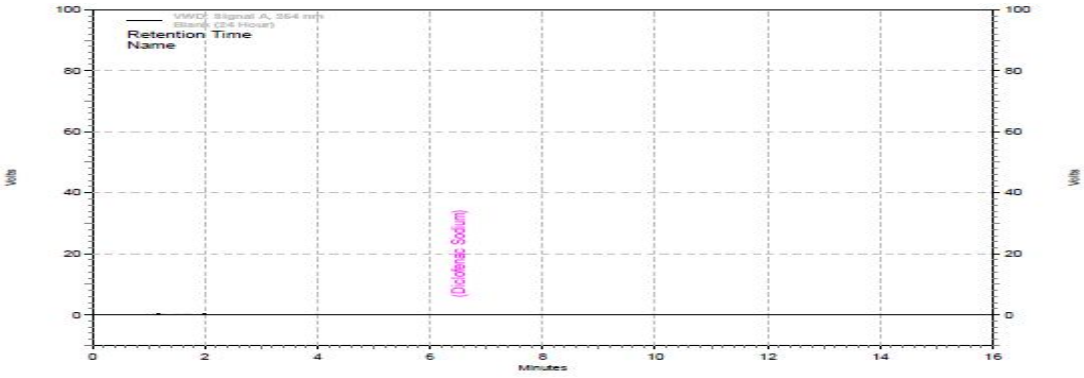

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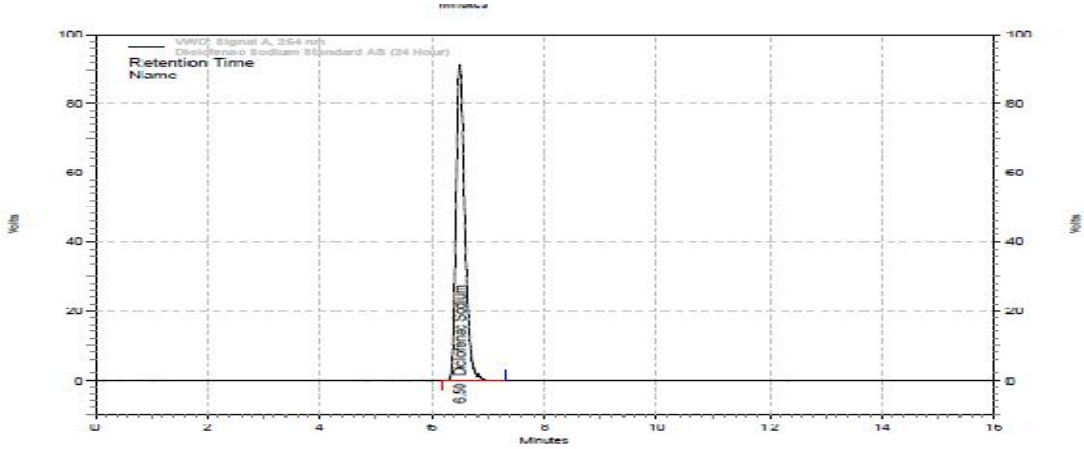

Chromatogram No – 8.153

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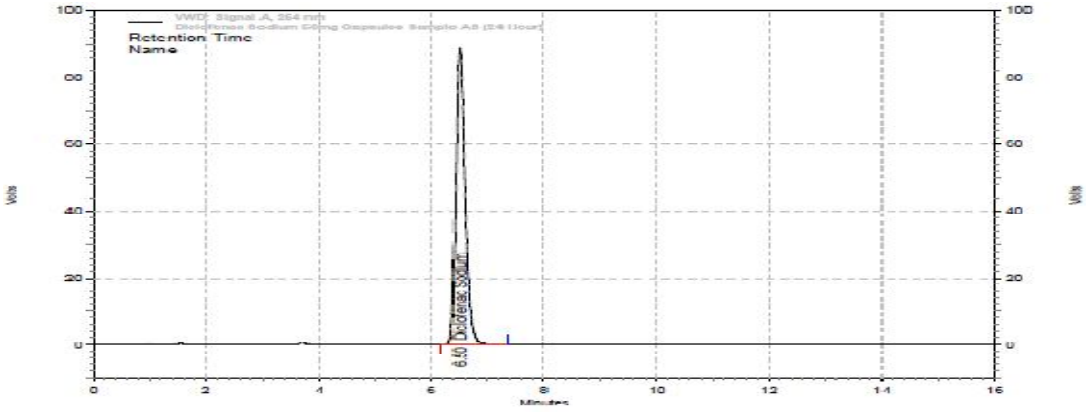

RESULTS AND DISCUSSION

A new isocratic reverse-phase high performance liquid chromatographic with UV-detection at 254 nm was developed for quantitative determination of Diclofenac Sodium in Pharmaceutical soft gelatin capsule dosage forms. The mobile phase used was of 400 volume of buffer (6.8 g of Potassium dihydrogen orthophosphate in 1000 mL of water and then adjust the pH to 3.00 with dilute Orthophosphoric acid.) and 600 volume of Acetonitrile . The chromatographic method was performed on C18, 15 cm x 4.6-mm, 5µm column at a flow rate of 1 ml/min. Column Temperature was set a 25ºC and injection volume was 20µl.

