BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF SELECTED CORTICOSTEROIDS IN RAT PLASMA USING RP-HPLC METHOD

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List of Abbreviations

S. No.	Abbreviation	Remarks
1	API	Active Pharmaceutical Ingredient
2	FPD	Fine Partial Dose/Diameter
3	IP	Induction Port
4	HLB	Hydrophilic-Lipophilic Balance
5	HPLC	High Performance Liquid Chromatography
6	IND	Investigational New Drug
7	µm	Micrometer
8	RSD	Relative Standard Deviation
9	SEM	Scanning Electron Microscopy

INTRODUCTION

This dissertation deals with the studies carried out by the writer in this laboratory on the development of bioanalytical method used for the study of validation parameters of selected corticosteroids in rat plasma by RP-HPLC method. Before discussing the experimental results, a brief introduction to bioanalytical methods of analysis, biopharmaceutical analysis, analysis of drugs in biological media, preliminary treatment of biological samples, estimation procedures for drugs and metabolites from biological samples, estimation of drugs in biological sample by RP-HPLC or LC-MS/MS methods.

1.1 Biopharmaceutical Analysis

1.1.1 *Need for Biopharmaceutical Analysis*

Methods for measurement of drugs in biological media are increasingly important problems related to following studies which are highly dependent on biopharmaceutical analytical methods.

- Bioavailability and Bioequivalence Studies
- New Drug Development
- Clinical Pharmacokinetics
- Research in Basic Biomedical and Pharmaceutical Sciences
- Therapeutic drug monitoring

1.1.2 *Analysis of drugs in various biological media*

The most common samples obtained for biopharmaceutical analysis are blood and urine. Faeces are also utilized, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized include saliva, breath, and tissue.

The choice of sampling media is determined largely by the nature of the drug study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and possibly saliva. A bioavailability study may require drug level data in blood and/or urine

Whereas a drug identification or drug abuse problem may be solved with only one type of biological sample.

Detection of a drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involving techniques such as solvent extraction and chromatography are employed to effectively separate drug components from endogenous biological material. The ultimate sensitivity and selectivity of the assay method may be limited by the efficiency of the clean up methodology.

Whole blood is usually collected by venipuncture with either a hypodermic syringe or a Vacutainers apparatus. The volume of blood collected at any one sampling time is usually limited to 5 to 15 ml (depending on the assay sensitivity and the total number of samples taken for a given study). If the blood is allowed to clot and is then centrifuged, about 30 to 50% of the original volume is collected as serum (upper level). Thus, plasma generally is preferred because of its greater yield from blood. The greater the yield, the greater the amount of drug and the fewer the problems with sensitivity. Blood, serum, or plasma samples can be utilized for drug studies and may require protein denaturation steps before further manipulation.

If plasma or serum is used for the analytical procedure, the fresh whole blood should be centrifuged immediately at 3000rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as a Pasteur pipette, to a clean container of appropriate size for storage. The remaining blood cells can then be discarded or stored for further studies such as drug binding.

Urine is the easiest to obtain from the patient and also permits collection of large and frequently more concentrated samples. The lack of protein in a healthy individual's urine obviates the need for denaturation steps. Because urine samples are readily obtained and often provide the greatest source of metabolites, they are frequently analyzed in drug metabolism studies.

Saliva and breath are biological media obtained from humans when constant ratio between plasma and salivary levels of certain drugs exists via non invasive sampling techniques. Saliva is advantageous in drug studies done with children. Although the concentrations of drugs in saliva are rarely equal to those in plasma, a constant ratio (over an effective therapeutic range) permits calculation of plasma levels based on salivary analysis.

Separation or isolation of drugs and metabolites from biological samples is performed in order to partially purify a sample. In this manner, an analyst can obtain the selectivity and sensitivity needed to detect a particular compound and can do so with minimum interference from components of the more complex biological matrix. The number of steps in a separation procedure should be kept to a minimum to prevent loss of drug or metabolite. Sometimes, the separation steps are preceded by a sample pretreatment.

1.1.3 *Storage requirements for biological samples*

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride, to blood samples immediately after collection helps to prevent drug decomposition.

When collecting and storing biological samples, the analyst should be aware of facts that from tubing or storage vessels that can contaminate the sample. For example, plastic ware frequently contains the high boiling liquid bis (2-ethylhexyl) phthalate; similarly, the plunger plugs of Vacutainers are known to contain tri-butoxyethyl phosphate, which can interfere in certain drug analysis.

1.1.4 *Bioavailability studies*

Bioavailability is defined as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action." This definition focuses on

the processes by which the active ingredients or moieties are released from an oral dosage form and move to the site of action.

BA studies provide pharmacokinetic information related to distribution, elimination, the effects of nutrients on absorption of the drug, dose proportionality, linearity in pharmacokinetics of the active moieties and, where appropriate, inactive moieties.

BA data may also provide information indirectly about the properties of a drug substance prior to entry into the systemic circulation, such as permeability and the influence of pre systemic enzymes and/or transporters (e.g., p-glycoprotein).

1.1.5 *Preliminary treatment of biological fluids*

In most cases, preliminary treatment of a sample is needed before the analyst can proceed to the measurement step. Analyses are required for drug in samples as diverse as plasma, urine, feces, saliva, bile, sweat, and seminal fluid. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method can be selected. Such factors as texture and chemical composition of the sample, degree of drug-protein binding, chemical stability of the drug, and types of interferences can affect the final measurement step.

1.1.6 *Extraction procedures for biological fluids and samples*

After pre treating biological material, the next step is usually the extraction of the drugs from the biological matrix. All separation procedures use one or more treatments of matrix-containing solute with some fluid. If the components are a liquid (extracting solvents) and a solid (e.g., lyophilized feces), it is an example of liquid-solid extraction. If the extraction involves two liquid phases, it is an example of liquid-liquid extraction.

1.1.7 *Liquid-Solid extraction (Solid phase extraction)*

Liquid - solid extractions occur between a solid phase and a liquid phase, either phase may initially contain the drug substance. Among the solids that have been used successfully in the

extraction (usually via adsorption) of drugs from liquid samples are XAD-2 resin, charcoal, alumina, silica gel, and aluminum silicate. Sometimes the drugs are contained in a solid phase, such as in lyophilized specimens. Liquid-solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water.

Factors governing the adsorption and elution of drugs from the resin column include solvent polarity; flow rate of the solvent through the column, and the degree of contact the solvent has with the resin beads. In the adsorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the resin surface while the hydrophilic portion of the solute remains in the aqueous phase. Alteration in the lipophilic / hydrophilic balance within the solute or solvent mix, and not within the resin, affects adsorption of the solute.

Biological samples can be prepared for cleanup by passing the sample through the resin bed where drug (metabolite) components are adsorbed and finally eluted with an appropriate solvent. The liquid solid extraction method provides a convenient isolation procedure for blood samples, thus avoiding solvent extraction, protein precipitation, drug losses, and emulsion formulation. It is possible; however, that strong drug-protein binding could prevent sufficient adsorption of the drug to resin.

1.1.8 *Liquid-liquid extraction*

Liquid-liquid extraction is probably the most widely used technique because

- The analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination.
- The technique is simple, rapid, and has a relatively small cost factor per sample.
- The extract containing the drug can be evaporated to dryness, and the residue can be redissolved in a smaller volume of a more appropriate solvent. In this manner, the sample becomes more compatible with a particular analytical methodology in the measurement step, such as a mobile phase in HPLC determinations.

- The extracted material can be redissolved in small volumes (e.g., 100 to 500 µl of solvent), thereby extending the sensitivity limits of an assay.
- It is possible to extract more than one sample concurrently.
- Near quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extractions.

1.2 Method Development

Method development involves considerable trial and error procedures. In general, one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water-soluble.

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

Elution of drug molecules can be altered by changing the polarity of the mobile phase. The elution strength of a mobile phase depends upon its polarity. Ionic samples (acidic or basic) can be separated only, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH. Whenever acidic or basic samples are to be separated it is strongly advisable to control mobile phase pH by adding a buffer. PH of the buffer is to be adjusted before adding organic phase. The buffer selected for a particular separation should be used to control pH over the range of pka ±1.0.

Optimizations can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram.

During method development selection of column can be streamlined by starting with shorter columns (50 mm, 100 mm or even 150 mm). By selecting a shorter column with an appropriate phase the run time can be minimized so that an elution order and an optimum mobile phase can be quickly determined. The internal diameter of the column is also one factor is to be considered. Many laboratories use 4.6 mm ID as standard one, but it is worth considering to use 4mm ID column as an alternative. This 4 mm column requires only 75 % of the solvent consumption than that of 4.6 mm column.

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C-8 phase (reversed phase) can provide a further time saving over a C-18 as it does not retain the analytes as strongly as the C-18 phase. For normal phase application Cyano phases are most versatile. C-18 (250 x 4.6 mm) column are more often used in laboratory. These columns are able to resolve a wide variety of compounds due to their selectivity and higher number of theoretical plates.

Selection of internal standard should be on the basis of structural similarity, physicochemical properties related to the analyte to be quantified. Method optimization or Performance of selected method is checked by running three or more precision and accuracy batches and evaluating the results for meeting acceptance criteria for within run precision, accuracy and between run precision and accuracy. Finally selected method has to be validated to see whether it does what it was intended to do; i.e. it must be validated through validation parameters.

1.3 Bio-Analytical Method Validation

1.3.1 *Principles of Bioanalytical Method Validation and Establishment*

- The fundamental parameters to ensure the acceptability of the performance of a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability.
- A specific, detailed description of the bioanalytical method should be written. This can be in the form of a protocol, study plan, report, and/or SOP.

- Each step in the method should be investigated to determine the extent to which environmental, matrix, material, or procedural variables can affect the estimation of analyte in the matrix from the time of collection of the material up to and including the time of analysis.
- It may be important to consider the variability of the matrix due to the physiological nature of the sample. In the case of LC-MS-MS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation.
- A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).
- Whenever possible, the same biological matrix as the matrix in the intended samples should be used for validation purposes. (For tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices can be substituted.)
- The stability of the analyte (drug and/or metabolite) in the matrix during the collection process and the sample storage period should be assessed, preferably prior to sample analysis.
- For compounds with potentially labile metabolites, the stability of analyte in matrix from dosed subjects (or species) should be confirmed.
- The accuracy, precision, reproducibility, response function, and selectivity of the method for endogenous substances, metabolites, and known degradation products should be established for the biological matrix. For selectivity, there should be evidence that the substance being quantified is the intended analyte.
- The concentration range over which the analyte will be determined should be defined in the bioanalytical method, based on evaluation of actual standard samples over the range, including their statistical variation. This defines the *standard curve*.
- A sufficient number of standards should be used to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous and reproducible. The number of standards used should be a function of the dynamic range and nature of the concentration-response relationship. In many cases, six to eight concentrations

(excluding blank values) can define the standard curve. More standard concentrations may be recommended for nonlinear than for linear relationships.

- The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation.

1.4 VALIDATION

1.4.1 *Types of validation process*

A. Full validation

Full validation is important when developing and implementing a bioanalytical method for first time. Full validation is important for a new drug entity. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification.

B. Partial validation

Partial validations are modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation.

C. Cross-validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator. The comparisons should be done both ways.

1.5 VALIDATION PARAMETERS

1.5.1 *Linearity*

Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample. The linear range of the method must be determined regardless of the phase of drug development. ICH guidelines recommend evaluating a minimum of five concentrations to assess linearity. The five concentration levels should bracket the upper and lower concentration levels evaluated during the accuracy study.

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the (accountability systems that ensure integrity of test articles), sample preparation and

analyticalal tools such as methods, reagents, equipments, instrumentation and procedures for quality control and verification of results.

The obtained values of slope **a** and intercept **b** is used in the Linear regression equation:

$$y = ax + b$$

1.5.2 *Calibration/Standard Curve*

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the 6 sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

- Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met: C The analyte response at the LLOQ should be at least 5 times the response compared to blank response. C Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

1.5.3 *Selectivity (Specificity)*

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analysis of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least five to six sources. Each blank sample should be tested for interference and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering

substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

- For every phase of product development, the analytical method must demonstrate specificity. The method must have the ability to unambiguously assess the analyte of interest while in the presence of all expected components, which may consist of degradants, excipients/sample matrix, and sample blank peaks. The sample blank peaks may be attributed to things such as reagents or filters used during the sample preparation.
- For identification tests, discrimination of the method should be demonstrated by obtaining positive results for samples containing the analyte and negative results for samples not containing the analyte.
- For assay/related substances methods, the active peak should be adequately resolved from all impurity/degradant peaks, placebo peaks, and sample blank peaks.
- The forced degradation studies should consist of exposing the API and finished product to acid, base, peroxide, heat, and light conditions, until adequate degradation of the active has been achieved. An acceptable range of degradation may be 10–30% but may vary based on the active being degraded. Overdegradation of the active should be avoided to prevent the formation of secondary degradants.
- The selectivity is defined as the lack of significant interfering peaks at the retention time of analyte and internal standard.
- Selectivity is evaluated by injecting extracted blank plasma samples (anticoagulant sodium citrate) and comparing any interference with the response of the extracted LOQ samples processed with internal standard.

1.5.4 *Accuracy*

Accuracy should be performed at a minimum of three concentration levels. For drug substance, accuracy can be inferred from generating acceptable results for precision, linearity, and specificity.

- For assay methods, the spiked placebo samples should be prepared in triplicate at 80, 100, and 120%. The weight of drug product may be varied in the sample preparation step of the analytical method to prepare samples at the three levels listed above. In this case, the accuracy study can be combined with method precision, where six

sample preparations are prepared at the 100% level, while both the 80 and 120% levels are prepared in triplicate.

- For assay methods, the change in active content must be controlled tightly to establish sample stability. If impurities are to be monitored in the method sample, solutions can be analyzed on multiple days and the change in impurity profiles can be monitored.
- For chromatographic robustness, all compounds of interest, including placebo related and sample blank components, should be present when evaluating the effect of modifying chromatographic parameters. For an HPLC impurity method, this may include a sample preparation spiked with available known impurities at their specification level or, alternatively, a forced degraded sample solution can be utilized. The analytical method should be updated to include defined stability of solutions at evaluated storage conditions and any information regarding sample preparation and chromatographic parameters, which need to be tightly controlled. Sample preparation and chromatographic robustness may also be evaluated during method development.
- The accuracy of an analyticalal method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.Accuracy should be measured using a minimum of five determinations per concentration.

The mean value should be within 15% of the actual value except, at LLOQ where it should not deviate by more than 20 %. The deviation of the mean from the true value serves as the measures of accuracy.

$$[\%]\ \text{Nominal} = 100 \times \left\{ \frac{\text{Mean Concentration}}{\text{Nominal Concentration}} \right\}$$

1.5.5 *Precision and repeatability*

Repeatability reflects the closeness of agreement of a series of measurements under the same operating conditions over a short interval of time. For a chromatographic method, repeatability can be evaluated by performing a minimum of six replicate injections of a single sample solution prepared at the 100% test concentration.

- Alternatively, repeatability can be determined by evaluating the precision from a minimum of nine determinations that encompass the specified range of the method. The nine determinations may be composed of triplicate determinations at each of three different concentration levels, one of which would represent the 100% test concentration.
- Intermediate precision reflects within laboratory variations such as different days, different analysts, and different equipments. Intermediate precision testing can consist of two different analysts, each preparing a total of six sample preparations, as per the analytical method. The analysts execute their testing on different days using separate instruments and analytical columns.
- The use of experimental design for this study could be advantageous because statistical evaluation of the resulting data could identify testing parameters (i.e., brand of HPLC system) that would need to be tightly controlled or specifically addressed in the analytical method. Results from each analyst should be evaluated to ensure a level of agreement between the two sets of data. Acceptance criteria for intermediate precision are dependent on the type of testing being performed.
- However, precision testing should be conducted by one analyst for early phase method qualification. Reproducibility reflects the precision between analytical testing sites. Each testing site can prepare a total of six sample preparations, as per the analytical method. Results are evaluated to ensure statistical equivalence among various testing sites. Acceptance criteria similar to those applied to intermediate precision also apply to reproducibility.
- Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intraassay precision. Repeatability is sometimes also termed within run or within day precision.
- The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.Precision should be measured using a minimum of five determinations per concentration.

The precision determined at each concentration level should not exceed 15 % of the Coefficient of variation (CV), except for the LLOQ where it should not exceed 20 % of the coefficient of variation.

[%] CV = 100 × standard deviation / Mean concentration

Good precision and accuracy can be obtained from methods with moderate recoveries, provided they have adequate sensitivity.

1.5.6 *Reproducibility*

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility only has to be studied, if a method is supposed to be used in different laboratories. Unfortunately, some authors also used the term reproducibility for within laboratory studies at the level of intermediate precision. This should, however, be avoided in order to prevent confusion. As already mentioned above, precision and bias can be estimated from the analysis of QC samples under specified conditions. As both precision and bias can vary substantially over the calibration range, it is necessary to evaluate these parameters at least at three concentration levels (low, medium, high).

1.5.7 *Stability*

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the 7 intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution.

- Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times,then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -700C during the three freeze and thaw cycles.

- Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

- Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

- Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen it for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

- Post-Preparative Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

1.5.8 *Recovery*

As already mentioned above, recovery is not among the validation parameters regarded as essential by the Conference Reports. Most authors agree that the value for recovery is not important as long as the data for LLOQ, LOD, precision and accuracy (bias) are acceptable. It can be calculated by comparison of the analyte response after sample workup with the response of a solution containing the analyte at the theoretical maximum concentration. Therefore, absolute recoveries can usually not be determined if the sample workup includes a derivatization step, as the derivatives are usually not available as reference substances.

- The recovery of an analyte in assay is the detector response obtained from an amount of the analyte added and extracted from the biological matrix, compared to the pure authentic standard. Recovery pertains to the extraction efficiency of an analyticalal method within the limits of variability.
- Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible.Recovery experiments should be performed by comparing the analyticalal results for extracted samples at three concentrations (low, medium and high) with unextracted standards that represent 100% recovery.

$$[\%]\ \text{Recovery} = 100 \times \left[\frac{\text{Mean Response of Extracted Samples}}{\text{Mean Response of Post Extracted Standards}} \right]$$

1.6 Application of Validated Method to Routine Drug Analysis

Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, biological samples can be analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. This is true for procedures where precision and accuracy variabilities routinely fall within acceptable tolerance limits. For a difficult procedure with a labile analyte where high precision and accuracy specifications may be difficult to achieve, duplicate or even triplicate analyses can be performed for a better estimate of analyte. A calibration curve should be generated for each analyte to assay samples in each analytical run and should be used to calculate the concentration of the analyte in the unknown samples in the run.

The following recommendations should be noted in applying a bioanalytical method to routine drug analysis:

- A matrix-based standard curve should consist of a minimum of six standard points, excluding blanks (either single or replicate), covering the entire range.
- Response Function: Typically, the same curve fitting, weighting, and goodness of fit determined during prestudy validation should be used for the standard curve within the study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation. Changes in the response

function relationship between prestudy validation and routine run validation indicate potential problems.

- The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
- System suitability: Based on the analyte and technique, a specific SOP (or sample) should be identified to ensure optimum operation of the system used. Any required sample dilutions should use like matrix (e.g., human to human) obviating the need to incorporate actual within-study dilution matrix QC samples.
- Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and acceptance criteria. This SOP or guideline should explain the reasons for repeating sample analysis. Reasons for repeat analyses could include repeat analysis of clinical or preclinical samples for regulatory purposes, inconsistent replicate analysis, samples outside of the assay range, sample processing errors, equipment failure, poor chromatography, and inconsistent pharmacokinetic data. Reassays should be done in triplicate if sample volume allows. The rationale for the repeat analysis and the reporting of the repeat analysis should be clearly documented.

1.7 Analytical Techniques in Bioanalysis

Chromatography is a separation technique by which solutes of two or more components are separated by a dynamic differential migrational process. In a system consisting of two phases, one of which moves continuously in a given direction and in which individual components exhibit differential mobility due to difference in their adsorption or partition or molecular size etc. Methods for separation of Drugs and their metabolites in biological sample can be developed, provided one has knowledge about the nature of the Drug, its molecular weight, polarity, pKa, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column to be used with what kind of mobile phase.

Interfacing mass spectrometers with chromatographic separation techniques is one of the most sophisticated and sensitive technique used in detection and quantization of Drug and its active metabolite in biological fluid.

1.7.1 Selection of Mobile Phase

Since mobile phase governs solute –stationary phase interaction

- Practical considerations dictate that it should not degrade the equipment or column packing .So strong acids; bases and halides should be avoided.
- Chemical purity of sample is important factor. Since large volume of solvents are pumped through the column. Trace amount of impurities can easily concentrate in column and eventually detrimental to the result. So HPLC grade solvents only recommended to use in the analysis
- Volatility should be considered if sample recovery is required and mass transfer between solvent and stationary phase will be reduced. Water, acetonitrile, ethanol, 2 propanol & methanol are widely used solvents.

1.7.2 Role of solvent type

Chromatographic separations thus vary with solvent properties and are related to sample solubility, polarity and solvent strength. Solvents that interact strongly with the sample will increase the sample ion exit in the solvents and are not able to equilibrate with adsorbent surface. Changing the organic solvent will change the selectivity. In reverse phase, less polar solvent exhibit greater solvent strength than polar solvents .The solvents water (most polar), methanol, Acetonitrile and Tetrahydrofuran placed in ascending order of their polarity.

1.7.3 Selection of buffer and role of pH

pH is another factor in resolution equation that will affect the selectivity of the separation. In reverse phase HPLC, sample retention increases when the analyte is more hydrophobic .Thus when an acid (HA) or base (B) is ionized (converted in the form of unionized free or base) it becomes more hydrophilic and is less interactive with column binding sites.

Thus when selecting a buffer for a given application the following considerations are important. They are,

- The buffer capacity is dependent on the buffer pH, pKa and buffer concentration.
- Other properties such as volatility, solubility, stability of the buffer and its reactivity to the analytes play important role in chromatographic system.

1.7.4 Selection of column

HPLC column is the important tool for separation of analytes .So the column must posse's good selectivity, efficiency and reproducibility to provide good separation of analytes. Commonly used column are C-18, C-8, Phenyl, Cyano columns. They are chemically different bonded phase having different selectivity with same mobile phase.

1.8 Chromatographic Method

The presence of metabolites or more than one drug in a biological sample usually demands a more sophisticated separation for their measurement especially, when two or more drugs are of similar physical and chemical nature. Chromatography is a separation technique that is based on differing affinities of a mixture of solutes between at least two phases. The result is a physical separation of the mixture into its various components. The affinities or interactions can be classified in terms of a solute adhering to the surface of a polar solid (adsorption), a solute dissolving in a liquid (partition), and a solute passing through or impeded by a porous substance based on its molecular size (exclusion).

In the following sections, individual chromatographic techniques are discussed in relation to their usefulness as separation tools for drugs or metabolites in biological samples.

1.9 High Performance Liquid Chromatography

HPLC is the chemistry based tool for quantifying and analyzing mixtures of chemical compounds. It is used to find the amount of a chemical compound of interest within a mixture of other chemicals. As the mobile phase used to separate the sample components is liquid (like water or alcohol), thus the technique is termed Liquid chromatography. Due to the use of reduced particle size (generally 5 μ) and closed packing of the adsorbent, a very high pressure of about 6000 psi (400 atm) is required to force the mobile phase through the column improving the performance of the instrument thus earning the name High Pressure/ Performance Liquid Chromatography.

1.9.1 *Basic principle of HPLC*

High performance liquid chromatography (HPLC) is a separation technique which is utilizing to differences in distribution of compounds in two phases called the stationary phase and the mobile phase. The stationary phase is a thin layer created on the surface of fine particles and the mobile phase is the liquid flowing over the particles. Under a certain dynamic condition, each component in a sample has a different distribution equilibrium depending upon the solubility in the phases and/ or molecular size. As a result, the components move at different speeds over the stationary phase and are thereby separated from each other. The column is a stainless steel (or resin) tube, which is packed with spherical solid particles. Mobile phase is constantly fed into the column inlet at a constant rate by a pump. The sample injected from a sample injector that are located near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and the mobile phases. Compounds move in the column only when they are in the mobile phase. Compounds that are tending to be distributed in the mobile phase therefore migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column. The separation process is monitored by the integrator from the time of injection to its elution, a graph is obtained. This graph is called a chromatogram.

1.9.2 *Elution Process*

There are two modes of elution processes: Isocratic elution and Gradient elution.

1.9.2.1 Isocratic Elution

In an isocratic elution, a sample is injected onto a given column and the mobile phase composition remains unchanged through the time required for the sample components to elute from the column. No single isocratic elution can separate a complex mixture with adequate resolution in a reasonable time and with good detectibility. The isocratic separation of samples with widely varying k' (partition ratio) values typically exhibits poor resolution of early-eluting bands, difficult detection of late-eluting bands, and unnecessarily long elution times.

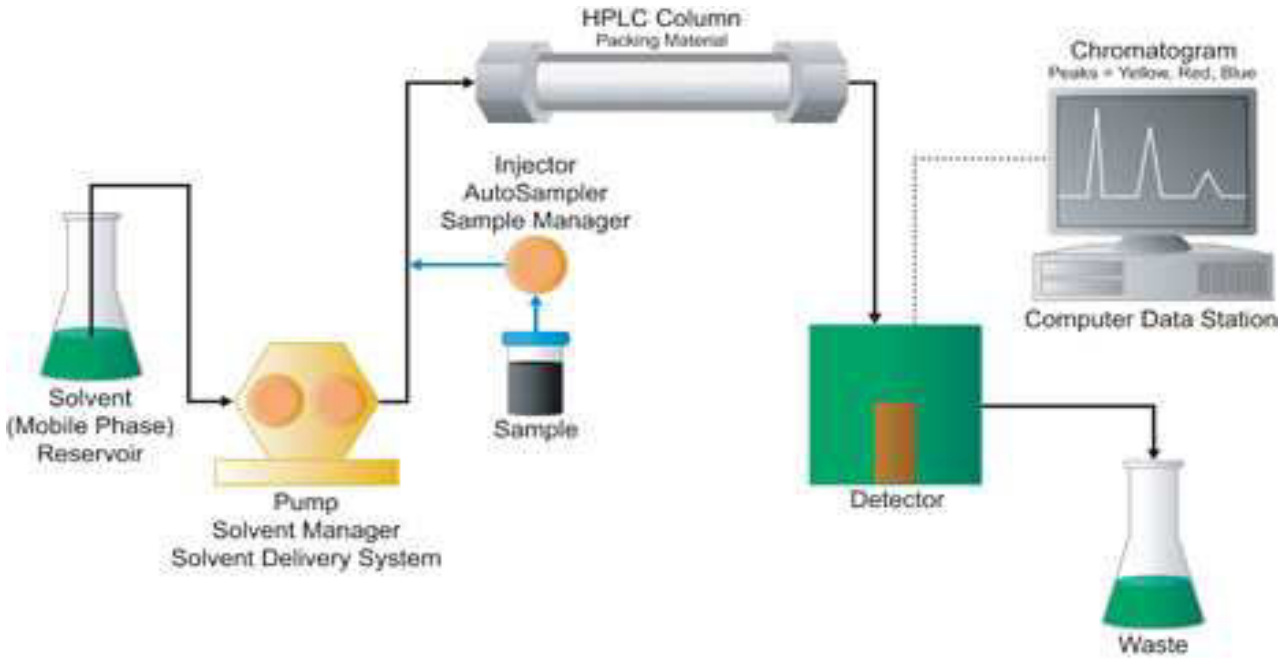


Figure 1 Isocratic LC System

1.9.2.2 Gradient Elution

Solvent Programming, also called gradient elution, involves changing the mobile-phase composition either stepwise or continuously as elution proceeds during the chromatographic run. The main purpose of gradient elution is to move strongly retained components of the mixture faster, but having the least retained component well resolved. Usually all the sample components are initially retained at the top of the column. Starting with the low content of the organic component in the eluent, the least retained componentgets separated. Strongly retained components will sit on the adsorbent surface on the top of the column, or will move very slowly.

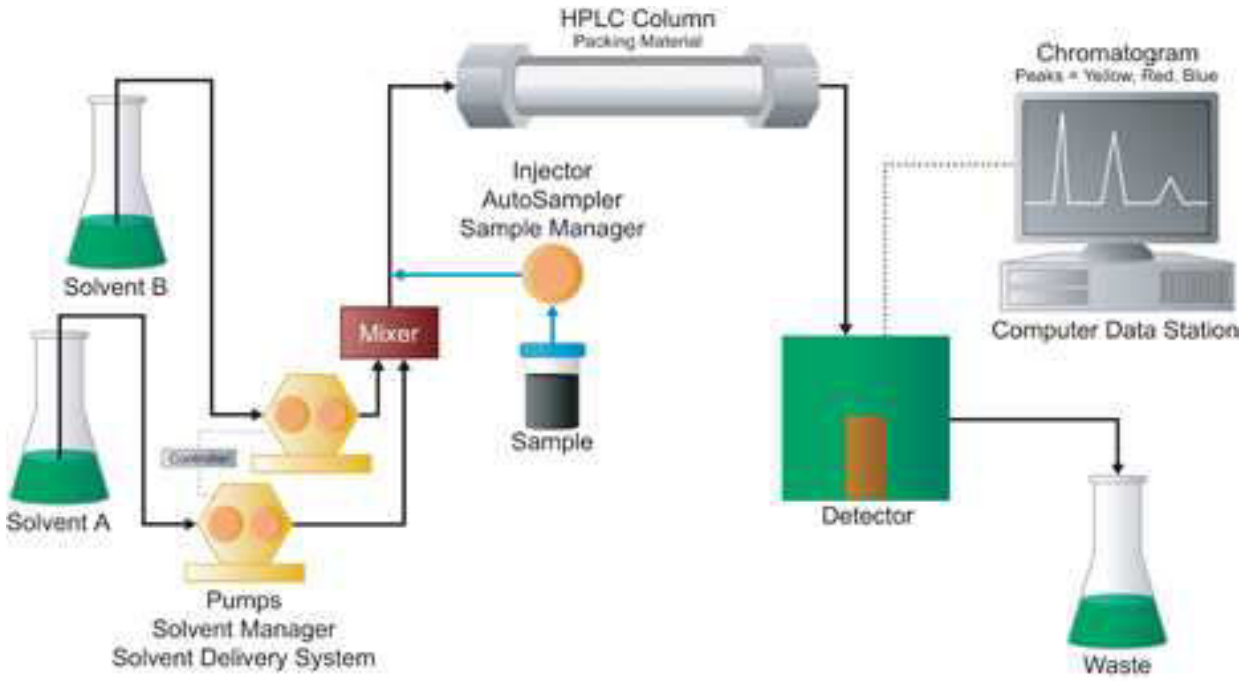


Figure: 2 High-Pressure-Gradient Systems

1.9.3 *The general instrumentation for HPLC incorporates the following components*

- There is a solvent reservoir for the mobile phase.
- The mobile phase must be delivered to the column by different types of pump. To obtain separations either based on short analysis time or under optimum pressure, a wide range of pressure and flow is desirable. The pumping system must be pulse-free or else have pulse damper to avoid generating baseline instability in the detector.
- Sampling valves or loops are used to inject the sample in the flowing mobile phase just at the head of the separation column. Samples should be dissolved in a portion of the mobile phase to eliminate an unnecessary peak.
- At the head of separation column there may be a guard column or an in-line filter to prevent contamination of the main column by small particulate.
- To measure column inlet pressure, a pressure gauge is inserted in front of the separation column.
- The separating column contains the packing needed to accomplish the desired HPLC separation. These packings may be of silica for adsorption chromatography, bonded phases for liquid-liquid chromatography, ion-exchange functional groups bonded to the stationary support for ion-exchange chromatography, gels of specific porosity for

exclusion chromatography, or some other unique packing for a particular separation method.

- A detector with different type of data handling device completes the basic instrumentation.[5]

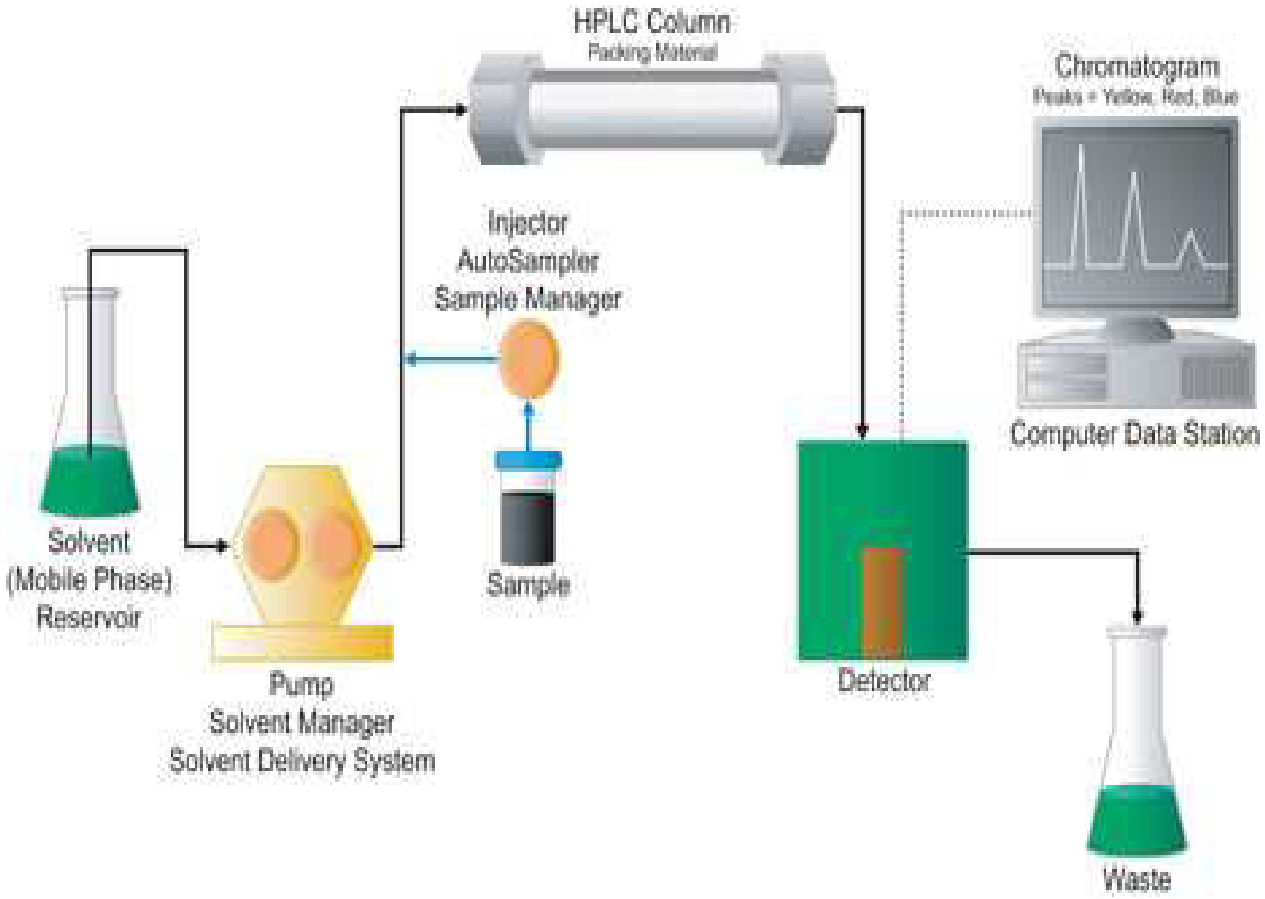


Figure 3 Schematic Diagram of HPLC Instrument

Figure 4 A Typical HPLC [Waters Alliance] System

1.9.4 *Mobile –Phase Delivery System (Pumps)*

The mobile phase must be delivered to the column over a wide range of flow rates and pressure. A degasser is needed to remove dissolved air and other gases from the solvent. Another desirable feature in the solvent-delivery system is the capability for generating a solvent gradient. A pump should be able to operate at least 100 atm (1500 psi), a pressure suited to less expensive chromatographs. However, 400 atm (6000 psi) is a more desirable pressure limit. For many analytical columns only moderate flow rates of 0.5-2.0 ml/min need to be generated.

There are two groups according to the manner in which they Works:

1.9.4.1 Constant flow rate pumps:

The two types of pumps in this category they are the reciprocating piston pump and syringe drive pumps. The reciprocating piston pump is the most widely used pump in modern HPLC. A filling and pumping cycle characterize this pump. During the filling cycle a piston is withdrawn from a syringe chamber. Two check valves are connected to this chamber such that during the piston withdrawal, solvent flows from the solvent reservoir but not into the

pump outlet. When the piston direction is reversed, the check valves operate to allow solvent to flow from the outlet valve only.

e.g. Milton Roy Reciprocating Pump, Dual Piston Pump, Triple Piston Pumps.

The syringe drive pump is a constant displacement pump in which all of the mobile phase contained within the pump. It is, in effect, a single stroke displacement pump. A screw free drive connected to a stepping motor actuates the piston inside the chamber. The voltage input to the stepping motor controls the volume displaced by the pump per unit time. This type pump produces a pulse less flow and requires no check valves.

1.9.4.2 Constant pressure pumps:

Constant pressure pump can deliver a constant-flow- rate if it operates against a constant column backpressure and if the viscosity of mobile phase remains constant. Consequently temperature should be controlled. Since the mobile phase is in direct contact with the pressurized gas a significant amount of gas may be dissolved. Thus a piston of mobile phase may contain sufficient dissolved gases to produce bubble in the detector. The pneumatic amplifier pump (e.g. The Haskell Pump) is a modification of a simple gas displacement pump. The gas pressure is applied to a large piston that is connected to a smaller diameter piston in contact with the mobile phase. The pressure on the gas piston is translated to a higher pressure (per unit area) on the solvent.[6]

1.9.5 *Columns*

The column is the heart of HPLC instrument. Columns are constructed of heavy-wall, glass-lined metal tubing or stainless steel tubing to withstand high pressures (up to 680 atm) and the chemical action of the mobile phase. Column end fittings and connector must be designed with zero void volume to avoid unswept corners or stagnant pockets of mobile phase that can contribute significantly to extra-column band broadening. Most column lengths range from 10 to 30 cm short, fast columns is 3 to 8 cm long. For exclusion chromatography, columns are 50 to 100 cm long.

1.9.6 *Standard columns*

Many HPLC separations are done on columns with an internal diameter of 4 to 5 mm. Such columns provide a good compromise between efficiency, sample capacity, and the amount of packing and solvent required. Column packing feature particles those are uniformly sized and mechanically stable. Particle diameters lie in the range 3-5 µm, occasionally up to 10 µm or higher for preparative chromatography. The columns are classified into various categories depending on their carbon loading.

The various column parameters to be considered are:

- Column length
- Column internal diameter
- Particle shape
- Particle shape
- Pore size
- Surface area
- End capping
- Carbon load

Figure: 1.5- HPLC Column Dimensions

Figure: 1.6-Column Hardware Examples

1.9.7 *Detector*

The choice of detector is an important consideration. There is little use in running a separation if the detector one uses cannot see all the components of interest, or conversely, if it sees too much. The sensitivity of universal detector for HPLC has not been devised yet. Thus it is necessary to select a detector on the basis of compound under consideration, [7]

Table: 1 List of detector

Detectors	Analytes	Solvent Requirement	Comments
UV-Visible	With any chromophore	UV-grade non-UV absorbing solvents	Has a high degree of selectivity & is useful for many HPLC application
Fluorescence	Fluorescent compounds	UV grade non UV absorbing solvents	Highly selective and sensitive. Often used to analyze derivatized compounds
Refractive Index (RI)	Compounds with different RI to the mobile phase	Mobile phase in gradient mode cannot be run	Virtually a universal detector but has limited sensitivity
Conductivity	Charged or polar compounds	Mobile phase must be conducting	Very selective and sensitive

Electrochemical	Readily oxidizable or reducible compounds, especially biological samples	Mobile phase must be conducting	Very selective and sensitive
Evaporative Light Scattering	Virtually all compounds	Fully gradient compatible, no limitation on solvent choice	A universal detector which is highly sensitive but not selective.
Mass Spectrometer	Broad range of compounds	Must be volatile solvents & volatile buffers	Highly sensitive and is powerful 2nd dimensional analytical tool.

1.9.8 *Normal Phase Liquid chromatography*

Normal-phase liquid-liquid chromatography uses a polar stationary phase and less polar mobile phase. To select an optimum mobile phase, it is best to start with a pure hydrocarbon mobile phase such as heptanes. If the sample is strongly retained, the polarity of the mobile phase should be increased, perhaps by adding small amounts of methanol or dioxane. In the normal phase mode, separation of oil-soluble vitamins, essential oils, nitrophenols, or more polar homologous series have been performed using alcohol/heptanes as the mobile phase.

1.9.9 *Reverse Phase Liquid chromatography*

Reverse phase chromatography uses hydrophobic bonded packing, usually with an octadecyl or octyl functional group and a polar mobile phase, often a partially or fully aqueous mobile phase. Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. Generally, the lower the polarity of the mobile phase, the higher is its eluent strength. The elution order of the classes of compounds in table is reversed (thus the name reverse-phase chromatography). Hydrocarbons are retained more strongly than alcohols. Also, the eluent strength of the various solvents in reverse-phase chromatography follows approximately the reverse order given in table. Thus water is the weakest eluent. Methanol and acetonitrile are popular solvents because they have low viscosity and are readily available with excellent purity.

1.9.10 Liquid chromatography and mass spectrometry

It is the way of interfacing the high vacuum domain of mass spectrometer with the condensed phase domain of liquid chromatography.

1.9.11 Need of LC system interfacing with mass spectrometer

The separation is done prior to mass analysis because the mass spectrometer is incapable of directly determining every analyte in all type of sample. The Liquid chromatography can be regarded as a part of preparative procedure required for sample clean up which improves linearity, accuracy and better sensitivity.

Mass spectrometer provides greater selectivity and sensitivity for chromatographic development because endogenous matrix can co elutes with analyte yet not interferes as long as these component posses' precursor masses. The main purpose of interface is to evaporate the mobile phase and transfer the analyte from the higher pressure/atmospheric pressure at which chromatographic separation is achieved to the lower pressure required for the mass analysis. LC/MS is highly effective interface for coupling liquid chromatography to the mass spectrometer.

1.9.12 *Instrumentation*

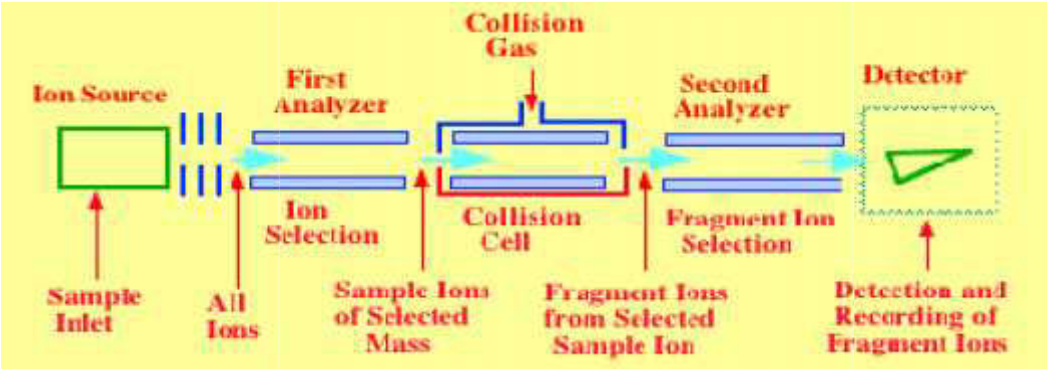


Figure 7 Instrumentation of mass spectrometry

1.9.13 *Sample inlet system*

There are two opinions of a sample inlet system

- The sample introduced as neutral species through a controlled vacuum leak followed by ionization in vacuum chamber.
- Create the ion at atmospheric pressure and then introduced the ion in to the mass spectrometer through a controlled vacuum leak with aid of electrostatic this process is called API (atmospheric pressure ionization) provide best way when a dynamic coupling of liquid chromatograph done.

1.9.14 *Ionization source*

Ionization proceeds by two fundamental processes:

- Loss/gain of an electron
- Loss/gain of a charge particle

An odd electron ions is generated by the gain/loss of an electron. In vacuum generating method ionic species of identical nominal molecular weight differ only by the mass of an electron to the neutral specie from which it was generated.

An even electron is produced by gain or loss of even electron specie from a molecule.

There are four common modes of ionization

- Electron ionization (EI)
- Chemical ionization (CI)
- Matrix-Assisted Laser desorption Ionization (MALDI)
- Atmospheric pressure ionization (API)

In LC/MS interfacing Atmospheric Pressure Ionization (API) is the potential Ionization techniques because

- It gives softer ionization
- It provide convenient interface with liquid chromatograph

1.9.15 *Type of API Source*

- Atmospheric Pressure Electro Spray Ionization
- Atmospheric Pressure Chemical Ionization

1.9.16 *Atmospheric Pressure Electro Spray Ionization*

It is an atmospheric ionization technique in which ions are generated in the solution phase by evaporation of carrier solvent and ions are produced in gas phase. An appropriate solvent from LC (liquid chromatographic) system is passed through a metal capillary to which a static DC voltage is applied to create ionization of effluents. When the solvent got evaporate the charge density increases creating columbic repulsion and subsequent dissociation of droplet. Further evaporation of droplet creates an environment in which charge transfer takes place from the solvent to the analyte. Typically a voltage of 2.5 to 5 kV will be applied to generate an even electron ion in gas phase. This method is commonly used for high molecular compounds. Low ionization is observed in this technique due to solvent clustering and analyte adduct formation, so this is most applicable for LC/MS system.

1.9.17 *Atmospheric Pressure Chemical Ionization*

It is an ionization technique in which the ionization occurs not in vacuum but at atmospheric pressure. It is gas phase ionization process whereby gas phase molecules are isolated from the carrier solvent before ionization. Generally less polar compounds are ionized by this method.

1.10 Glossary

Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed *trueness*.

Analyte: A specific chemical moiety being measured, which can be intact drug, biomolecule or its derivative, metabolite, and/or degradation product in a biologic matrix.

Analytical run (or batch): A complete set of analytical and study samples with appropriate number of standards and QCs for their validation. Several runs (or batches) may be completed in one day, or one run (or batch) may take several days to complete.

Biological matrix: A discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues.

Calibration standard: A biological matrix to which a known amount of analyte has been added or *spiked*. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.

Internal standard: Test compound(s) (e.g. structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

Limit of detection (LOD): The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.

Lower limit of quantification (LLOQ): The lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

Matrix effect: The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Method: A comprehensive description of all procedures used in sample analysis.

Precision: The closeness of agreement (*degree of scatter*) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Quantification range: The range of concentration, including ULOQ and LLOQ, that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship.

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

Reproducibility: The precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time.

Sample: A generic term encompassing controls, blanks, unknowns, and processed samples, as described below:

Blank: A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.

Quality control sample (QC): A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

Unknown: A biological sample that is the subject of the analysis.

Selectivity: The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants, or matrix components.

Stability: The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Standard curve: The relationship between the experimental response value and the analytical concentration (also called a *calibration curve*).

System suitability: Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.

Upper limit of quantification (ULOQ): The highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.

3. REVIEW OF LITERATURE

I. Salem, Musab Alkhatib, Naji Najib *et al* done LC-MS/MS determination of betamethasone and its phosphate and acetate esters in human plasma after sample stabilization.

Liljana Bogdanovska, Mirjana Popovska, Aneta Dimitrovska *et al* Studied the development and validation of RP-HPLC method for determination of betamethasone dipropionate in gingival crevicular fluid.

Song Ja-park, Yun-je Kim *et al* done the Analysis of corticosteroids in urine by HPLC and thermospray LC-MS method.

Mahesh N. Samtani, Willam J *et al* studied for quantification of dexamethasone and corticosterone in rat biofluids and fetal tissue using highly sensitive LC/MS/MS method.

Valerie A. Frerichs, Kathleen. M. Tornatore *et al* performed determination of the glucocorticoids such as prednisone,prednisolone,dexamethasone and cortisol in human serum using liquid chromatography coupled to tandem mass spectrometry**.**

Kumar V., Mostafa S., Kayo M.*, et al* done HPLC determination of dexamethasone in human plasma and its applications to an in vitro release study from endovascular stents.

N. Goyal, E. Goldberg et al done the determination of dexamethasone, dexamethasone 21-acetate and paclitaxel in a simulated biological matrix by RP-HPLC method.

Isam I. Salem, Musab Alkhatib, Naji Najib et al done the LC–MS/MS determination of betamethasone and its phosphate and acetate esters in human plasma after sample stabilization.

S.A. Doppenschmitt, B. Scheidel, J.P. Surmann *et al* Performed simultaneous determination of prednisolone, prednisolone acetate and hydrocortisone in human serum by high performance liquid chromatography.

Majid, Oneeb, Akhlaghi, Fatemeh *et al* performed simultaneous determination of plasma prednisolone, prednisone and cortisol levels by high performance liquid chromatography.

Robert L Taylor, Dwaine Machacek *et al* validation of high throughput liquid chromatography-mass spectrometry method for prednisolone.

Syed Naeem Razzaq, Islam Ullah Khan et al done the Stability indicating HPLC method for the simultaneous determination of moxifloxacin and prednisolone in pharmaceutical formulations.

Raval Kashyap, E.V.S. Subrahmanyam et al done the Development and validation of UV spectroscopy method for the estimation of prednisolone in bulk and dosage form.

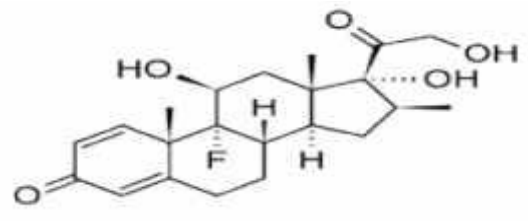
Mohammed Shahid Ali, Mohsin Ghori et al done the Simultaneous Determination of Ofloxacin, Tetrahydrozoline Hydrochloride, and Prednisolone Acetate by High-Performance Liquid Chromatography.

Ahi Shobha, Dubey Sachin et al done the Identification of Prednisolone, Methylprednisolone and Their Metabolites in Human urine using HPLC (+) ESI-MS/MS and Detection of Possible Adulteration in Indian Herbal Drug Preparations.

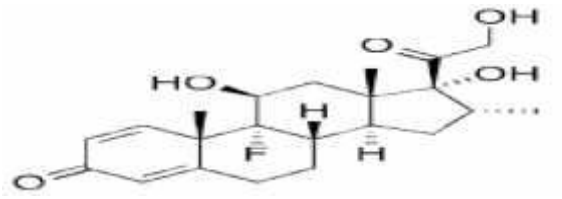
Robert L. Taylor, Stefan K. Grebe, Ravinder J. Singh et al done the Quantitative, highly sensitive liquid chromatography-tandem mass spectrometry method for detection of synthetic corticosteroids.