Estimation of Diclofenac Sodium in Pharmaceutical soft gelatin capsule dosage form dosage forms by RP-HPLC method was carried out using optimized chromatographic conditions. The standard and sample solutions were prepared. The chromatograms were recorded. The peak area ratio of standard and sample solutions was calculated. The capsule shows percentage purity values ranging from 97% to 99% for Diclofenac Sodium respectively.

The method was validated according to the Q2 specifications of the ICH guidelines. The validated parameters were system suitability, Precision, Accuracy, Specificity, linearity, Ruggedness Robustness and. LOD, LOQ.

The resulting chromatograms exhibited retention time at 6.50 min for Diclofenac Sodium .The number of theoretical plates were more than 2000, for Diclofenac Sodium it was 8496 respectively. The tailing factor was less than 2 that is 1.29 for Diclofenac Sodium . Hence all the system suitability parameters were with the specified limits.

The specificity of the method was established by injecting blank, placebo and standard . It was observed that there was no interference caused due to the placebo. Results were presented in **Table 7.2** and figure was shown in **Chromatogram No. 8.1 - 8.10.**

System suitability result passes and the results obtained for specificity are found within the acceptance criteria. The obtained results proved that there will not be blank and placebo interference in the Diclofenac Sodium peak by this assay method. The linearity for the drugs from concentration range of 30 - 80 µg/ml was established by constructing the calibration curve with concentration on x-axis and peak area on y-axis with the correlation coefficient of 0.999 for the drug which were within specified limits. Results were shown in **Table 7.6 - 7.10** and **Chromatogram No. 8.1 - 8.27.**

System suitability result passes and the results obtained for linearity are found within the acceptance criteria. Hence it is concluded that the range of concentrations, 60 % to 160 % with respect to 100 % working concentration for assay method is linear for Diclofenac Sodium .

System Precision was determined by preparing the standard solution at working concentration and analysis was carried for six replicate injections. The percentage relative standard deviation (% RSD) was calculated for the peak areas of Diclofenac Sodium which was found to be 0.055 respectively which were within the acceptance criteria of not more than 2.0%. The percentage relative standard deviation (% RSD) was calculated for the peak retention time of Diclofenac Sodium which was found to be 0.03 respectively which were within the acceptance criteria of not more than 1.0%. Results were presented in **Table 7.13** and figure were shown in **Chromatogram No. 8.28 - 8.33** All the system suitability parameters were well within the desirable limits, it indicates that the prescribed method is suitable to perform the estimation of Diclofenac Sodium from Diclofenac Sodium Soft gelatin Capsules 300 mg. Further there was no deviation in the given method

Method precision was determined by preparing six different samples solutions from the capsules of same batch at working concentration and analysis was carried out for six replicate injections. The percentage relative standard deviation (% RSD) was calculated for the peak areas of Diclofenac Sodium which was found to be 1.452 for the drug which is with in the acceptance criteria of not more than 2.0%. These results give the assurance of method repeatiility. Results were presented in **Table 7.16** and figures

were shown in **Chromatogram No. 8.34 -8.51 .**

System suitability parameters were well within the prescribed limits which revealed that the prescribed procedure is capable to perform Method precision using sample preparation. All the performed samples showed results between 90.0 % and 110.0 %.The Method precision parameter complies as per In-House specification.

Accuracy of the method was determined by performing recovery studies at 50%, 100%, 150%. Percentage recovery Diclofenac Sodium were found to be % of recovered with in the range of 98.91% - 100.52% and mean of % recovered within the range of 99.08% - 99.86% respectively which were with in the acceptance criteria of 98 - 102% respectively. Result of accuracy were shown in the **Table 7.19** and figures were shown in **Chromatogram No. 8.52 – 8.78.**

System suitability result passes and the results obtained for accuracy are found within the acceptance criteria. Hence, it is concluded that the assay method for Diclofenac Sodium Soft gelatin Capsules 50 mg is proficient to recover between 50 % and 150 % of Diclofenac Sodium drug material when spiked in placebo.