4. DRUG PROFILE

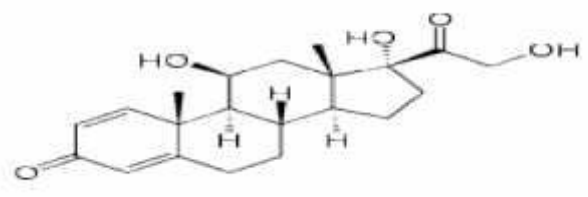
Betamethasone

S.No.	Parameter	Description
1	Structure of Betamethasone	
2	Drug category	Corticosteroid
3	Chemical formula	**$C_{22}H_{29}FO_5$**
4	Molecular weight	392.461
5	IUPAC Name	(8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*S*,17*R*)-9-fluoro- 11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro- 3*H*-cyclopenta[*a*]phenanthren-3-one
6	Bioavilability	-
7	Half Life	36-54 hours
8	Metabolism	hepatic CYP3A4
9	Routes	Oral,Topical,IM
10	Excretion	Renal (in urine)

Dexamethasone

S.No.	Parameter	Description
1	Structure of Dexamethasone	
2	Drug category	Corticosteroid
3	Chemical formula	$C_{22}H_{29}FO_5$
4	Molecular weight	392.461 g/mol
5	IUPAC Name	(8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9- Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17- dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one
6	Bioavilability	80-90%
7	Half Life	190 mins
8	Metabolism	Hepatic
9	Routes	Oral, IV, IM, SC and IO
10	Excretion	Urine (65%)

Prednisolone

S.No.	Parameter	Description
1	Structure of Prednisolone	
2	Drug category	Corticosteroid
3	Chemical formula	$\mathbf{C_{21}H_{28}O_5}$
4	Molecular weight	360.444 g/mol
5	IUPAC Name	(11β)-11,17,21-trihydroxypregna-1,4-diene-3,20-dione
6	Bioavilability	-
7	Half Life	2-3 hours
8	Metabolism	Hepatic
9	Routes	Oral, Nasal, Rectal, IV, Injection
10	Excretion	Urine

5. MATERIALS AND METHODS

5.1 List of materials used

1) **API of Betamethasone, Dexamethasone & Prednisolone respectively.**
2) **Rat plasma**
3) **Solvents used for extraction process:**
   - Water for HPLC grade (Millie Q or equivalet)
   - Ethyl acetate (HPLC grade)
   - Diethyl ether
   - Chloroform
   - Dichloromethane

5.2 Steps followed in developmental study

- Study of literature review
- Proposed protocols or parameters for extraction process & validation were established
- Find out the probable correct Cmax for our drug.
- Calculations to decide LLOQ, ULOQ/HQC, MQC.
- Selection of internal standard
- Procurement of plasma sample
- Method optimization of sample with different solvent to get maximum %recovery
  - Approximately analysis with 5 diff. solvents.
- Individual reading for Blank plasma.
- Individual reading for internal std.
- Individual runs of LQC, MQC, HQC, LLOQ, ULOQ.

- Readings for calibration curve
  - Singlet or doublet could be taken for each concentration.
- Validation
  - For Accuracy: 5 replicates of LQC, MQC and HQC for 5 days.
  - Selectivity: 6 plasma obtained from 6 different sources.
- Stability Studies
  - Freeze-thaw method: LQC and HQC, 5 samples of each were freeze-thaw for 3 cycles of 12, 24 and 48hrs.
  - Bench-top: Short term analysis between 2-24hrs.
  - Long term analysis
  - Plane stock solution stability.
- Experimental studies were conducted
- Analytical results were evaluated
- Statistical evaluation was carried out
- Report was prepared documenting all the results

Fig. 5.1 Instrument information

System: HPLC Binary Gradient System

Model no.: HPLC 3000 Series

Company: Analytical Technologies Ltd.

Detector: UV-3000-M

Pump: P-3000-M Reciprocating (40MPa)

Column: Grace C18 (250mm x 4.6ID, Particle size: 5 micron)

Software: HPLC Workstation

Balance: Wenser High Precision Balance

Model: PGB 100

Max: 100gm Min: 0.001gm

Sonicator**:** Wenser Ultra Sonicator

Model: WUC- 4L

Capacity: 4 Liter

5.3 Methods used & optimization parameters

Name of Drug-Betamethasone

- **Development of chromatographic condition**

  The chromatogram was developed initially using separation condition such as mobile phase (methanol:water in the ratio of 10:90 in increasing order). The system was used Analytical technologies Lts. Model no. 3000Series as mentioned above. The optimized chromatographic conditions were optimized using a mobile phase methanol:water in the ratio of 60:40 at flow rate 0.9ml/min for betamethasone with the stationary phase was used as Grace C18column(250mm x 4.6ID,partical size:5 micron).

- **Standard solution preparation**

  Standard solutions were prepared by using HPLC grade methanol and water in the ratio 1:1. Initially 10 mg of Betamethasone was weighted and transferred into the standard flask.In that combined solvent (methanol and water) added and finally made the volume with the same up to 100ml to get 100ppm stock solution. The stock solution further serialy diluted and was used for the analysis. The stock solution was maintained refrigerated at 8ºC.

- **Extraction method**

  In this process first take 1 ml of plasma from sample which is previously stored at 5-7^{0}C. In this add 0.0125 milliliter of 1ppm of drug(Betamethasone) which is prepared in methanol:water combination & 0.125 milliliter of 1ppm of internal standard. After this vortex the above prepared mixture for 3 mins. Also in this add 0.200 milliliter of 1% of hydrochloric acid to provide acidic nature to the plasma. Again vortex the above mixture for 3-5 mins. In this add ethyl acetate which act as a extracting solvent & again vortex the mixture for 3-5 mins. Now withdraw 2ml of ethyl acetate in which drug is extracted in fresh tube & finally allow to evaporate the solvent which will leave dried drug in tube & dilute it with 0.500 milliliter of mobile phase.

Table no 5.1 Summary for Older methods

Name of Authors (Literature review)	Analytical method	Mobile phase	Column used	Flow rate
Liming peng,tivader farcas etl	HP 1100 LC	Formic acid:water	Gemini 5µm,C18 150X3.0 mm	0.6ml/min
Isam salem,musab alkhatib etl	LC-MS/MS	-	C8 column	-
Robert taylor,Stefan geebe etl	LC-MS/MS		Reversed phase column	

Table no 5.2 Summary for Optimized method

Column used	Mobile phase	Flow rate	Wavelength	Observation	Result
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (10:90)	0.9ml/min	242nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (20:80)	0.9ml/min	242nm	Very low resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (40:60)	0.9ml/min	242nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (80:20)	0.9ml/min	242nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle	Methanol:water (50:50)	0.9ml/min	242nm	Improved resolution	Method rejected

size: 5 micron)					
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	**Methanol:water (60:40)**	**0.9ml/min**	**242nm**	**Good resolution**	**Method accepted**

Table no 5.3 Different ranges of concentrations for preparation of standards for Betamethasone

S.No	Sample	Concentration of Drug (ng/ml)	Concentration of Internal standard (ng/ml)
1	Std-A	10	80
2	Std-B	10	80
3	Std-C	20	80
4	Std-D	60	80
5	Std-E	120	80
6	Std-F	160	80

Name of Drug-Dexamethasone

- **Development of chromatographic condition**

  The chromatogram was developed initially using separation condition such as mobile phase (methanol:water in the ratio of 10:90 in increasing order). The system was used Anaytical technologies Lts. Model no. 3000Series as mentioned above. The optimized chromatographic conditions were optimized using a mobile phase methanol:water in the ratio of 40:60 at flow rate 1.0ml/min for dexamethasone with the stationary phase used as Grace C18column(250mm x 4.6ID,partical size:5 micron)

- **Standard solution preparation**

  Standard solutions were prepared by using HPLC grade methanol and water in the ratio 1:1. Initially 10 mg of drug was weighted and transferred into the standard flask.In that combined solvent (methanol and water) added and finally made the volume with the same up to 100ml to get 100ppm stock solution. The stock solution further serialy diluted and was used for the analysis. The stock solution was maintained refrigerated at 8ºC.

- **Extraction method**

  In this process first take 1 ml of plasma from sample which is previously stored at 5-7^{0}C. In this add 0.0125 mililitre of 1ppm of drug (Dexamethasone) which is prepared in methanol:water combination & 0.125 mililiter of 1ppm of internal standard. After this vortex the above prepared mixture for 3 mins. Also in this add 0.200 mililiter of 1% of hydrochloric acid to provide acidic nature to the plasma.Again vortex the above mixture for 3-5 mins. In this add ethyl acetate which act as a extracting solvent & again vortex the mixture for 3-5 mins. Now withdraw 2ml of ethyl acetate in which drug is extracted in fresh tube & finaly allow to evaporate the solvent which will leave dried drug in tube & dilute it with 0.500 mililiter of mobile phase.

Table no 5.4 Older methods

Name of Authors (Literature review)	Analytical method	Mobile phase	Column used	Flow rate
Kumar V,Mosta S, etl	HPLC	Acetonitrile: triple distilled water (28:72)	C18 column	1.2 ml/min
Goyal N, El Achchabi etl	LC-MS/MS	Acetonitrile: phosphoric acid: water	C18 column	-

Table no 5.5 Summary for Optimized method

Column used	Mobile phase	Flow rate	Wavelength	Observation	Result
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (10:90)	1.0 ml/min	240nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (20:80)	1.0 ml/min	240nm	Very low resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (30:70)	1.0 ml/min	240nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (80:20)	1.0 ml/min	240nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (50:50)	1.0 ml/min	240nm	Improved resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	**Methanol:water (40:60)**	**1.0 ml/min**	**240nm**	**Good resolution**	**Method accepted**

Table no 5.6 Different ranges of concentrations for preparation of standards for Dexamethasone

S. No	Sample	Concentration of Drug (ng/ml)	Concentration of Internal standard (ng/ml)
1	Std-A	70	480
2	Std-B	70	480
3	Std-C	140	480
4	Std-D	420	480
5	Std-E	840	480
6	Std-F	1120	480

Name of Drug- Prednisolone

- **Development of chromatographic condition**

  The chromatogram was developed initially using separation condition such as mobile phase (methanol:water in the ratio of 10:90 in increasing order). The system was used Analytical technologies Lts. Model no. 3000Series as mentioned above. The optimized chromatographic conditions were optimized using a mobile phase methanol:water in the ratio of 70:30 at flow rate 0.9ml/min with the stationary phase used as Grace C18column(250mm x 4.6ID,partical size:5 micron)

- **Standard solution preparation**

  Standard solutions were prepared by using HPLC grade methanol and water in the ratio 1:1. Initially 10 mg of Prednisolone was weighted and transferred into the standard flask.In that combined solvent (methanol and water) added and finally made the volume with the same up to 100ml to get 100ppm stock solution. The stock solution further serialy diluted and was used for the analysis. The stock solution was maintained refrigerated at 8ºC.

- **Extraction method**

  In this process first take 1 ml of plasma from sample which is previously stored at 5-7^{0}C. In this add 0.0125 milliliter of 1ppm of drug (Prednisolone) which is prepared in methanol:water combination & 0.125 milliliter of 1ppm of internal standard. After this vortex the above prepared mixture for 3 mins. Also in this add 0.200 milliliter of 1% of hydrochloric acid to provide acidic nature to the plasma. Again vortex the above mixture for 3-5 mins. In this add ethyl acetate which act as a extracting solvent & again vortex the mixture for 3-5 mins. Now withdraw 2ml of ethyl acetate in which drug is extracted in fresh tube & finally allow to evaporate the solvent which will leave dried drug in tube & dilute it with 0.500 milliliter of mobile phase.

Table no 5.7 Older methods

Name of Authors (Literature review)	Analytical method	Mobile phase	Column used	Flow rate
Ahi Shobha,Dubey sachin, etl	LC-MS/MS	Formic acid:Acetonitrile (50:50)	C-18,ODS-3 (3µm,50mm× 4.6mm)	0.6ml/min
Liming peng,Tivadar farcas, etl	HP 1100 LC system	Formic acid:Water	Gemini 5µm,C18 150X3.0 mm	0.6ml/min
Majid O,Alhlaghi F, etl	HPLC	Isopropanol:Water	Supelcosil LC-18-DB(5µm,150 mm× 4.6mm)	1.2ml/min

Table no 5.8 Summary for Optimized method

Column used	Mobile phase	Flow rate	Wavelength	Observation	Result
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (10:90)	0.9ml/min	238nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (20:80)	0.9ml/min	238nm	Very low resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (40:60)	0.9ml/min	238nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (50:50)	0.9ml/min	238nm	Poor resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	Methanol:water (60:40)	0.9ml/min	238nm	Improved resolution	Method rejected
Grace C18 (250mm x 4.6ID, Particle size: 5 micron)	**Methanol:water (70:30)**	**0.9ml/min**	**238nm**	**Good resolution**	**Method accepted**

Table no 5.9 Different concentration ranges for preparation of standards for Prednisolone

S. No	Sample	Concentration of Drug (ng/ml)	Concentration of Internal standard (ng/ml)
1	Std-A	100	80
2	Std-B	100	80
3	Std-C	200	80
4	Std-D	600	80
5	Std-E	1200	80
6	Std-F	1600	80

- **Statistical calculations**

  Following formulas were used for different statistical calculations in the proposed work.

1) **Coefficient of variation**:

$$CV = (SD/ Mean) \times 100$$

Where,

CV= Coefficient of variation

SD = Standard deviation

2) **Standard deviation:**

$$S^2 = \Sigma(X-M)^2/n-1$$

Where,

Σ = Sum of

X = Sample value

M = Mean value of samples

N = Sample size (number of samples)

3) **Relative Standard deviation:**

$$RSD = S^2 / X_{1*100}$$

4) Resolution:

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

Where,

R = Resolution between a peak of interest (peak 2) and peak preceding it (peak 1)

W_2 = width of the base of component peak 2

W1 = width of the base of component peak 2

t_2 = retension time of the second peak measured from point of injection

t_1 = retension time of the second peak measured from point of injection

5) Peak asymmetry:

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

Where,

T = Peak asymmetry or tailing factor W0.05 = Distance from the leading edge to the tailing edge of the peak, measured at a point 5% of the peak height from the baseline.

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

6) Theoretical plate per meter:

$$n = (5.54\ Vr_2) / Wh_2$$

Where,

n = Number of theoretical plates per meter.

Vr = the distance along the base line between the point of injection and a perpendicular dropped from the maximum of the peak of interest.

Wh = the width of the peak of interest at half peak height.

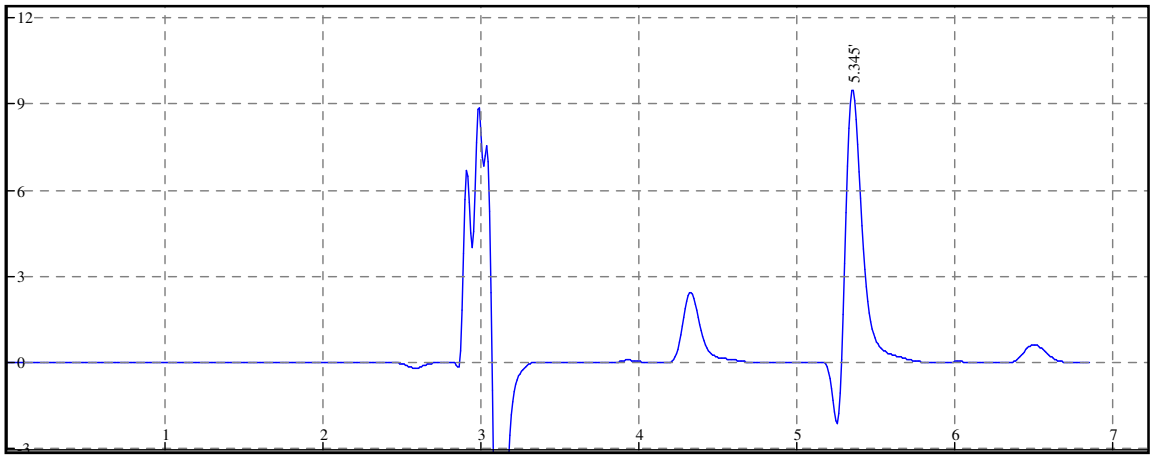
6. RESULTS AND ANALYSIS

Name of Drug: Betamethasone

Trial graphs for Betamethasone

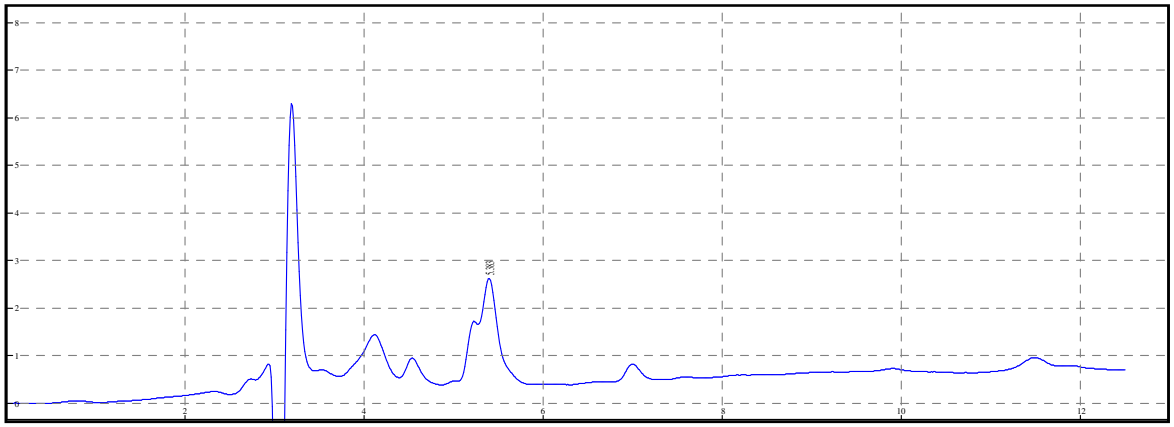
Sample name: Blank

Run time: 6.86min



Sample name: Blank+IS

Run time: 12.49min

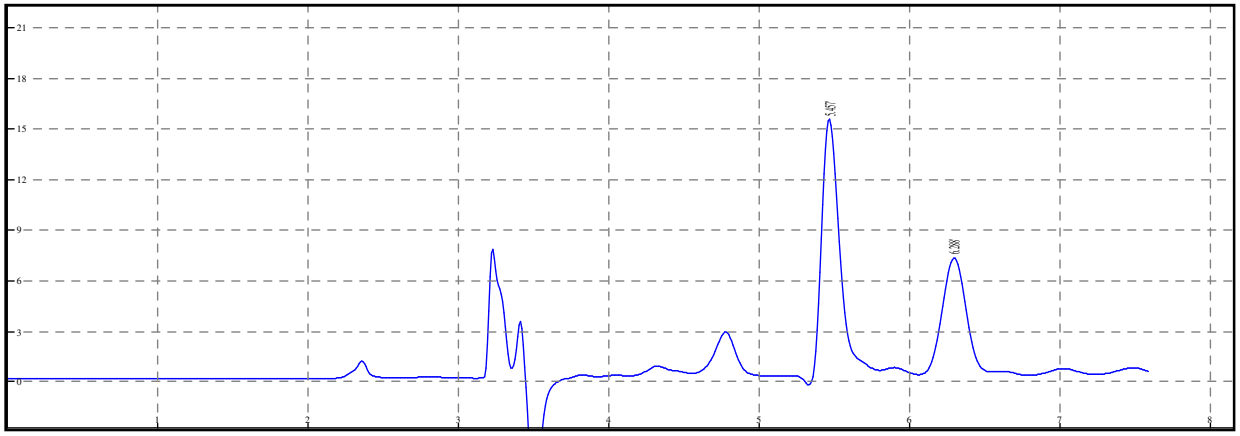


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.383	7919	0.00	13023	0.99

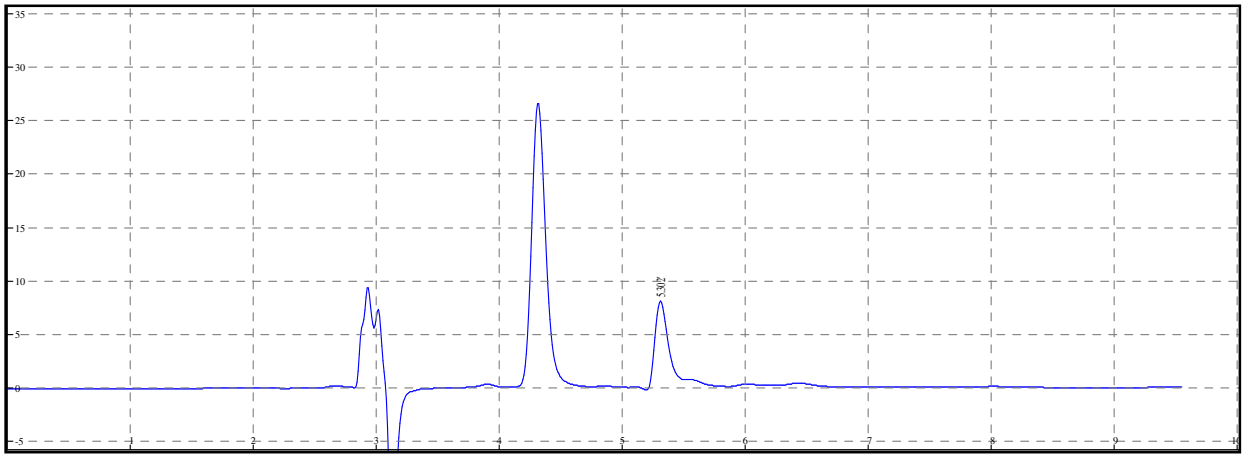
Sample name: LQC Trial

Run time: 7.59 min



Sample name: Betamethasone extraction trial

Run time: 9.55min

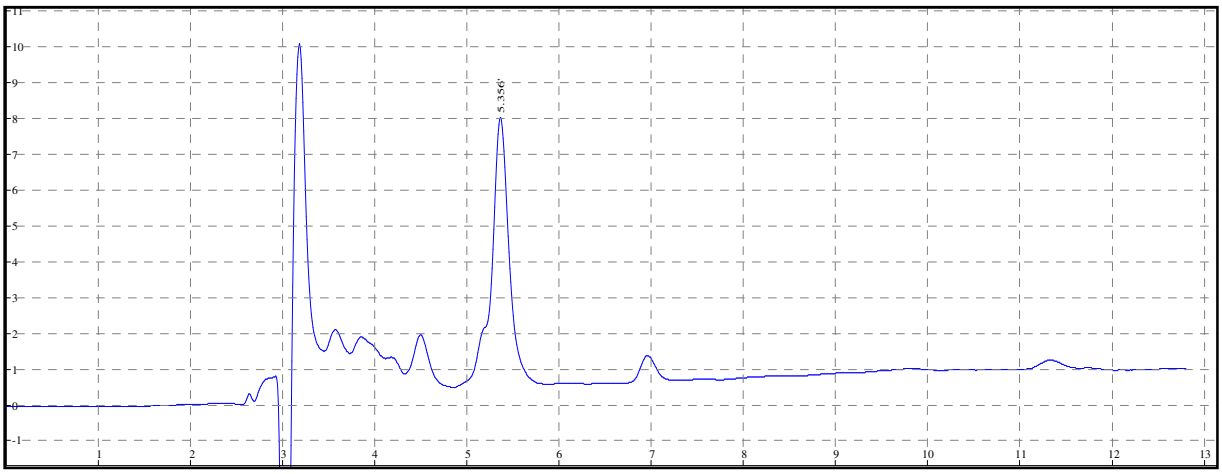


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.302	52372	0.00	14303	2.44

Selectivity

The desired method used RP-HPLC method for separation of betamethasone from paracetamol (IS) as well as plasma sample and was shown to be selective for the analyte and its IS (retention times for betamethasone and paracetamol were 5.80 and 7.20 minutes respectively).No interfering peaks were observed with the same retention time of the analyte when different plasma samples were analysed. The chromatograms of blank plasma and plasma sample spiked with drugs respectively are given below.



Chromatographic conditions

Sample Name: Blank + Paracetamol as an internal standard

Wavelength: 242nm

Mobile Phase: Methanol:Water (60:40)

Sample volume: 100µlFlow rate: 0.9 ml/min

Pressure: 10-11MPa

Run time: 12.80min

Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.356	34003	0.00	12491	1.06

FOR BLANK PLASMA SAMPLE

Chromatographic conditions

Sample Name: Blank Plasma Sample

Wavelength: 242nm

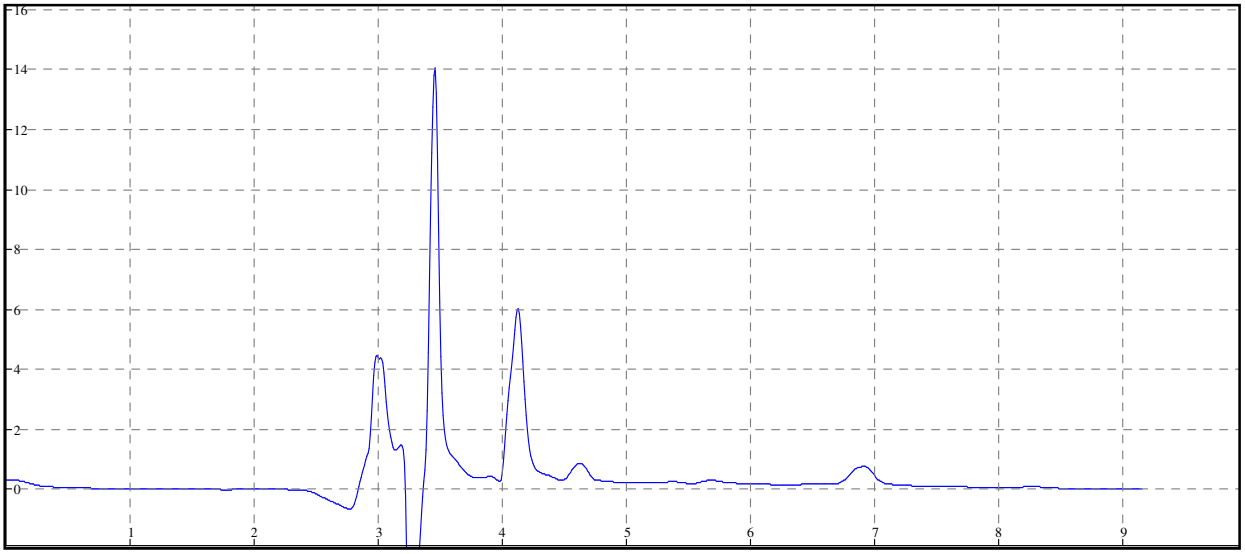
Mobile Phase: Methanol:Water (60:40)

Sample volume: 100µl

Flow rate: 0.9 ml/min

Pressure: 10-11MPa

Run time: 9.97min

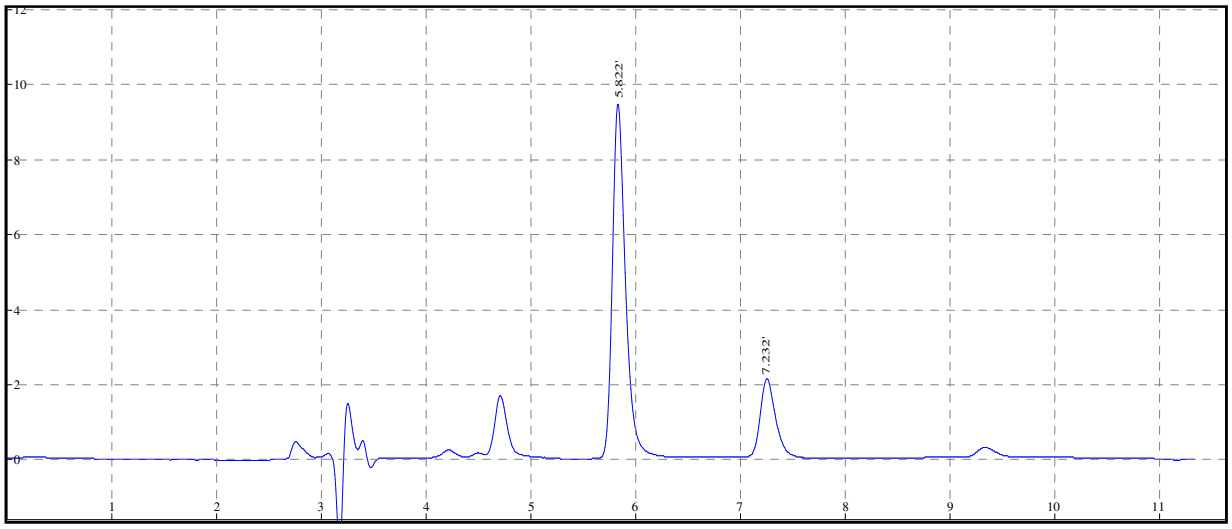


Linearity

Linearity was demonstrated by taking sample concentration ranges from 10.0-160 ng/ml. Fig. given below shows calibration curve of betamethasone. The calibration curve includes 6 calibration standards which are distributed 0.998 with goodness of fit.

Sample Name: Betamethasone Std A

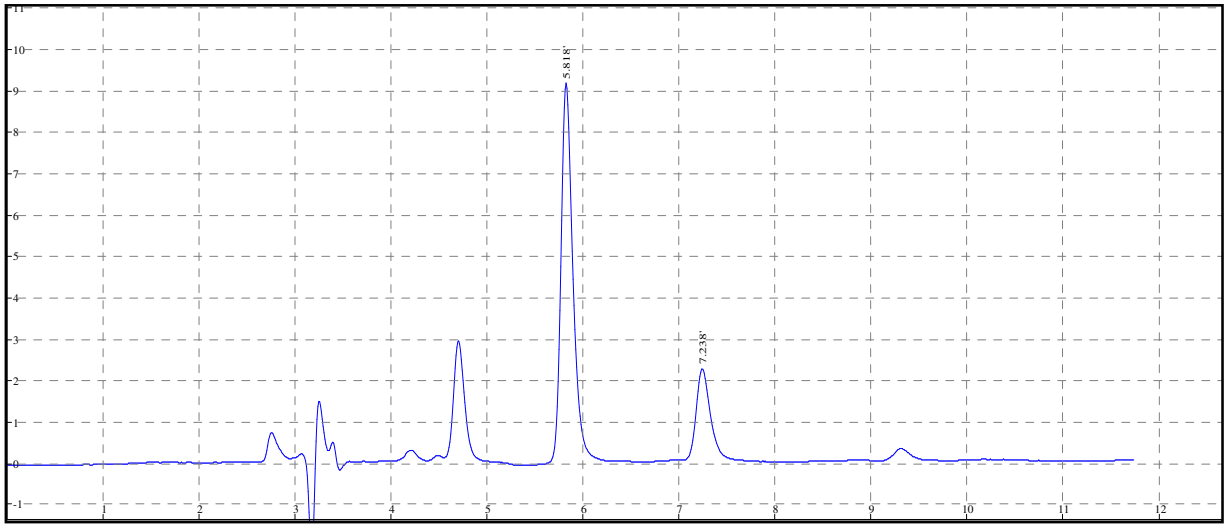
Run time: 11.34min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.822	52172	4.05	7363	1.35
7.232	11678	0.00	32496	1.27

Sample Name: Betamethasone Std B



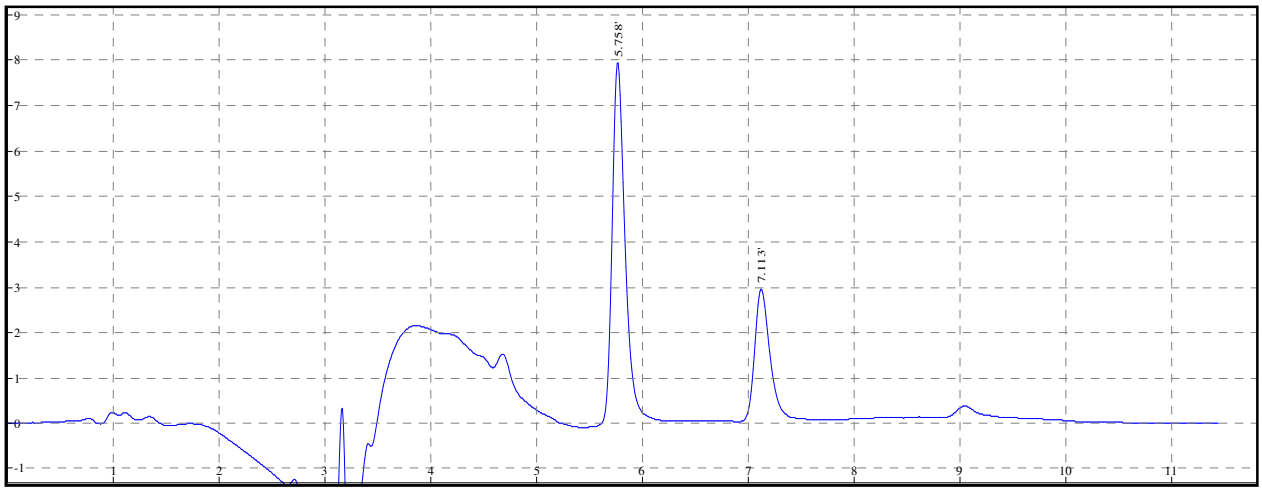
Run time: 11.74min

Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.818	57698	4.12	5463	1.33
7.238	12670	0.00	7443	1.32

Sample Name: Betamethasone Std C

Run time: 11.44min

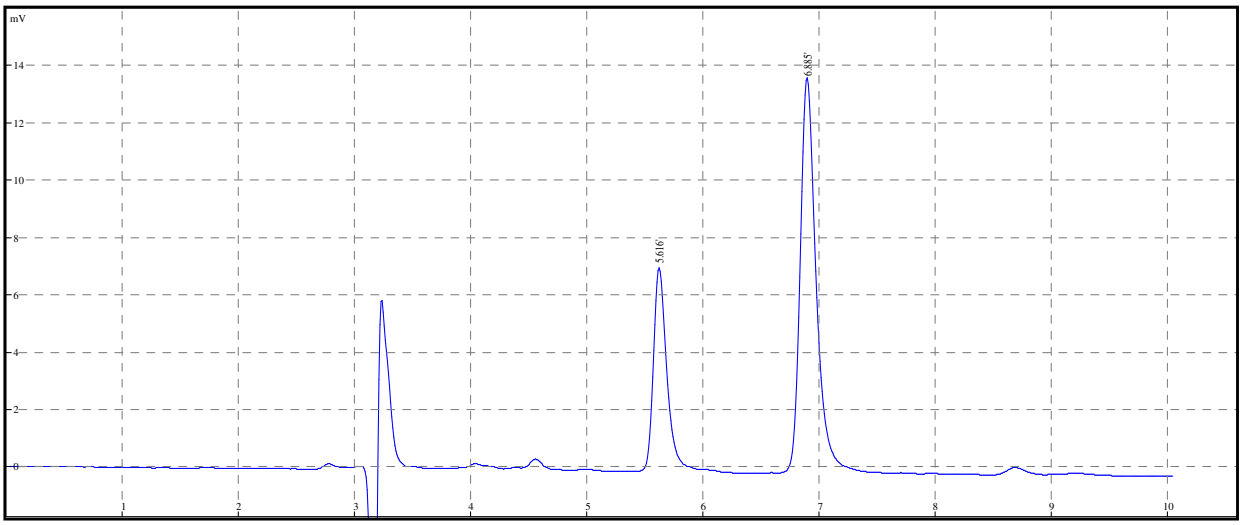


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.758	52910	2.02	3229	1.36
7.113	24459	0.00	3842	1.35

Sample Name: Betamethasone Std D

Run time: 10.05min

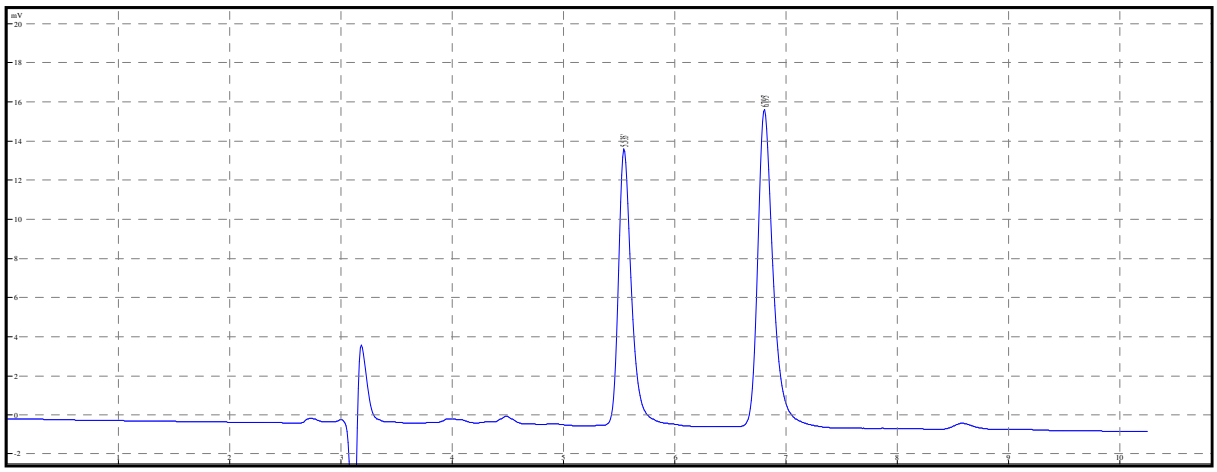


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.616	50116	3.67	3507	1.40
6.885	65371	0.00	3087	1.37

Sample Name: Betamethasone Std E

Run time: 10.24min

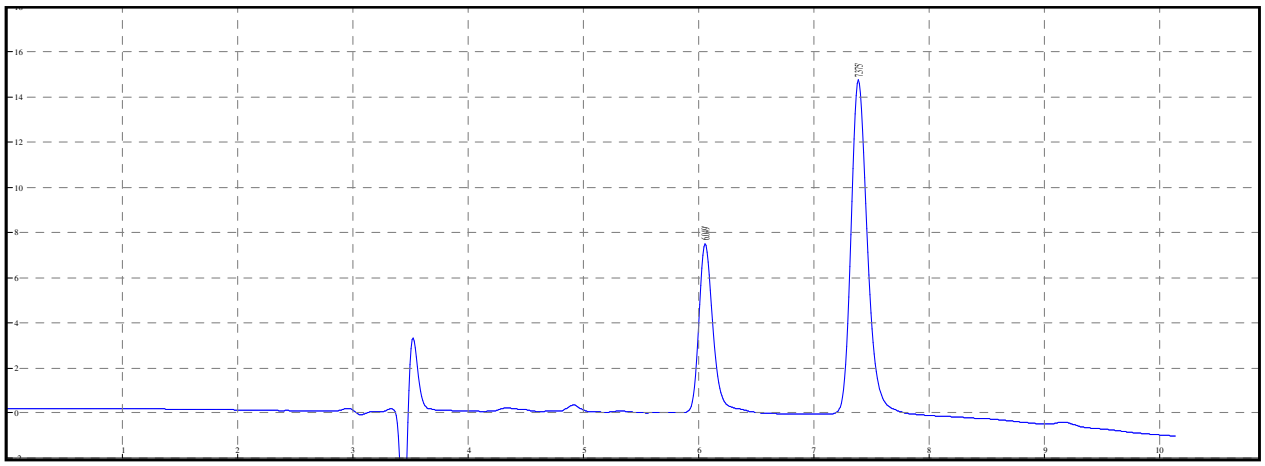


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.535	58350	5.26	10336	1.41
6.795	85899	0.00	11483	1.37

Sample Name: Betamethasone Std F

Run time: 10.14min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.049	54399	5.11	4468	1.32
7.375	129176	0.00	5148	1.27

Table no. 6.1: Observation table for linearity graph

Sample Name	Concentration ratio of sample and internal standard	Area ratio of sample and internal standard
Std-A	0.125	0.225
Std-B	0.125	0.225
Std-C	0.25	0.383
Std-D	0.75	0.983
Std-E	1.5	1.752
Std-F	2.0	2.425

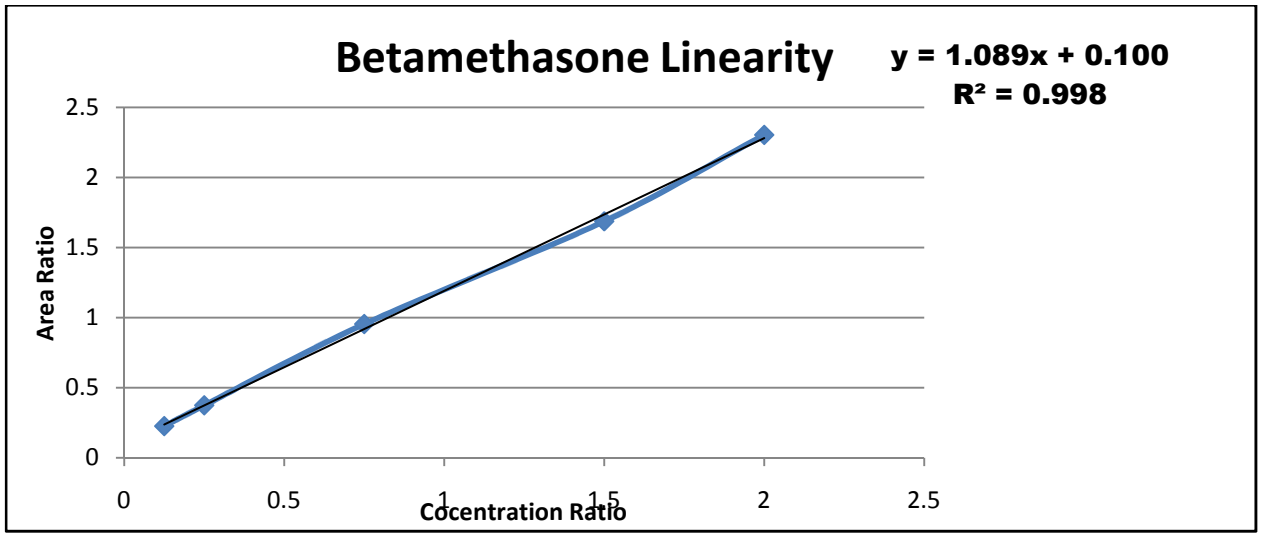


Fig No.6.1: Linearity graph

(Limit: The R^2 value should be near to 1)

FOR LLOQ

Chromatographic conditions

Sample Name: Betamethasone 5ng/ml + Paracetamol 80ng/ml

Wavelength: 242nm

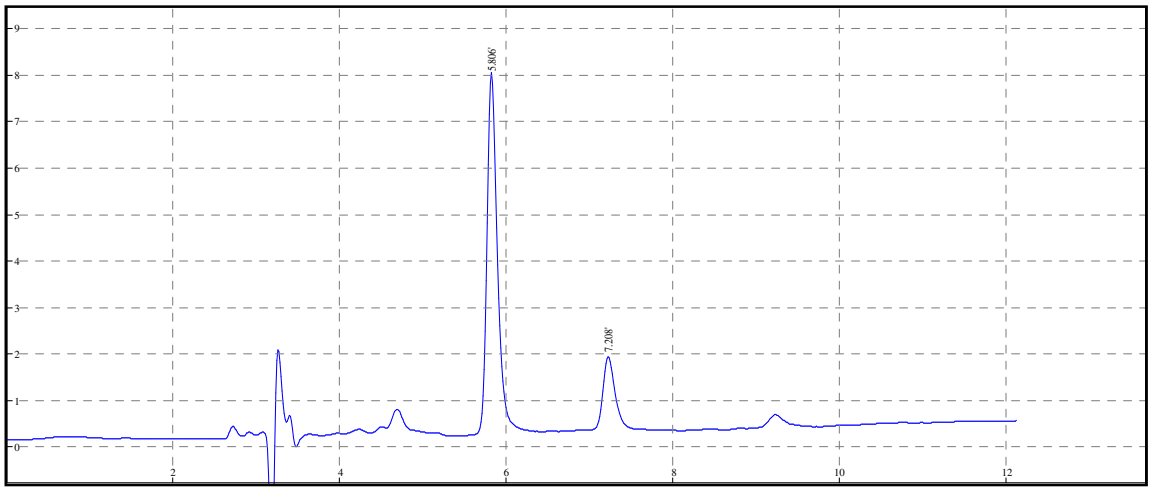
Mobile Phase: Methanol:Water (60:40)

Sample volume: 100µl

Flow rate: 0.9 ml/min

Pressure: 10-11MPa

Run time: 12.11min



Observation Table

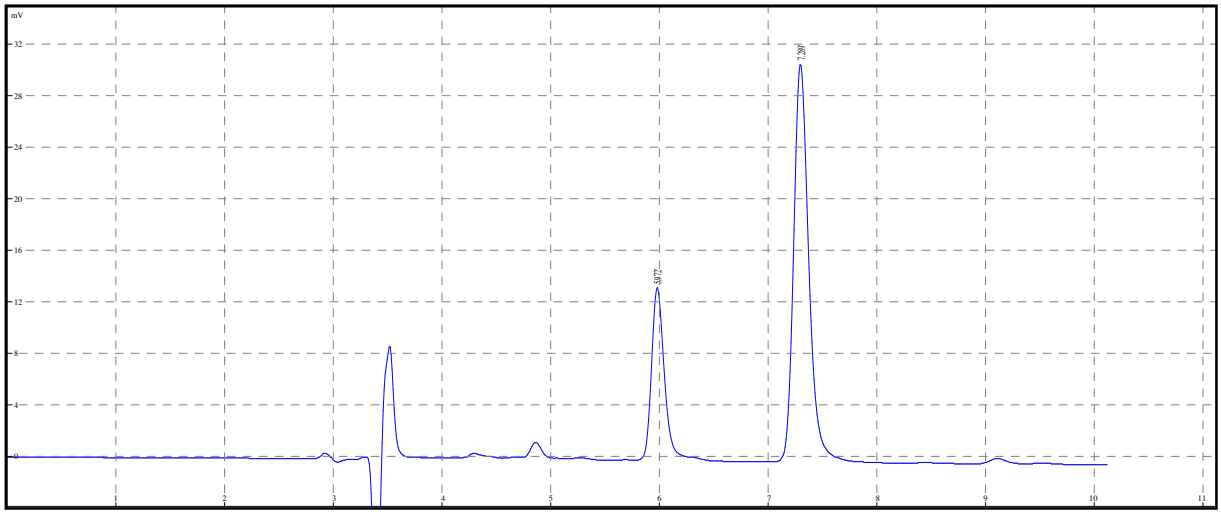
Time	Area	Resolution	T.Plate no.	Asymmetry
5.806	59299	5.06	12643	1.35
7.208	7321	0.00	12680	1.32

ACCURACY AND PRECISION

Accuracy and Precision was evaluated by analyzing 5 bathches. Each batch consist of five replicates of LQC, MQC and HQC.