Ruggedness was determined by performing the same assay by different analyst on the different days and the results were checked for the reproducibility. The %RSD was found to be 1.177 – 1.332 Diclofenac Sodium which were with in the acceptance range of not more than 2 %. Results of ruggedness were shown in **Table 7.22 and 7.23** and figures were shown in **Chromatogram No. 8.78 – 8.90.**

System suitability result passes and the results obtained for Intermediate precision are found within the acceptance criteria. Combined Intermediate precision results and Method precision results are meets the prescribed limit as per In-House specification. Hence, it is concluded that the assay method is capable to generate, repeatable assay results for Diclofenac Sodium Soft gelatin Capsules 50 mg in multiple preparations of a unique batch, besides by a different analyst

The Robustness of the method was established changing the parameters like wave length and flow rate. The changes in system suitability parameters were with in the limits which ensures that the method developed can withstand slight changes in the experimental conditions and produce results with good reproducibility and repeatability. Results were presented in **Table 7.24-7.26** and figures were shown in **Chromatogram No. 8.97 – 8.132**

System suitability result passes in all the deliberately changed methods and the results obtained for all deliberately changed methods are found within the acceptance criteria. Combined deliberately changed methods results and Method precision results are well within the desirable limit. It is concluded that the deliberately changed assay methods results are remains unaffected in small variations which confirmed that all the methods are proficient to estimate Diclofenac Sodium in Diclofenac Sodium Soft gelatin Capsules 50 mg.

Standard and sample solutions to be used in the analytical method are scrutinized for their solution's stability. This study was performed by injecting standard and sample solution for the period of 24 hours.

Stability Studies was carried out at 0,4,8,12,16,20 hours and 24 hours time lapse of solutions preparation. The %RSD for Diclofenac Sodium standard were found to be with in the 0.019% and 0.785% and The %RSD for Diclofenac Sodium sample were found to be with in the 0.035% and 0.080% which were with the acceptance criteria of not more than 2%. Results of stability were shown in **Table 7.28** and **7.29** and figures were shown in **Chromatogram No. 8.133 – 8.159.**

System suitability result passes and the results obtained for stability of standard solution and sample solution are found within the acceptance criteria for the minimum period of 24 hours study.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) was determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3).The detection limit

(LOD) was found to be 0.77µg/ml for Diclofenac Sodium respectively.

The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10).The quantitation limit (LOQ) was found to be 2.34 µg/ml for Diclofenac Sodium respectively. In case of LOD and LOQ the S/N ratio were found to be within the limits. Results of LOD and LOQ were parented in **Table 7.30** .

S.NO	Parameter	Specifications	Inference
1	Specificity	No interference	No interference
2	Similarity factor	0.98 – 1.02	0.99
3	System precision	Area of %RSD NMT 2.0	0.30
4	Method precision	%RSD NMT 2.0	1.45
5	Linearity range	Correlation coefficient NLT 0.999	0.9999
6	Accuracy	% Mean Recovery 98 –10 2 %	98.91% - 100.52%
7	Limit of Detection	Signal noise ratio should be more than 3:1 for the conc	0.77µg/ml
8	Limit of Quantitation	Signal noise ratio should be more than 10:1 for the conc	2.34µg/ml
9	Robustness by Change in flow rate and columntemperature	No effect on system suitability parameters	No effect on system suitability parameters

10. SUMMARY AND CONCLUSION

A RP-HPLC method is developed and validated as per ICH guidelines for simultaneous estimation of Diclofenac Sodium in soft gelatin dosage form.

In present study an attempt has been made to modify experimental condition, in order to estimate the drugs. The mobile phase was selected after trying various combinations of polar solvents. The proportion of solvents and variation of buffers was found to be quite critical as slight variation in it adversely affected the resolution of peaks. Considering all the fact the following parameter were finally fixed for this method

Chromatographic conditions:

Equipment	: High performance liquid chromatography
Column	: C18, 15 cm x 4.6 mm, 5 µm
Flow Rate	: 1.0 mL/minute
Pump mode	: Isocratic
Detector wavelength	: 254 nm
Injection volume	: 20 µL
Column Temperature	: 25.0˚C
Sample Temperature	: 20.0˚C
Run time	: 7 Min

The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. This method is also having an advantage than reported method that the retention time of the drugs is below 8 mins and the drugs can be assayed with the short time. Thus the method is not time consuming and can be used in laboratories for the routine analysis of combination drugs.

11. BIBLIOGRAPHY

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