Sample Name: Betamethasone HQC 01

Run time: 10.12min

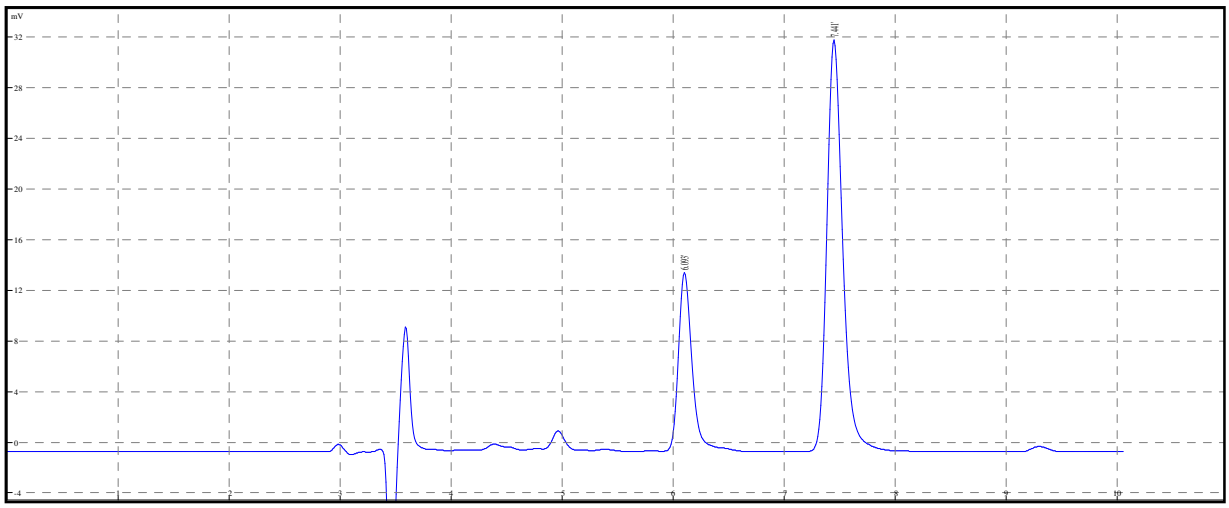


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.972	58181	2.40	4101	1.30
7.289	163368	0.00	7378	1.26

Sample Name: Betamethasone HQC 02

Run time: 10.05 min

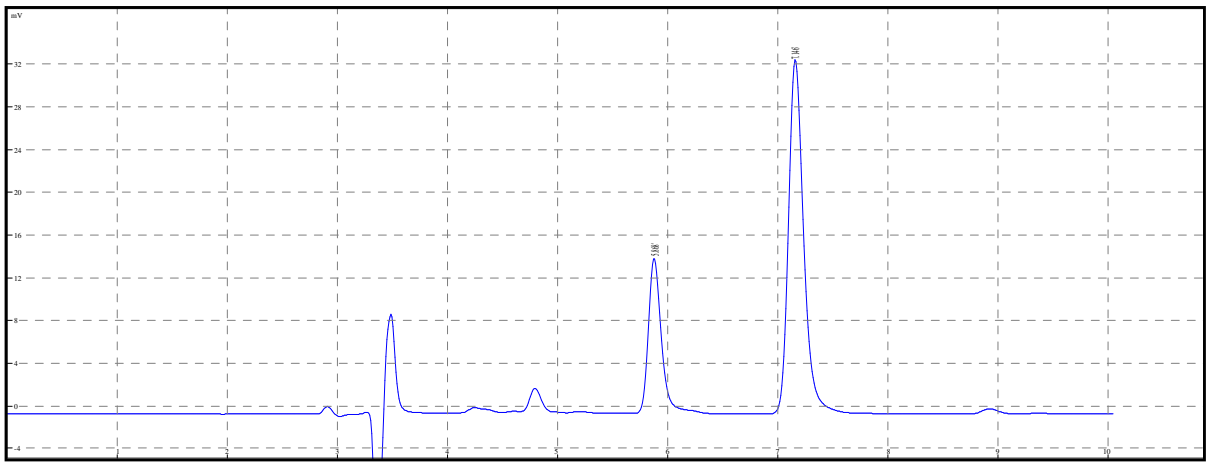


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.093	58302	3.04	5129	1.30
7.438	148297	0.00	4884	1.27

Sample Name: Betamethasone HQC 03

Run time: 10.05min

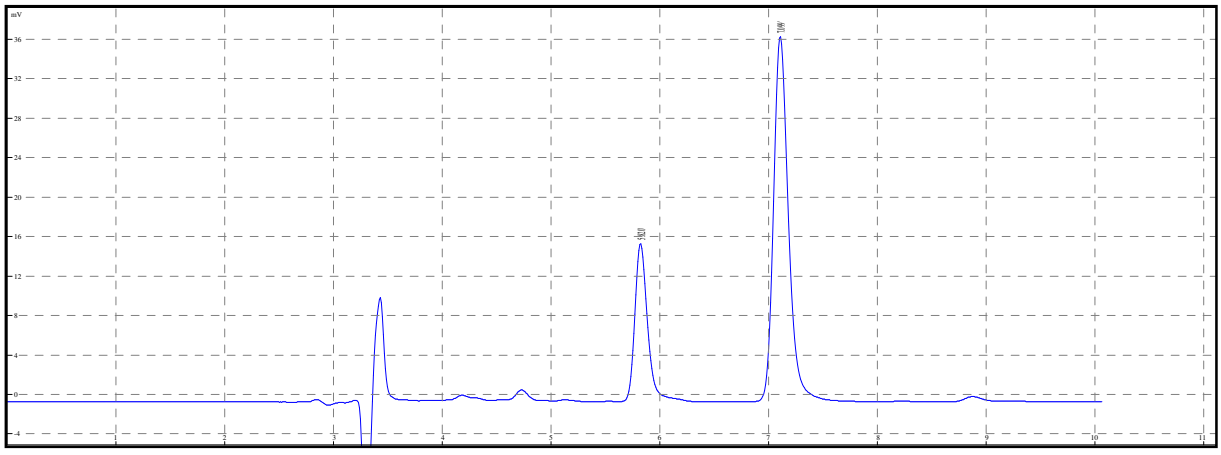


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.868	59297	3.65	4982	1.33
7.146	162071	0.00	6824	1.29

Sample Name: Betamethasone HQC 04

Run time: 10.06 min

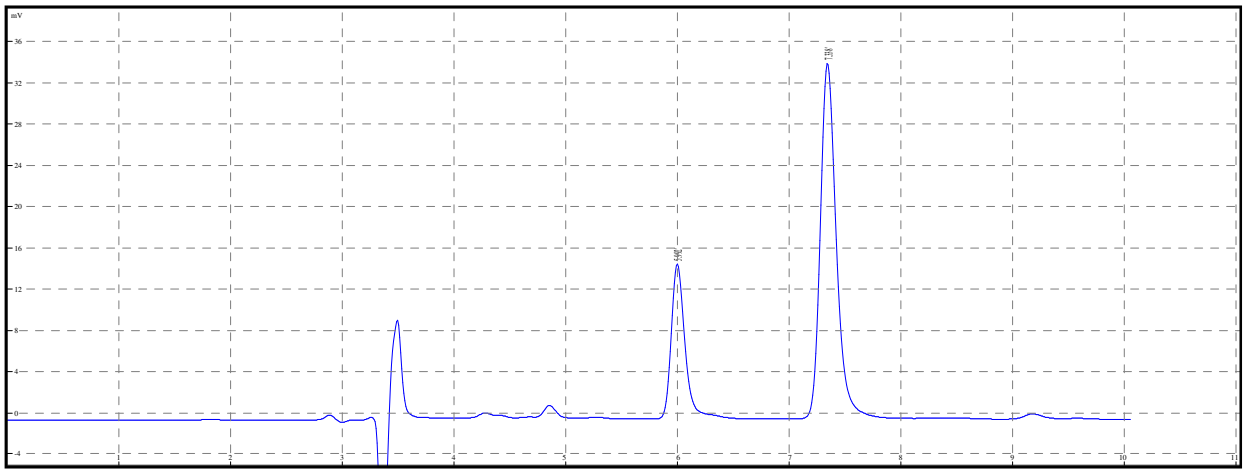


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.820	60388	3.50	4422	1.31
7.099	144867	0.00	7398	1.26

Sample Name: Betamethasone HQC 05

Run time: 10.05min

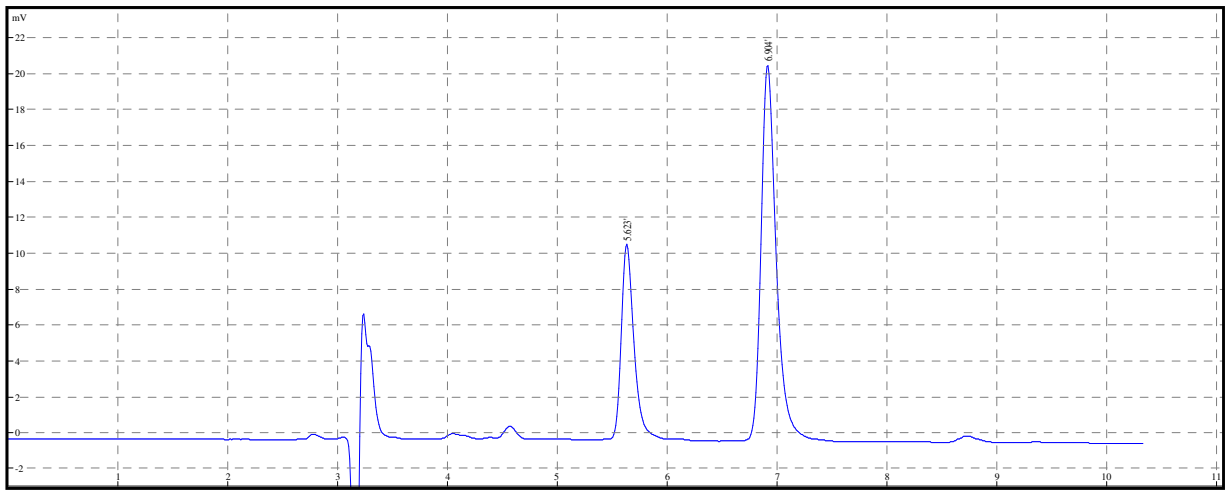


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.992	53038	3.40	7210	1.34
7.338	157923	0.00	6123	1.28

Sample Name: Betamethasone MQC 01

Run time: 10.32min

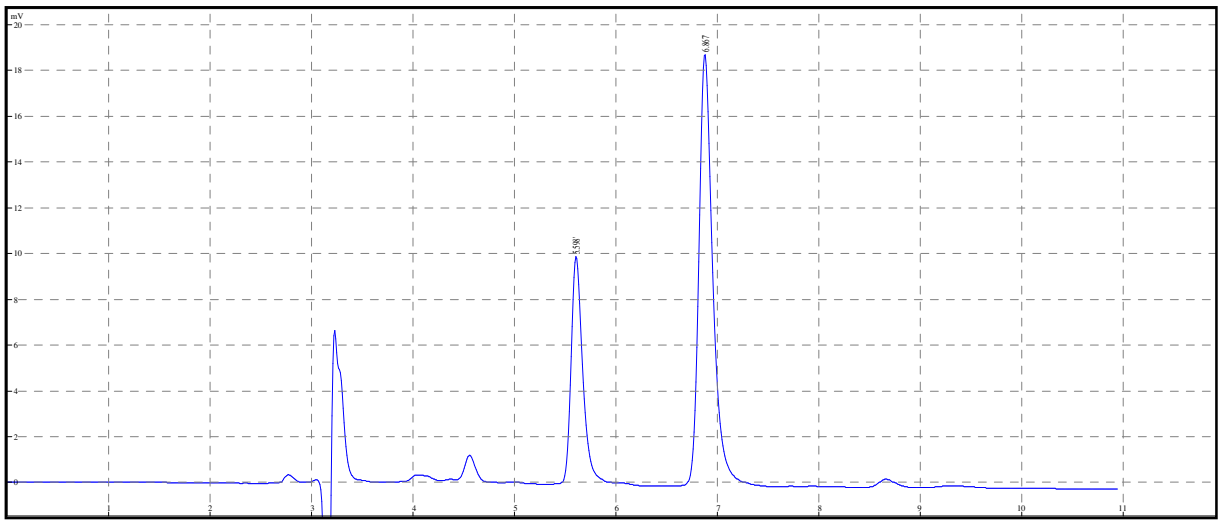


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.623	51057	2.60	5492	1.39
6.904	82197	0.00	7400	1.36

Sample Name: Betamethasone MQC 02

Run time: 10.94min

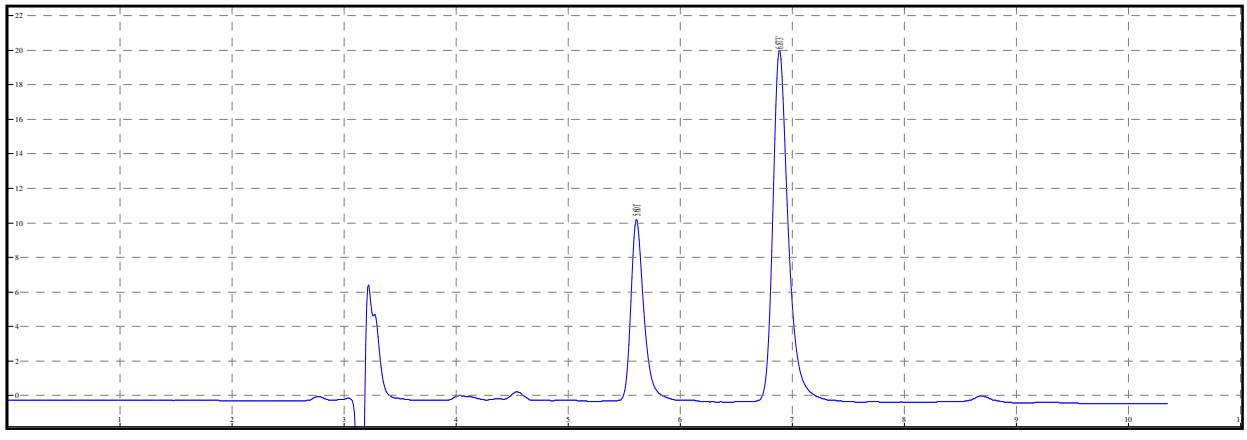


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.598	50713	2.57	6509	1.40
6.867	84128	0.00	8095	1.37

Sample Name: Betamethasone MQC 03

Run time: 10.34min

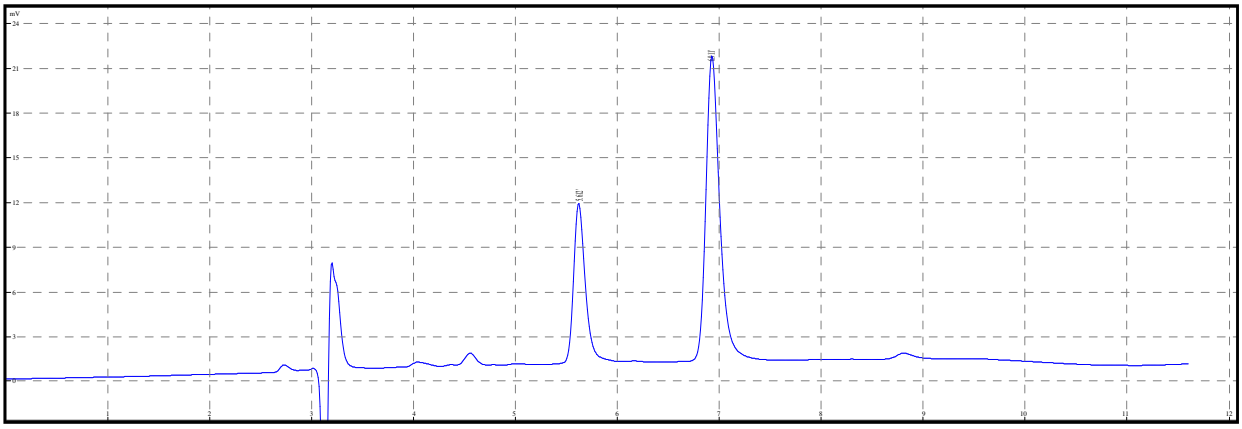


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.601	53708	3.40	5955	1.41
6.873	82287	0.00	6114	1.38

Sample Name: Betamethasone MQC 04

Run time: 11.60min

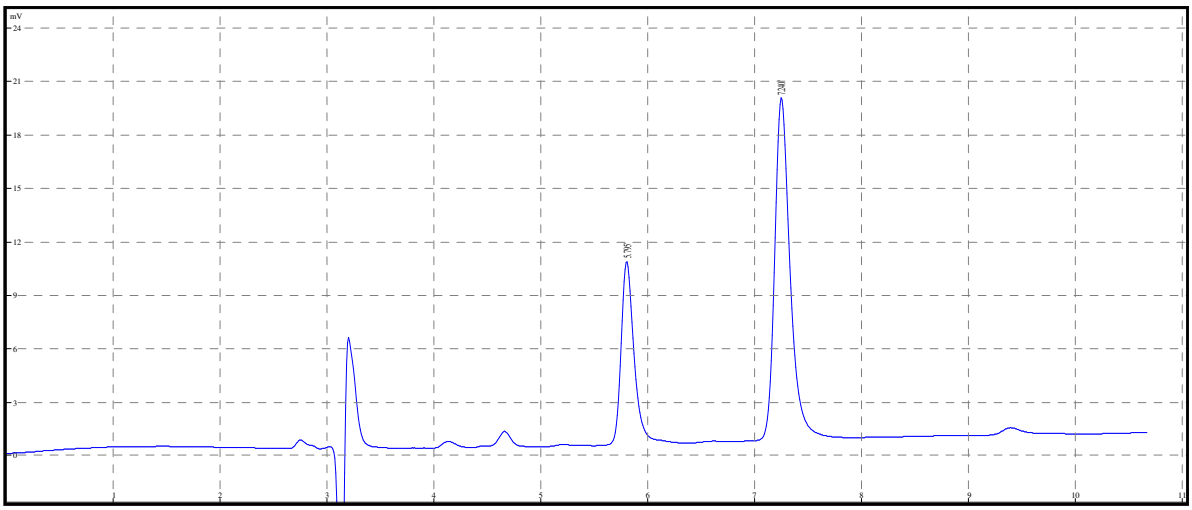


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.612	56502	4.30	9594	1.34
6.911	80273	0.00	5267	1.44

Sample Name: Betamethasone MQC 05

Run time: 10.66min

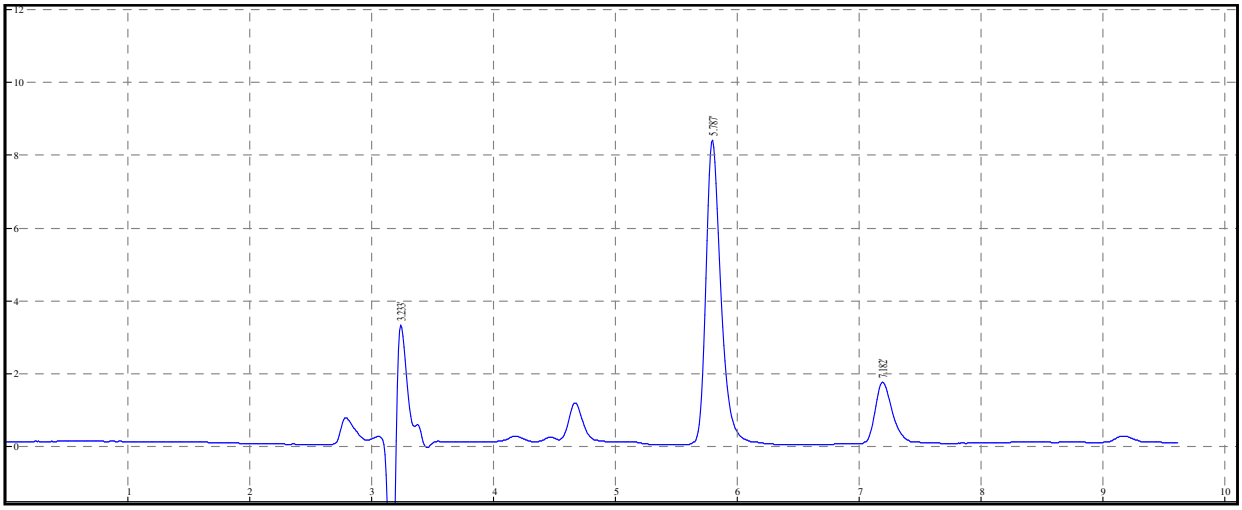


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.795	58389	2.96	7546	1.36
7.240	87071	0.00	6090	1.35

Sample Name: Betamethasone LQC 01

Run time: 9.61min

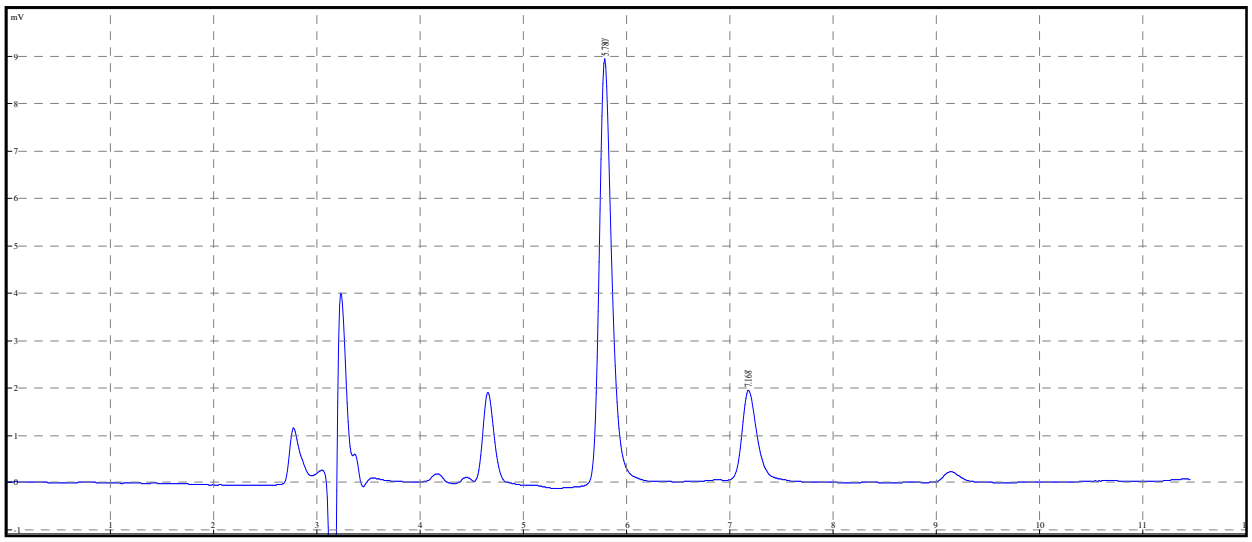


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.787	53775	2.87	6785	1.36
7.182	16708	0.00	8460	1.33

Sample Name: Betamethasone LQC 02

Run time: 11.45min

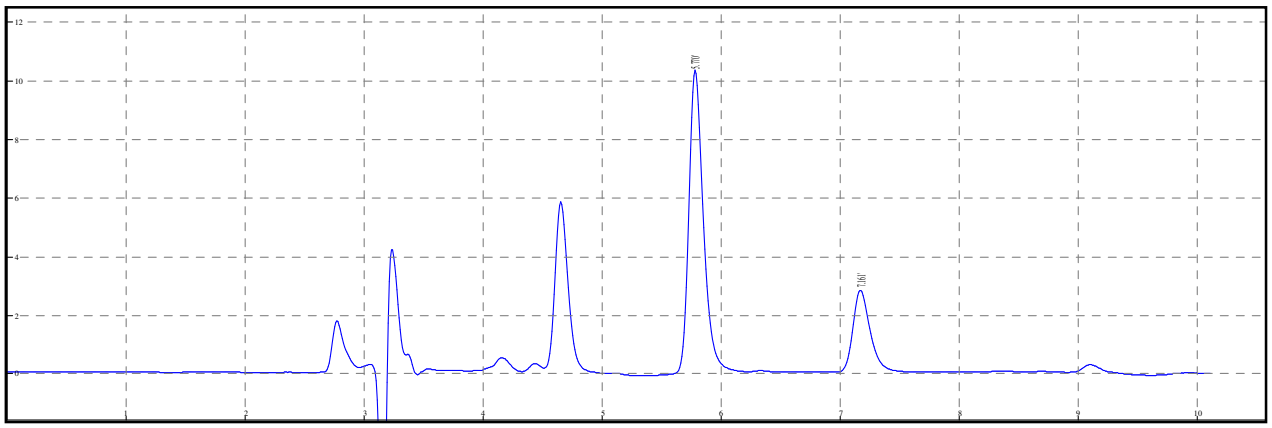


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.780	55937	2.98	6459	1.33
7.168	15903	0.00	9706	1.29

Sample Name: Betamethasone LQC 03

Run time: 10.11min

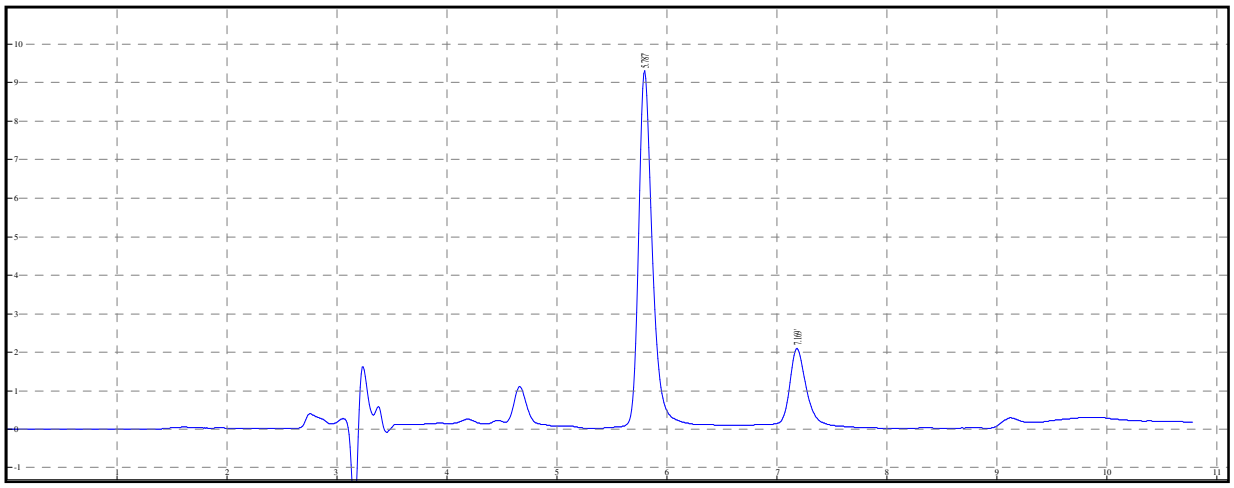


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.770	48969	4.12	8354	1.34
7.161	18158	0.00	5001	1.33

Sample Name: Betamethasone LQC 04

Run time: 10.78min

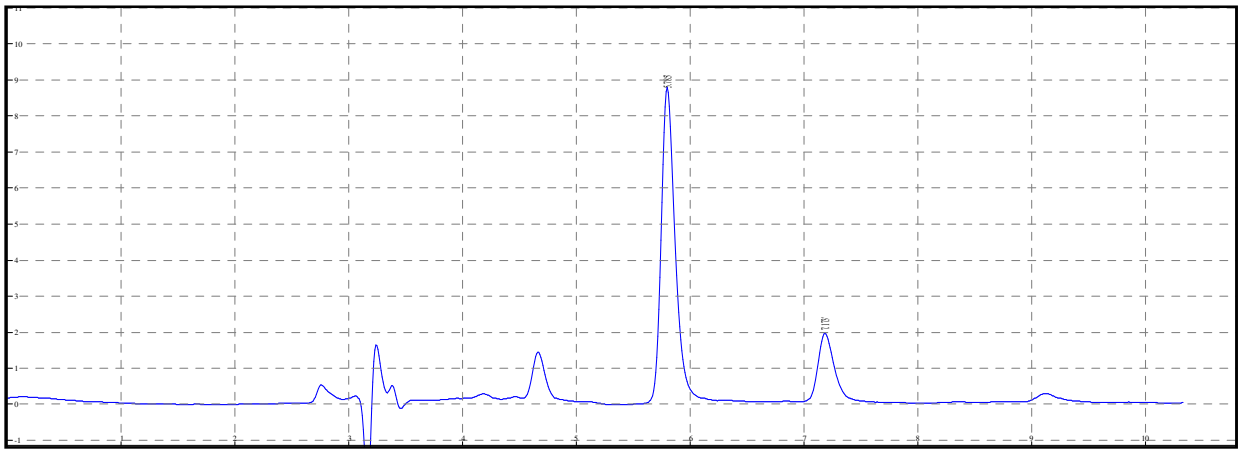


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.787	53350	3.60	7942	1.32
7.169	51329	0.00	6531	1.21

Sample Name: Betamethasone LQC 05

Run time: 10.32min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.785	51072	4.12	7569	1.34
7.173	16098	0.00	7739	1.33

The precision and accuracy of the method for each concentration levels are represented in following Table.

Table No.6.2 Observation table for Accuracy & Precision

			Standard Deviation		Accuracy	Precision
Conc.	**Conc. Ratio**	**Area Ratio**	**Mean**	**SD**	**%SD**	**%RSD**
LQC	0.1875	0.3107	0.322708	0.031953	9.9014	9.9014
	0.1875	0.2843				
	0.1875	0.3708				
	0.1857	0.3325				
	0.1857	0.3152				
MQC	1.5	1.6099	1.54257	0.094421	6.1214	6.1214
	1.5	1.6589				
	1.5	1.5321				
	1.5	1.4207				
	1.5	1.4912				
HQC	2.5	2.8079	2.69222	0.226147	8.4	8.4
	2.5	2.5436				
	2.5	2.7332				
	2.5	2.3989				
	2.5	2.9775				

(Standard Limit-%SD & % RSD should be less than 15%)

Table No.6.3 Observation table for Coefficient Variance (CV)

Sr.No	Concentration	Concentration ratio	Coefficient Varience
1	LQC	0.1875	0.178753
2	MQC	1.5	0.307289
3	HQC	2.5	0.475549

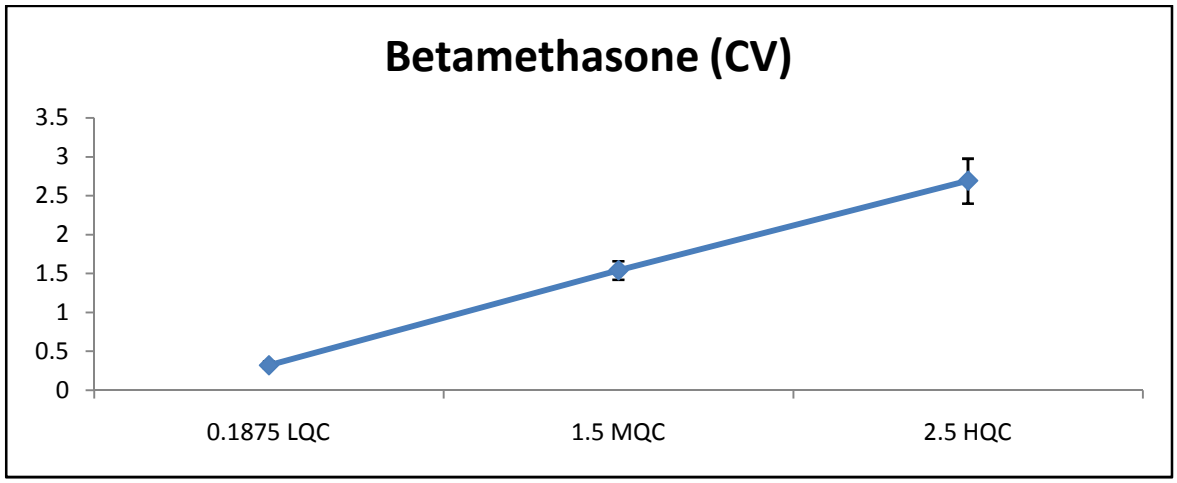


Fig No. 6.2: Coefficient Variance graph

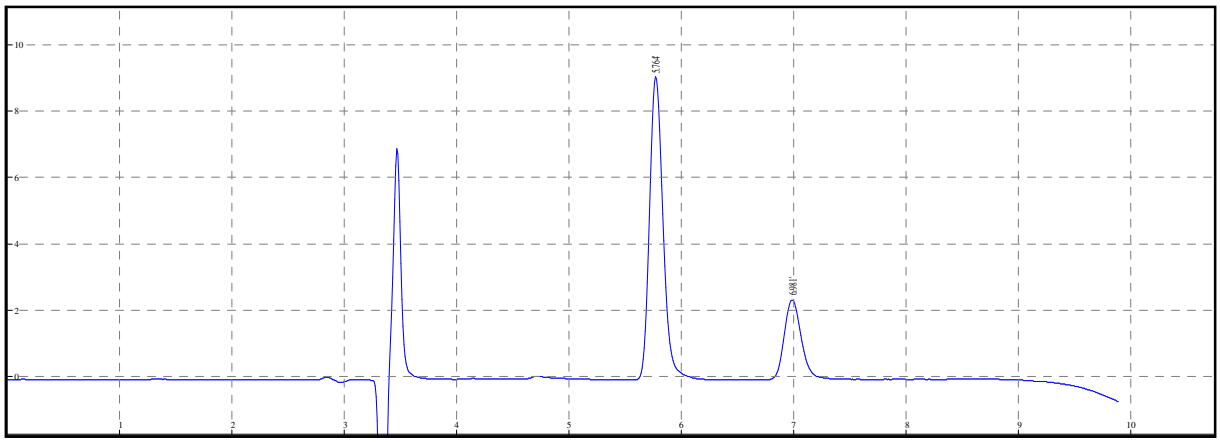
RECOVERY

The recovery was evaluated by comparing response of extracted and unextracted samples.The average recovery for Betamethasone in plasma was found to around 73.2%.

UNEXTRACTED SAMPLE

Sample Name: Betamethasone LQC

Run time: 9.88min



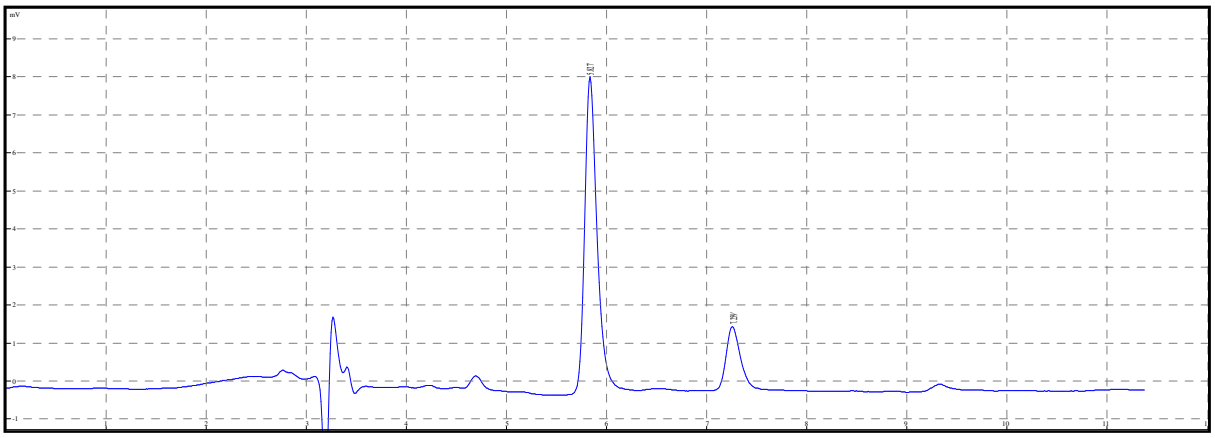
Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.764	70467	2.76	6490	1.22
6.981	24325	0.00	7223	1.21

Extracted sample

Sample Name: Betamethasone LQC

Run time: 11.37min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.827	58241	3.60	7205	1.35
7.259	15576	0.00	8698	1.33

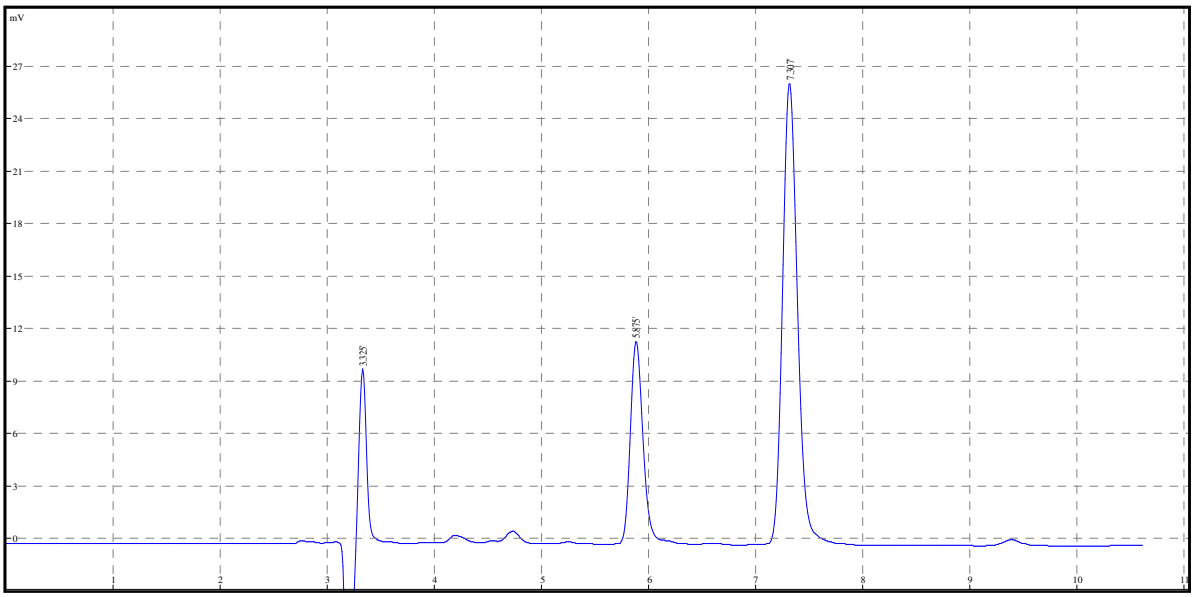
For stability study

Stability studies were performed to evaluate the stability of Betamethasone both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include bench top stability, freeze thaw stability, long term and short term stock stability. Betamethasone was found to be stable for three freeze and thaw cycles.

1. **Bench top Stability:**
    A. **Short Term Analysis: 2, 12 and 24hrs.**
    B. **Long Tem Analysis: 10, 20 and 30days**

Sample Name: Betamethasone HQC 2hrs

Run time: 10.62min

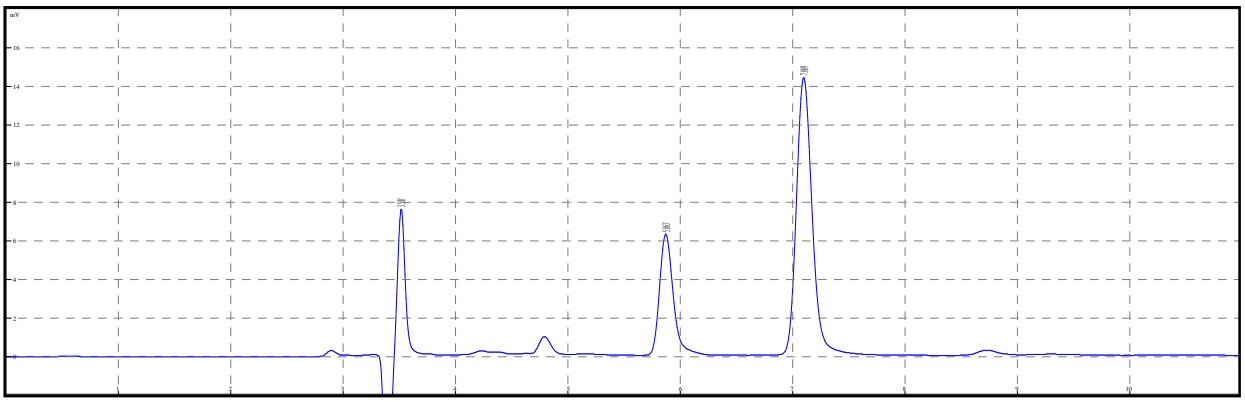


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.875	52260	3.26	7020	1.21
7.307	160343	0.00	5278	1.17

Sample Name: Betamethasone HQC 12hrs

Run time: 13.36min

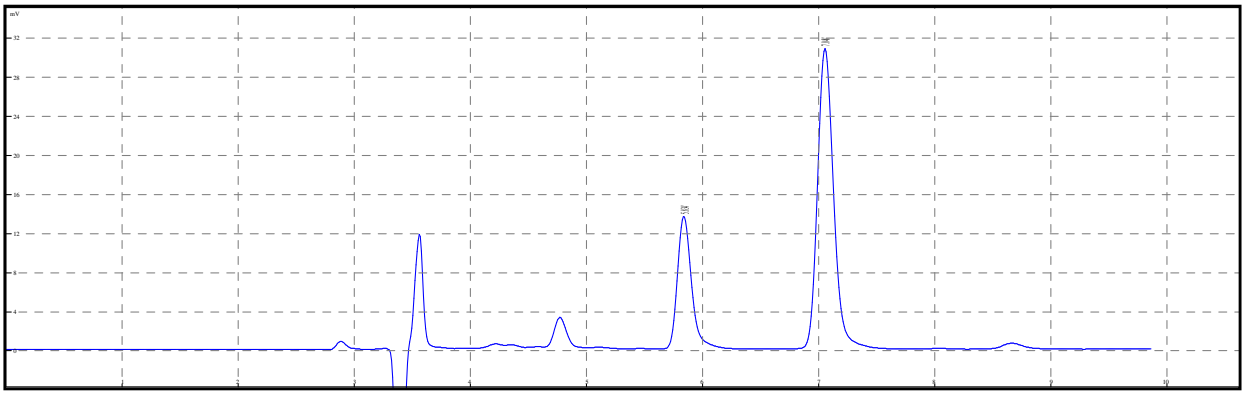


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.863	50613	3.39	6790	1.34
7.089	146555	0.00	7999	1.19

Sample Name: Betamethasone HQC 24hrs

Run time: 9.86min

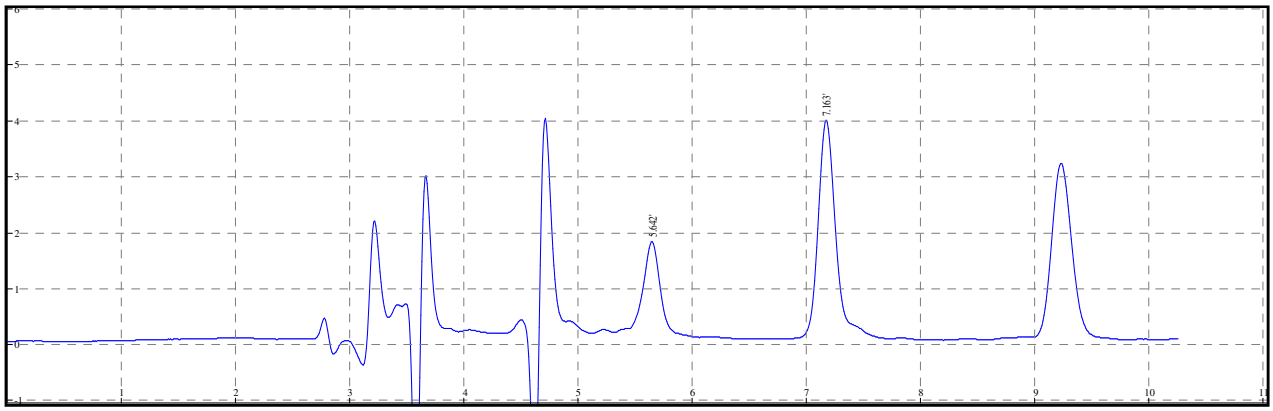


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.834	42589	3.42	5465	1.33
7.048	148994	0.00	8866	1.19

Sample Name: Betamethasone HQC 10days

Run time: 10.26min

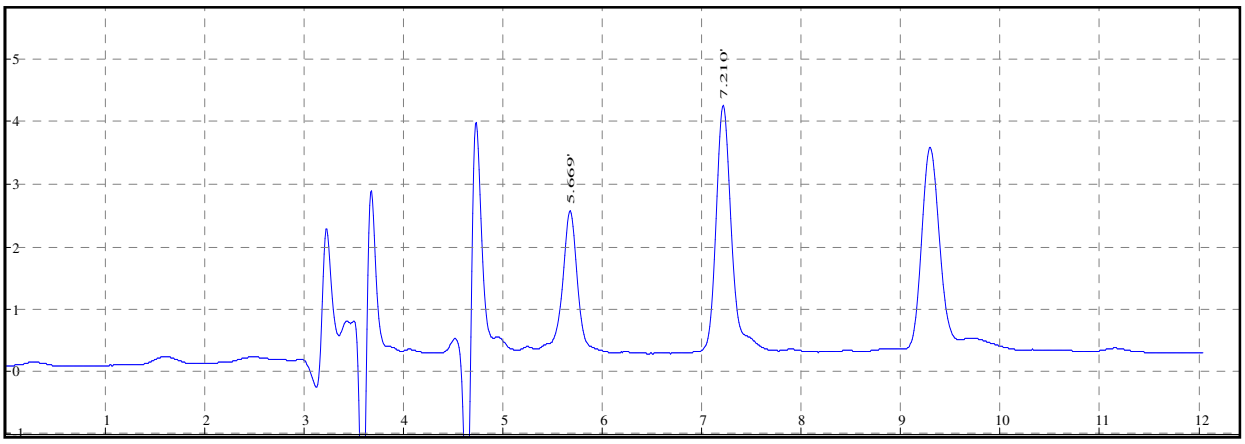


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.642	26804	3.50	7791	1.02
7.163	53669	0.00	4033	1.42

Sample Name: Betamethasone HQC 20 days

Run time: 12.04min

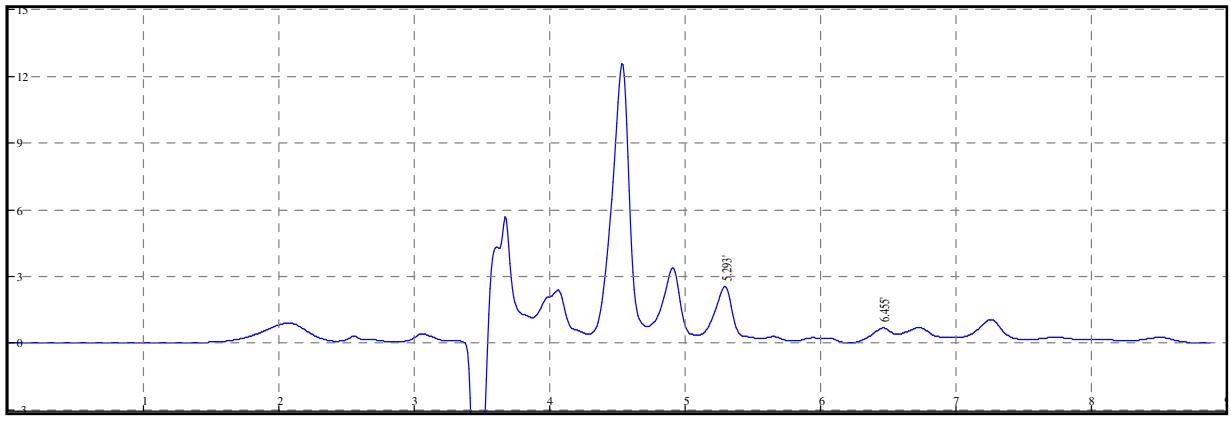


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.669	20940	2.00	4832	1.14
7.210	45603	0.00	6551	1.40

Sample Name: Betamethasone HQC 30days

Run time: 8.88min

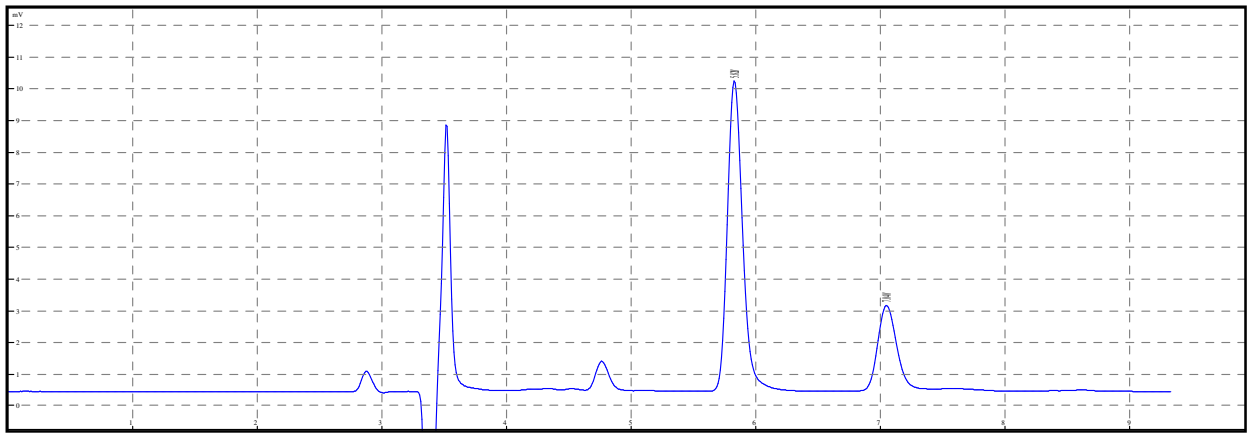


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.293	7988	1.68	3020	0.92
6.455	1568	0.00	2011	1.05

Sample Name: Betamethasone LQC 2hrs

Run time: 9.33min

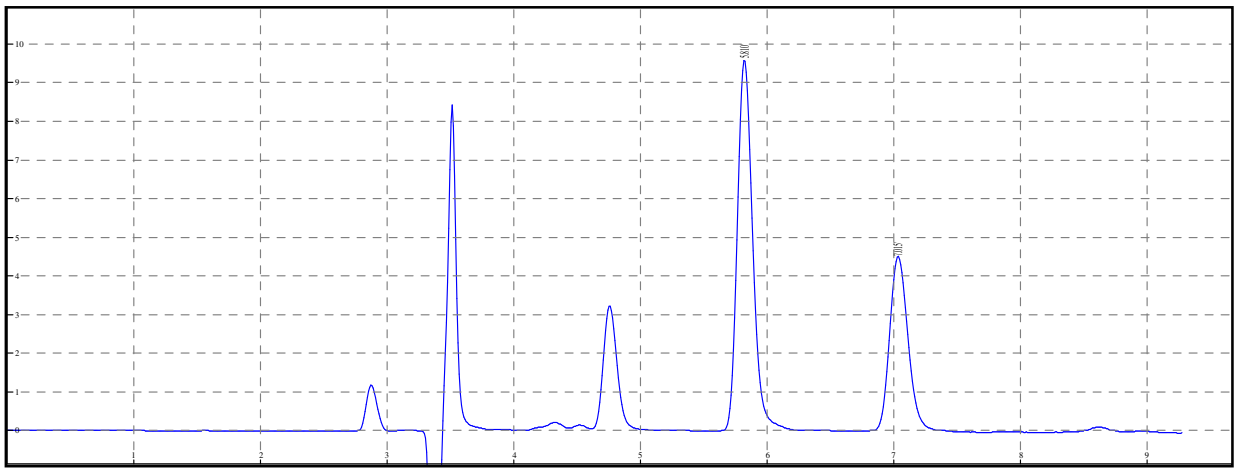


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.820	52185	3.54	5969	1.22
7.040	16299	0.00	6194	1.19

Sample Name: Betamethasone LQC 12hrs

Run time: 9.27min

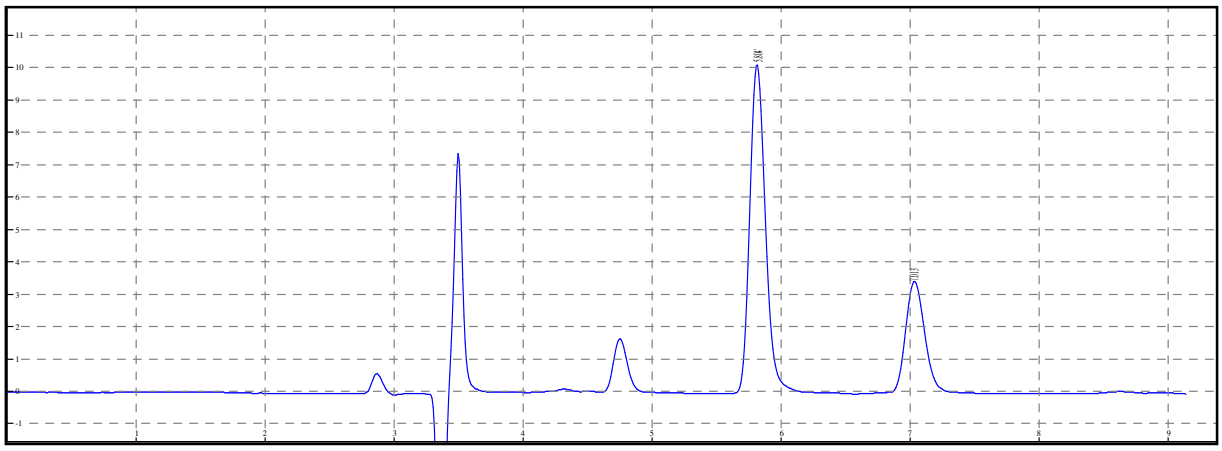


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.810	51139	2.74	7610	1.21
7.015	14362	0.00	8333	1.18

Sample Name: Betamethasone LQC 24hrs

Run time: 9.14min

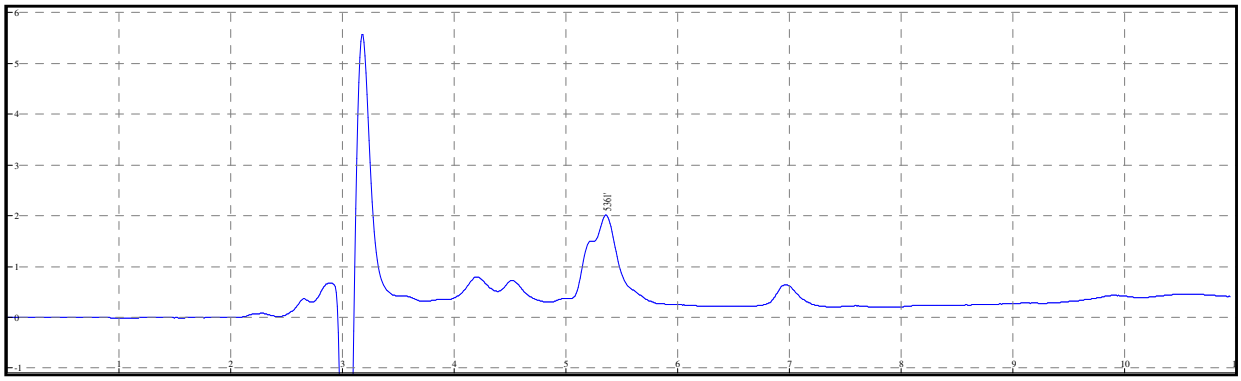


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.804	49264	3.12	4279	1.21
7.013	9487	0.00	4590	1.19

Sample Name: Betamethasone LQC 10days

Run time: 10.94min

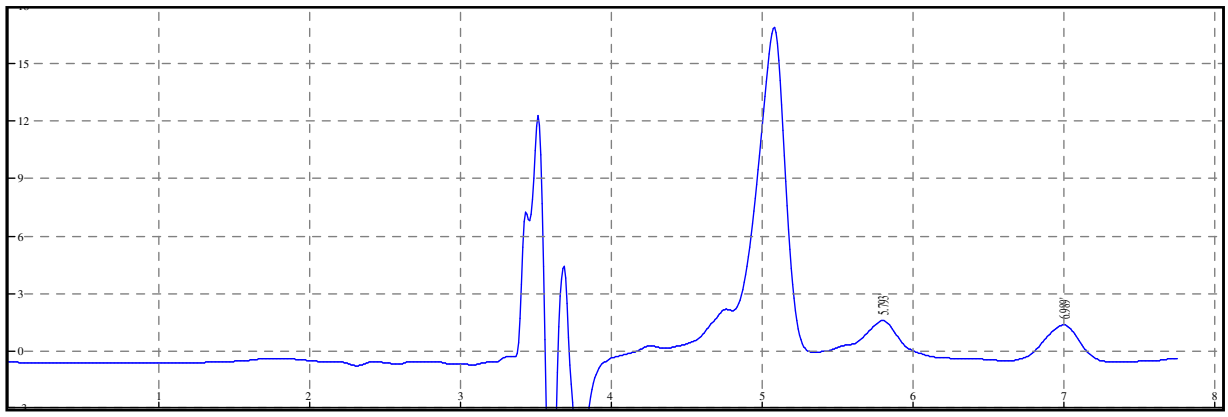


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.361	8235	1.40	3847	1.04
6.983	2981	0.00	2654	1.15

Sample Name: Betamethasone LQC 20days

Run time: 7.75min

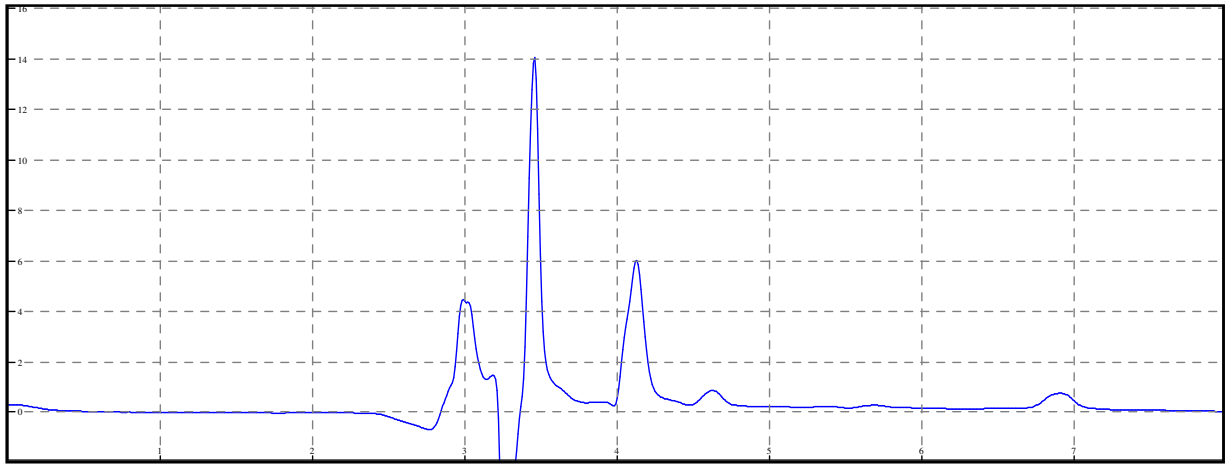


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.793	1505	1.49	1387	0.97
6.989	1521	0.00	1590	0.98

Sample Name: Betamethasone LQC 30days

Run time: 9.16min



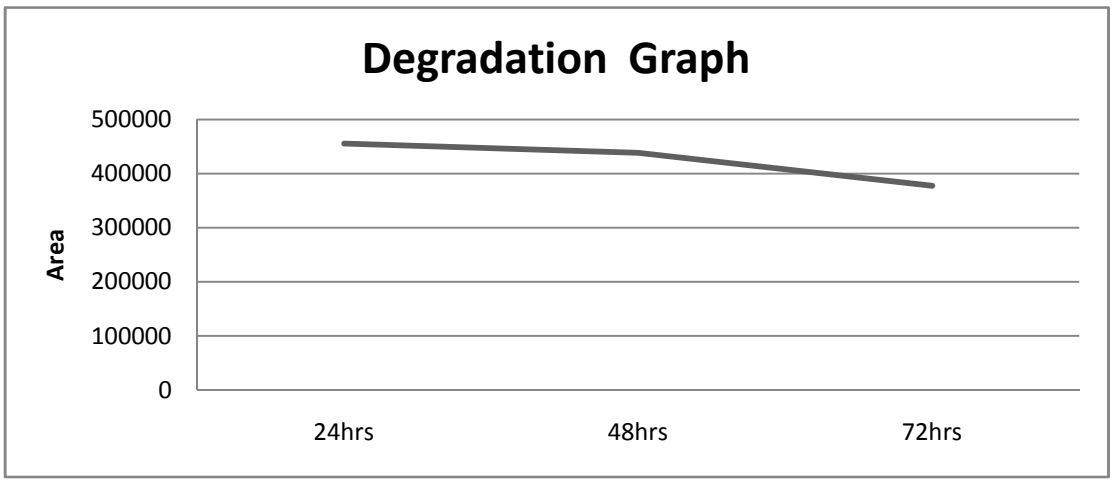
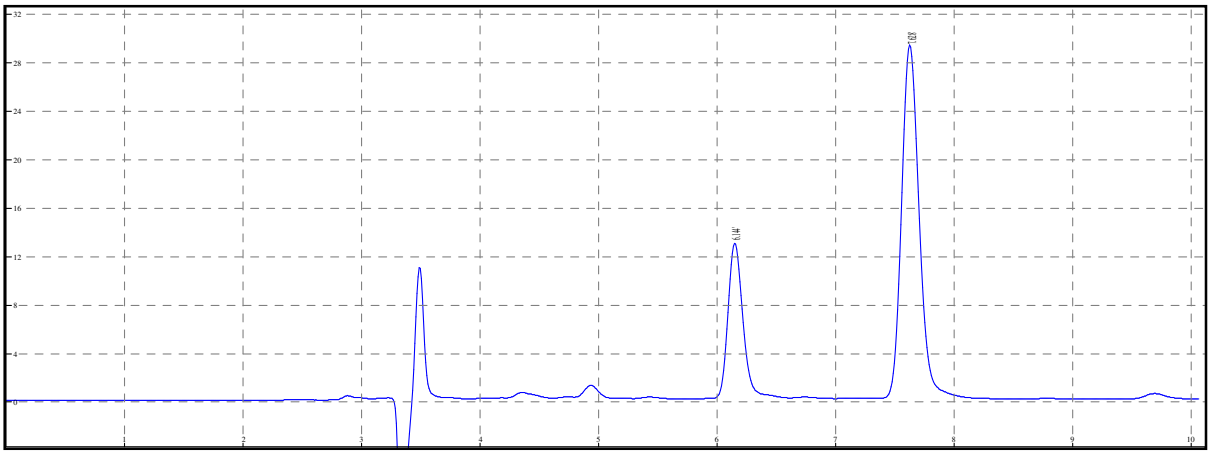


Fig No 6.3 Degradation graph for Bench top stabilty

2. **Freeze thaw Cycles Stability: Each Cycle at 12, 24 and 48hrs.**

   Sample Name: Betamethasone HQC 12hrs

   Run time: 10.06min

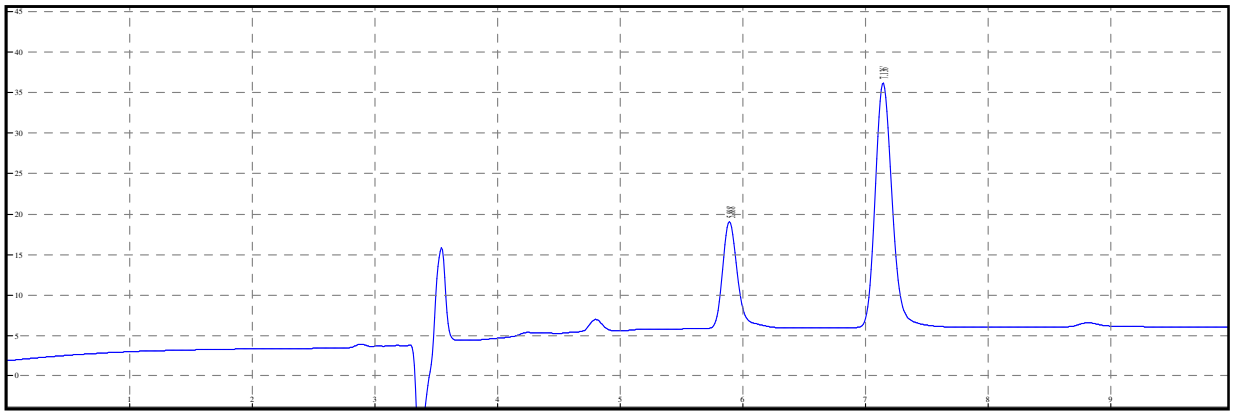


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.144	54736	2.89	6806	1.22
7.628	142033	0.00	5973	1.17

Sample Name: Betamethasone HQC 24hrs

Run time: 10.09min

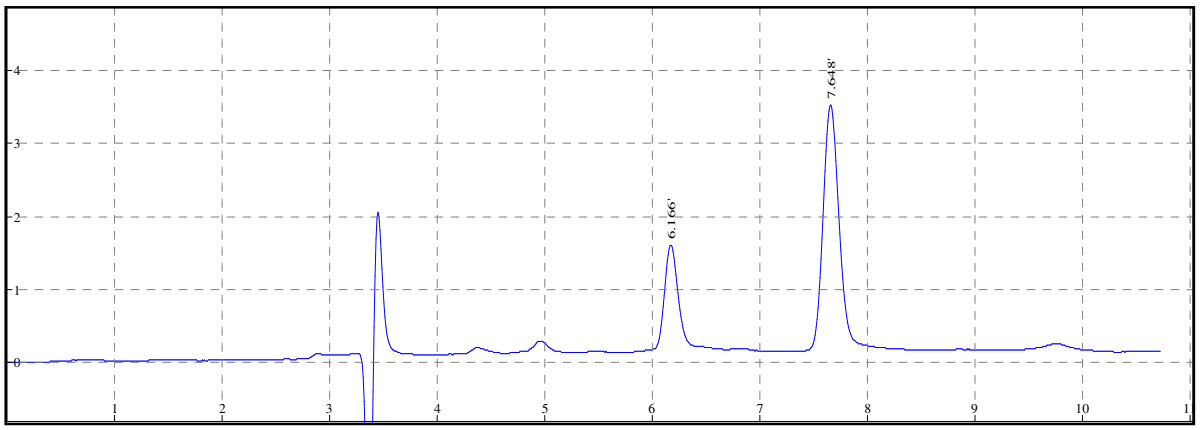


Observation Table

Time	**Area**	**Resolution**	**T.Plate no.**	**Asymmetry**
5.888	42534	2.60	7167	1.33
7.136	135780	0.00	5270	1.19

Sample Name: Betamethasone HQC 48hrs

Run time: 10.72min

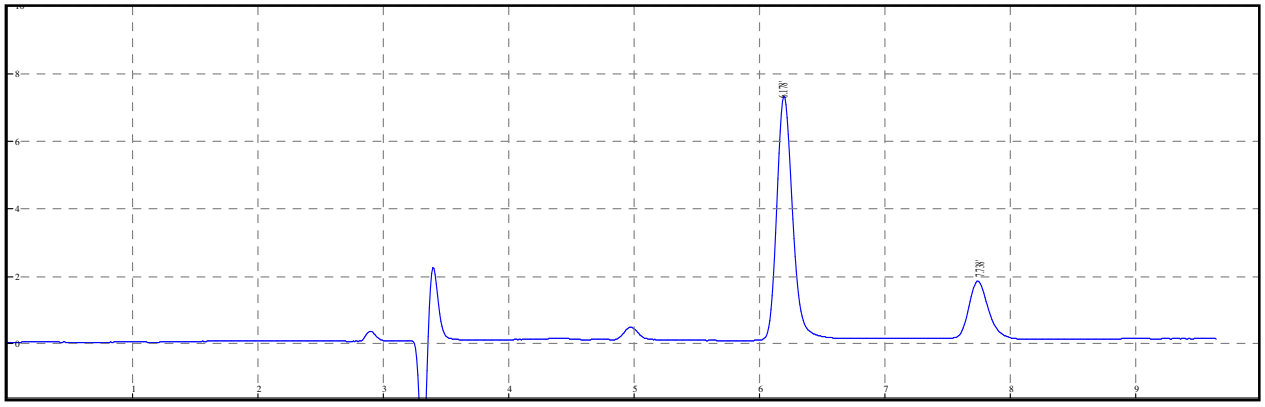


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.166	27886	3.06	6605	1.24
7.648	63152	0.00	5483	1.18

Sample Name: Betamethasone LQC 12hrs

Run time: 9.64min

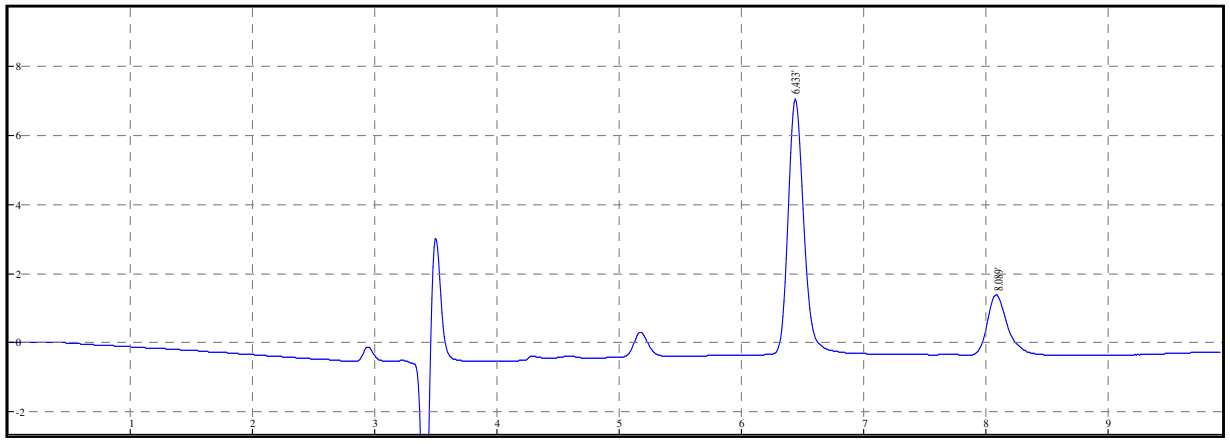


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.178	50286	4.32	6005	1.21
7.738	15745	0.00	6312	1.19

Sample Name: Betamethasone LQC 24hrs

Run time: 9.91min

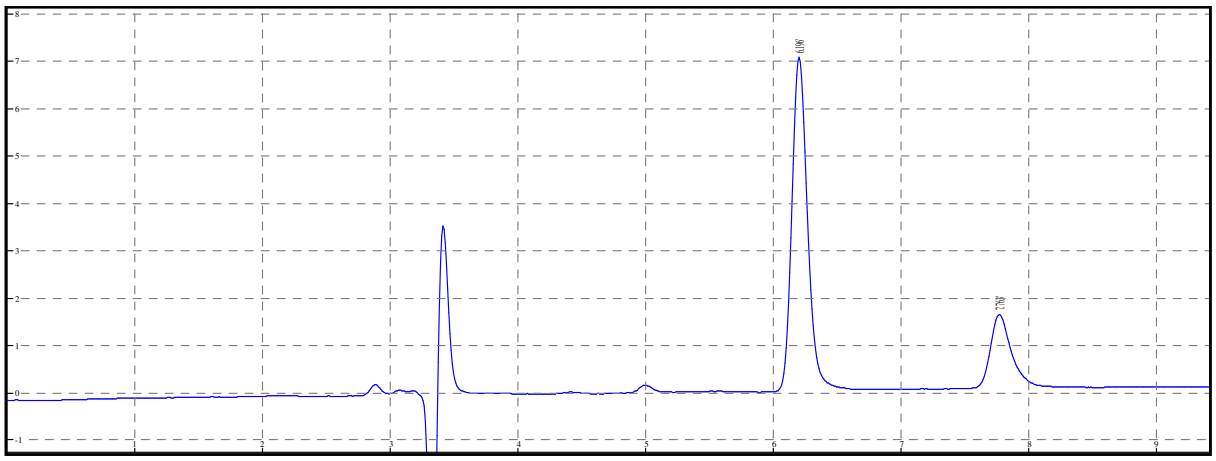


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.433	53134	3.04	5618	1.22
8.089	12958	0.00	5116	1.34

Sample Name: Betamethasone LQC 48hrs

Run time: 9.41min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.196	36084	3.16	8532	1.21
7.763	7002	0.00	7835	1.40

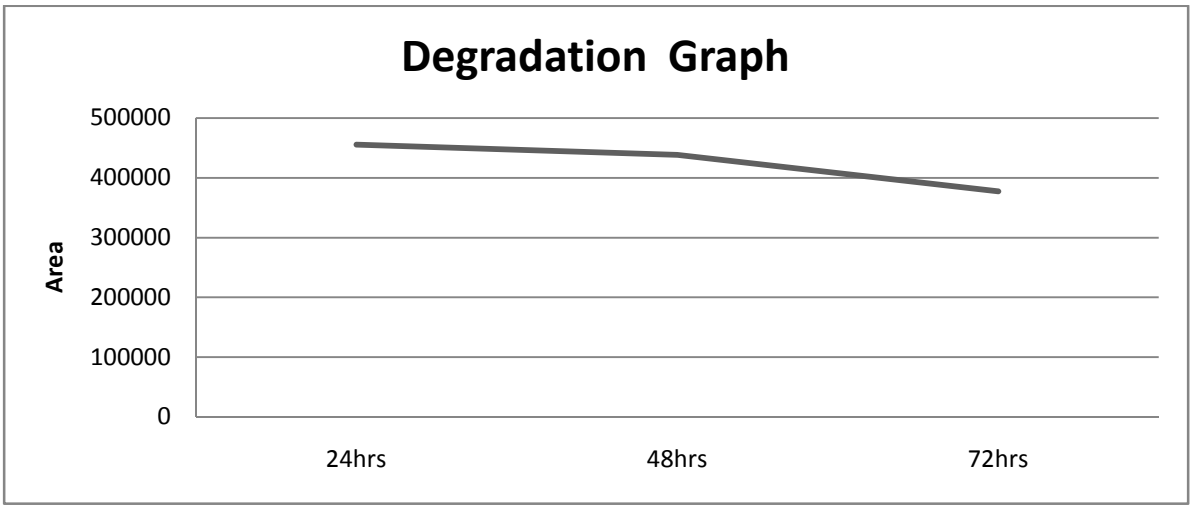
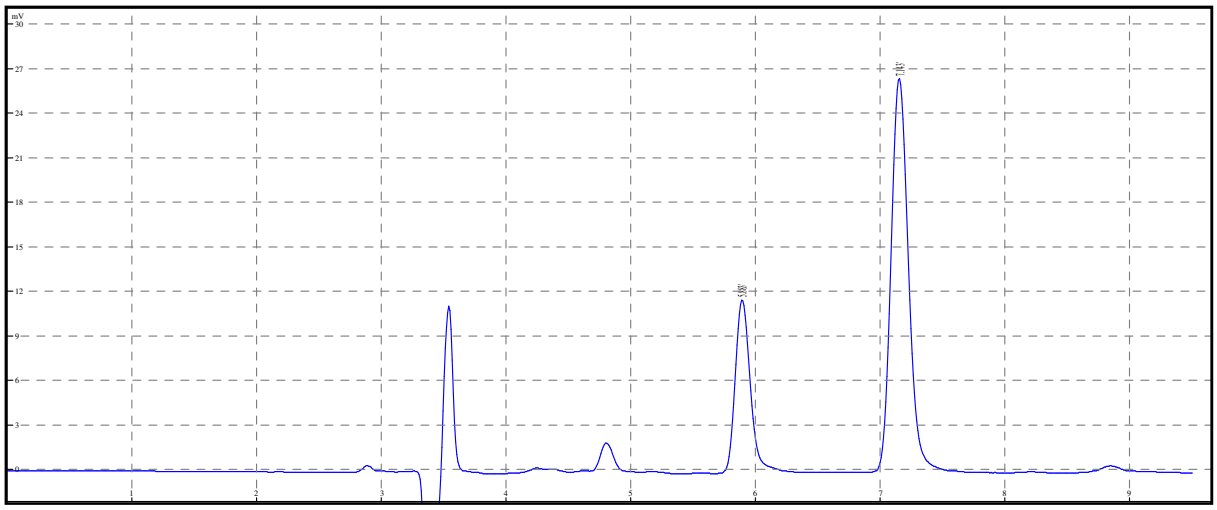


Fig No.6.4: Degradation graph for Freeze thaw stabilty

3. Plane Solution Stability: 24, 48 and 72hrs.

Sample Name: Betamethasone HQC 24 hrs

Run time: 9.50min

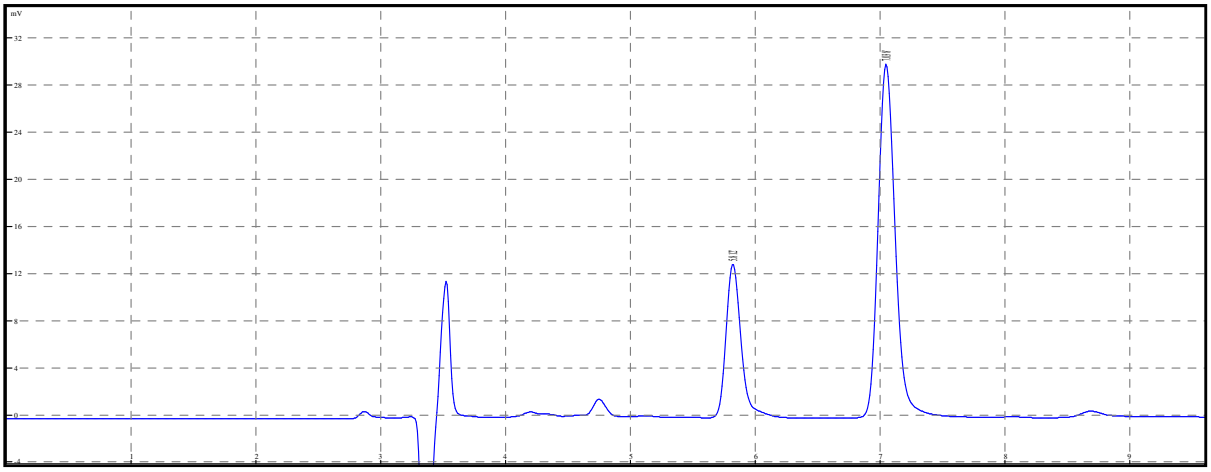


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.883	68704	2.69	5480	1.28
7.143	225871	0.00	5779	1.18

Sample Name: Betamethasone HQC 48 hrs

Run time: 9.60min

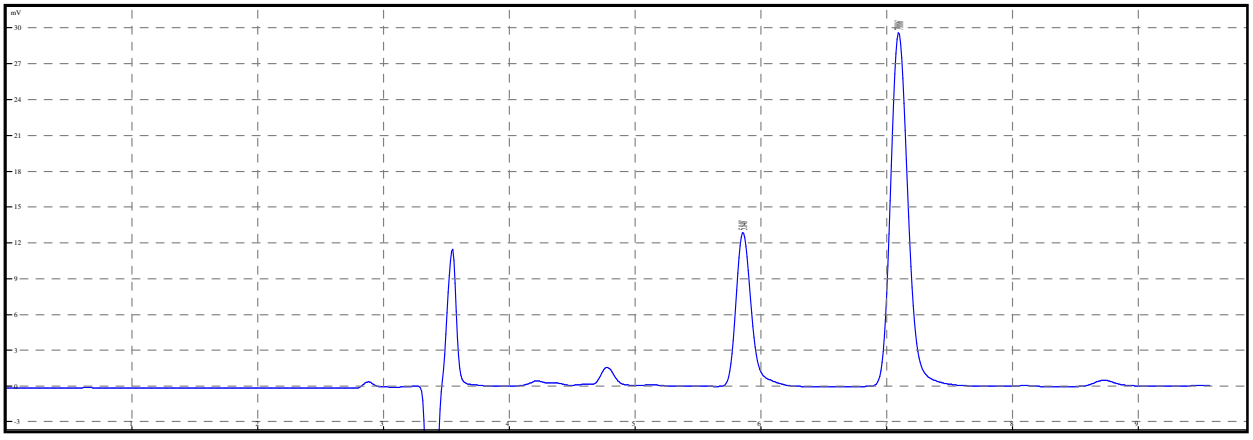


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.812	65333	2.25	5484	1.34
7.038	216633	0.00	7595	1.19

Sample Name: Betamethasone HQC 72 hrs

Run time: 9.57min

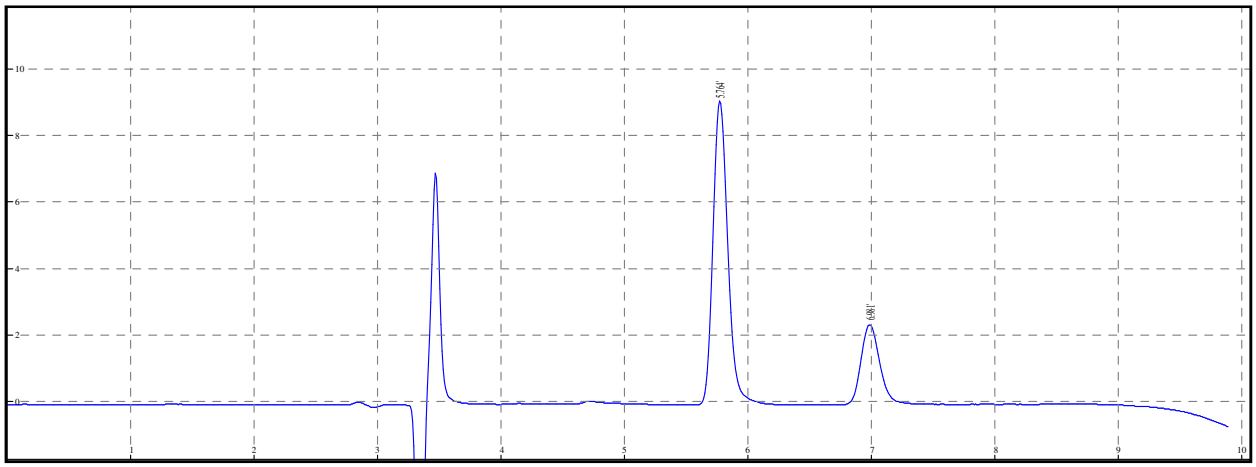


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.848	641017	2.86	6036	1.33
7.086	215088	0.00	8144	1.19

Sample Name: Betamethasone LQC 24hrs

Run time: 9.88min

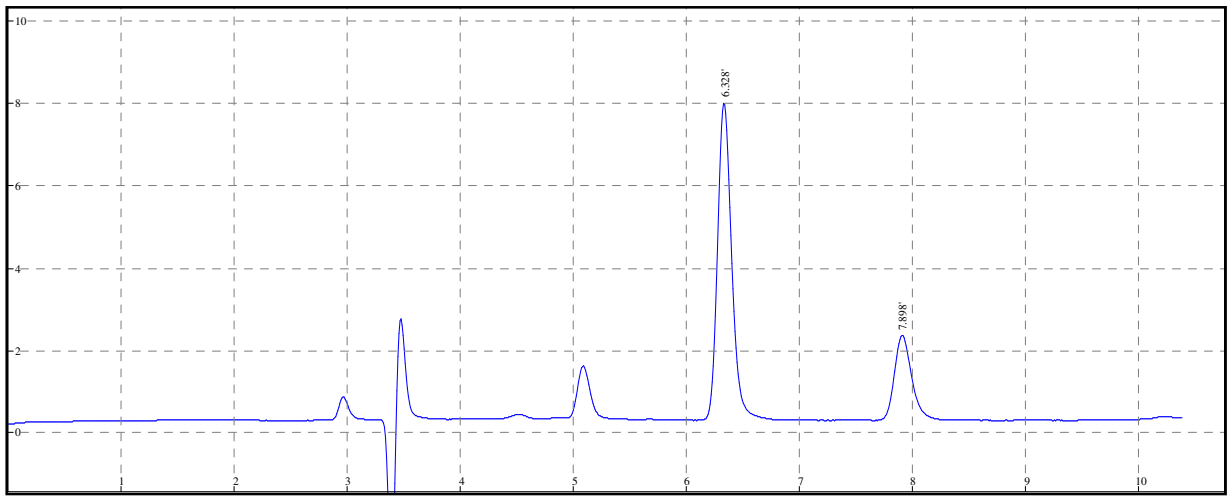


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.764	70467	2.76	6490	1.22
6.981	24325	0.00	7223	1.21

Sample Name: Betamethasone LQC 48hrs

Run time: 10.38min

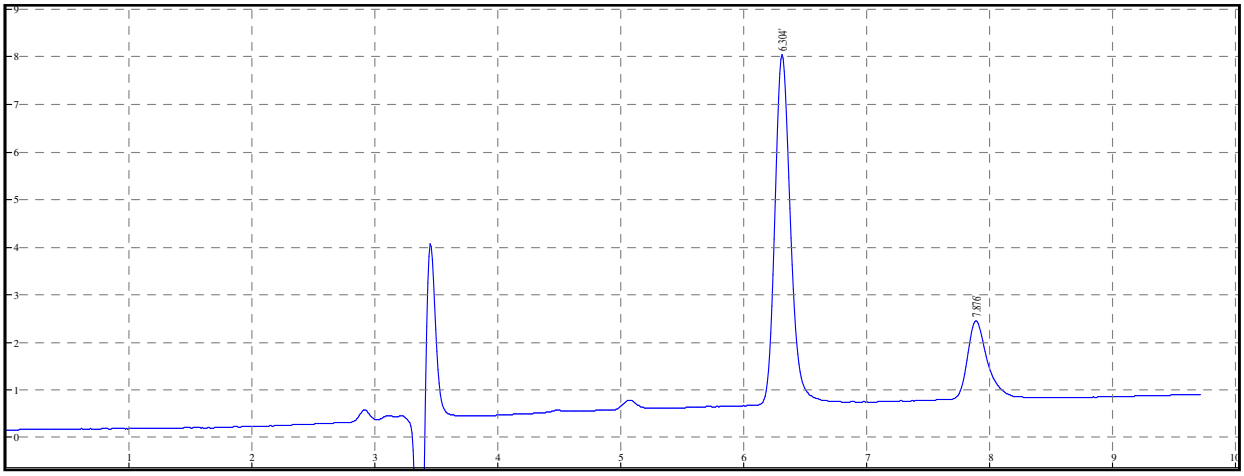


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.328	62638	3.08	5902	1.21
7.898	21167	0.00	9576	1.26

Sample Name: Betamethasone LQC 72hrs

Run time: 9.72min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
6.304	58222	2.32	6561	1.21
7.876	19434	0.00	5950	1.42

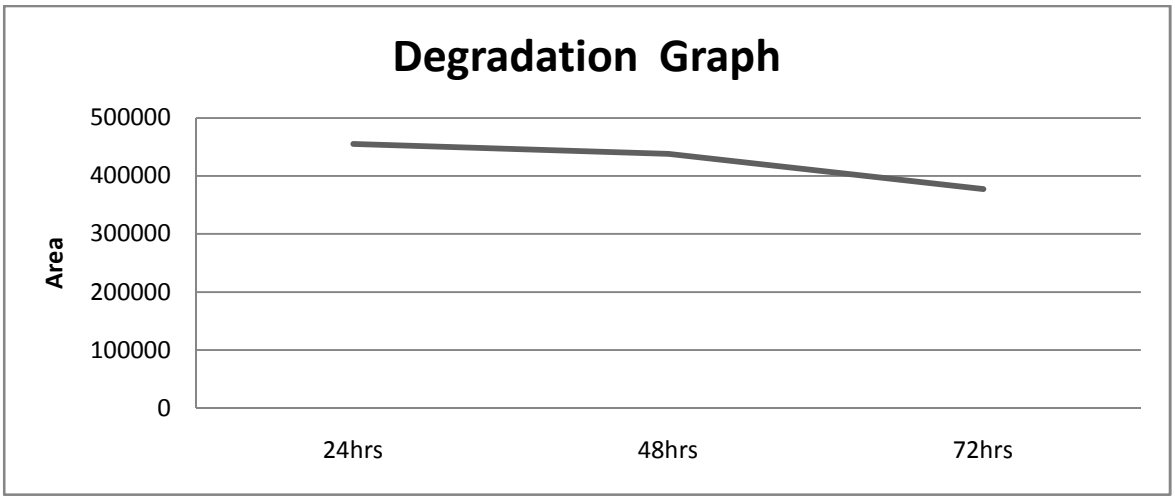


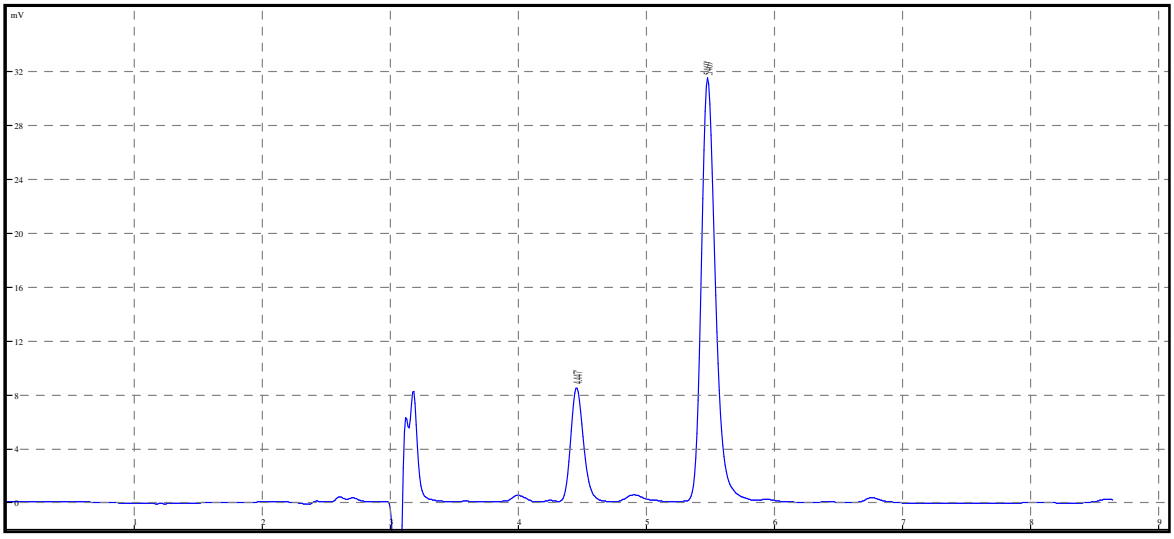
Fig No.6.5: Degradation graph for plane solution stability

Name of Drug: Dexamethasone

Trial graphs for Dexamethasone

Sample name: Dexamethasone Plasma Recovery

Run time: 8.64min

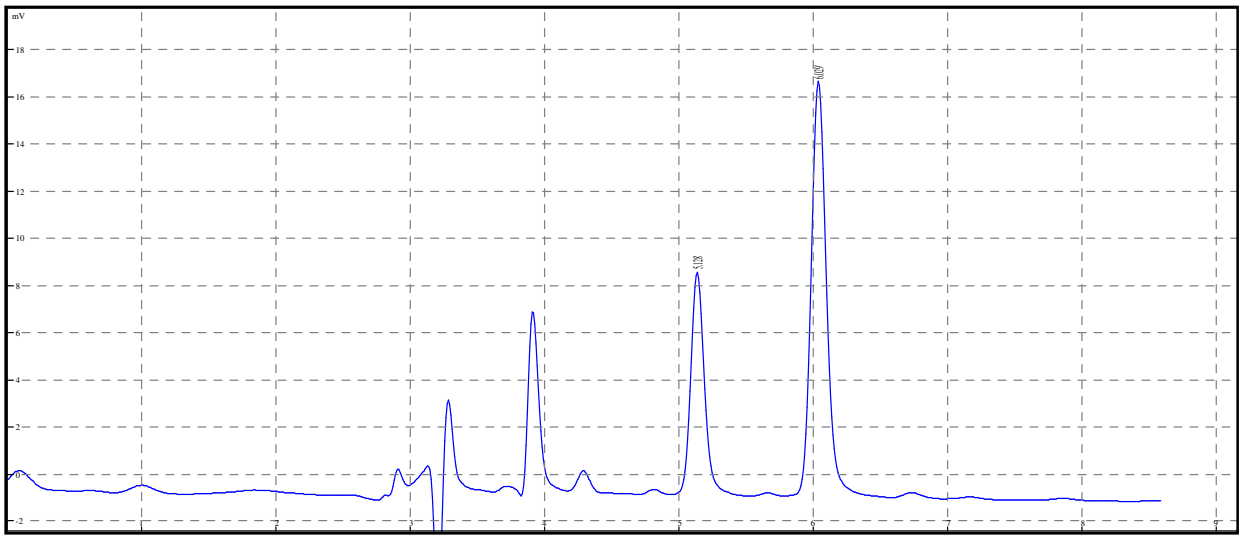


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.447	378082	0.78	3418	1.21
5.469	609582	0.90	3106	1.27

Sample Name: Dexamethasone LQC

Run time: 8.59min



Observation Table

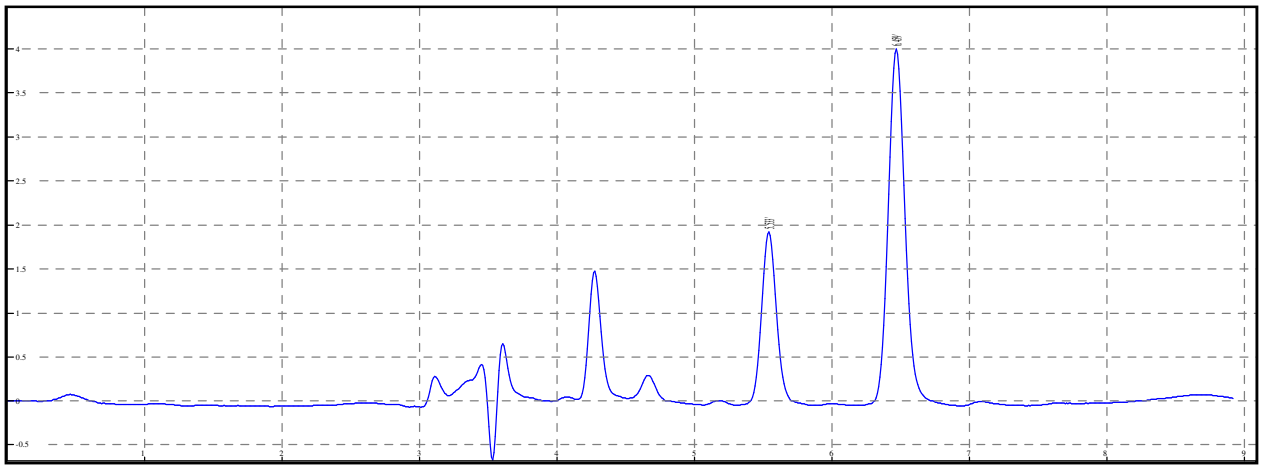
Time	Area	Resolution	T.Plate no.	Asymmetry
5.128	49651	2.52	5971	1.16
6.029	202636	0.00	5384	1.16

Selectivity

The desired method used RP-HPLC method for separation of Dexamethasone from Felodipin (IS) as well as plasma sample and was shown to be selective for the analyte and its IS (retention times for dexamethasone and felodipin were 5.44 and 6.33 minutes respectively).No interfering peaks were observed with the same retention time of the analyte, when different plasma samples were analysed.

Sample name: Drug+IS (Felodipin)

Run time: 8.92min



Observation Table

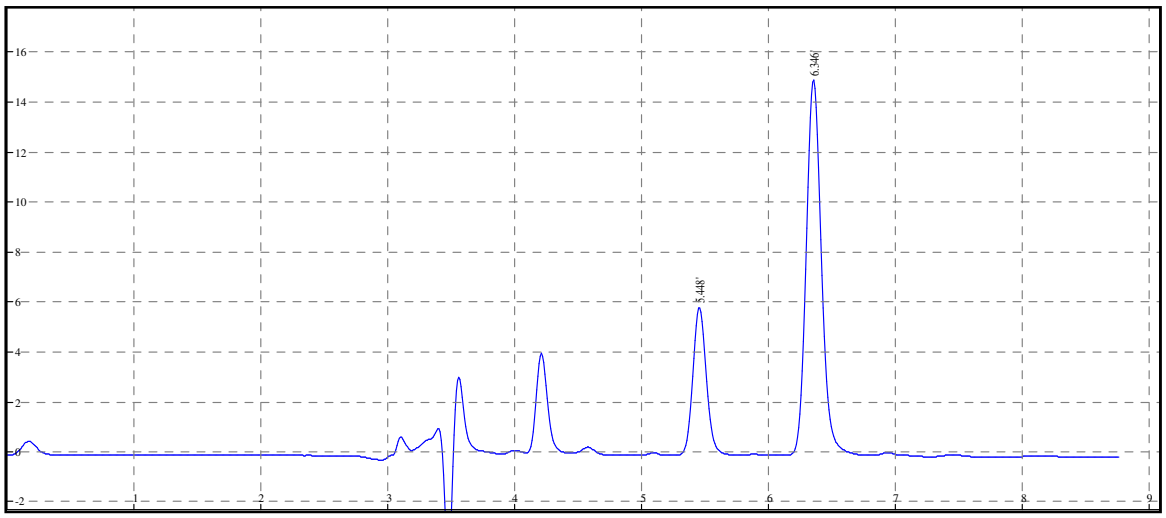
Time	Area	Resolution	T.Plate no.	Asymmetry
5.533	27054	3.15	5263	1.10
6.459	44529	0.00	8960	1.13

Linearity

Linearity was demonstrated by taking sample concentration ranges from 70-1120 ng/ml. Fig. given below showes calibration curve of dexamethasone.The calibration curve includes 6 calibration standards which are distributed 0.998 with goodness of fit.

Sample Name: Dexamethasone Standard A

Run time: 8.76min

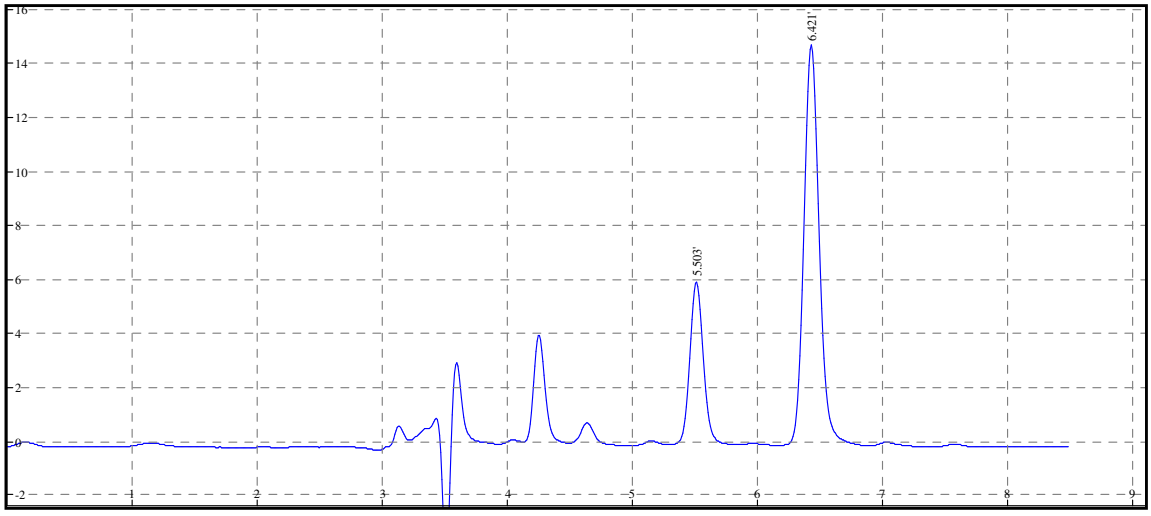


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.448	35019	2.66	8380	1.12
6.346	208242	0.00	7625	1.15

Sample Name: Dexamethasone Standard B

Run time: 8.48min

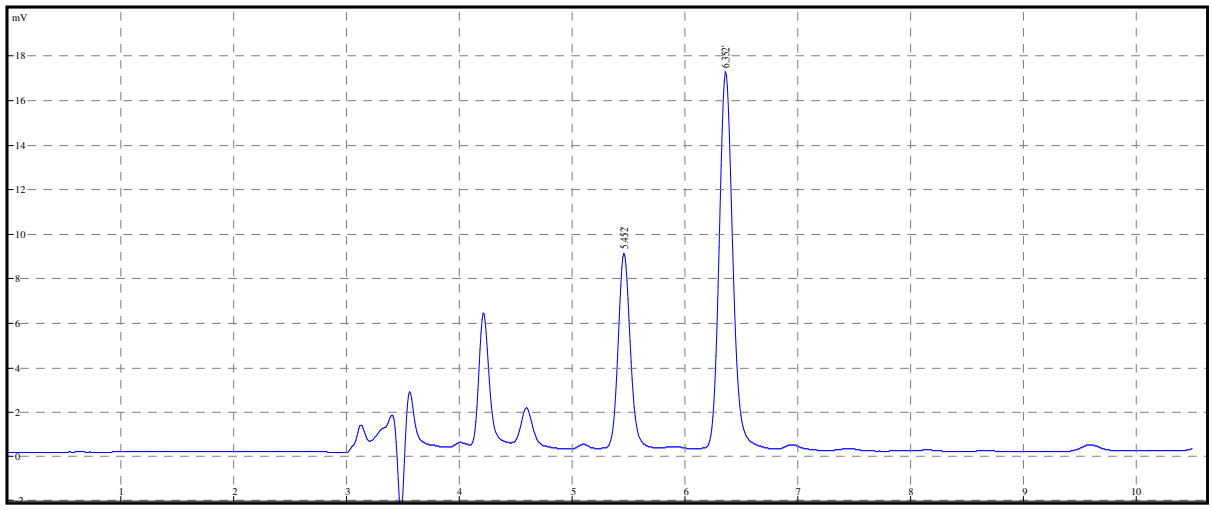


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.503	34870	2.50	5937	1.10
6.421	201735	0.00	6559	1.14

Sample Name: Dexamethasone Standard C

Run time: 10.49min

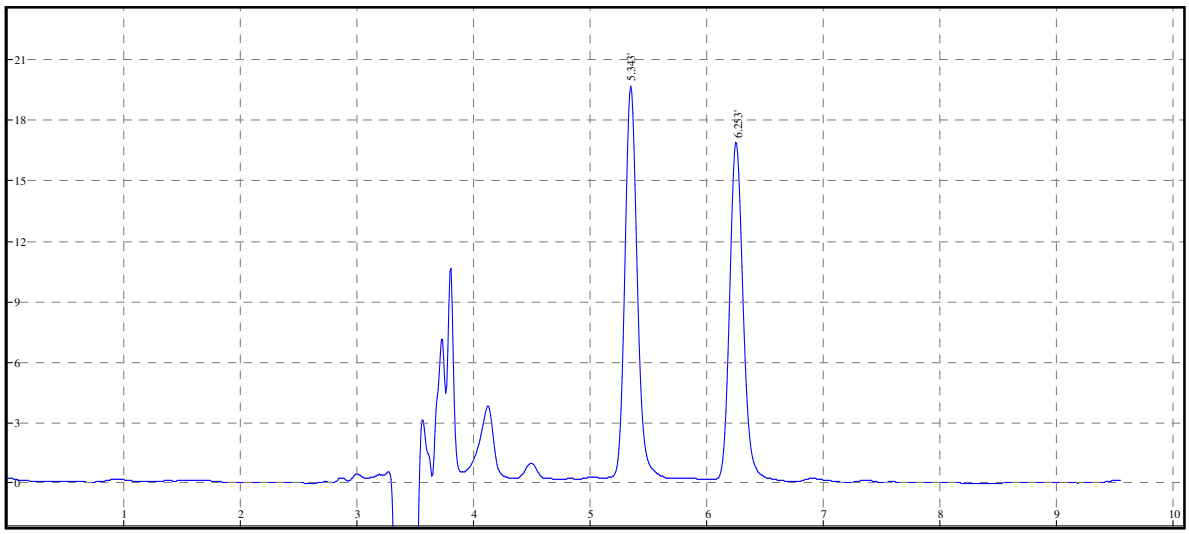


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.452	32875	2.87	6644	1.12
6.352	211506	0.00	7301	1.15

Sample Name: Dexamethasone Standard D

Run time: 9.55min

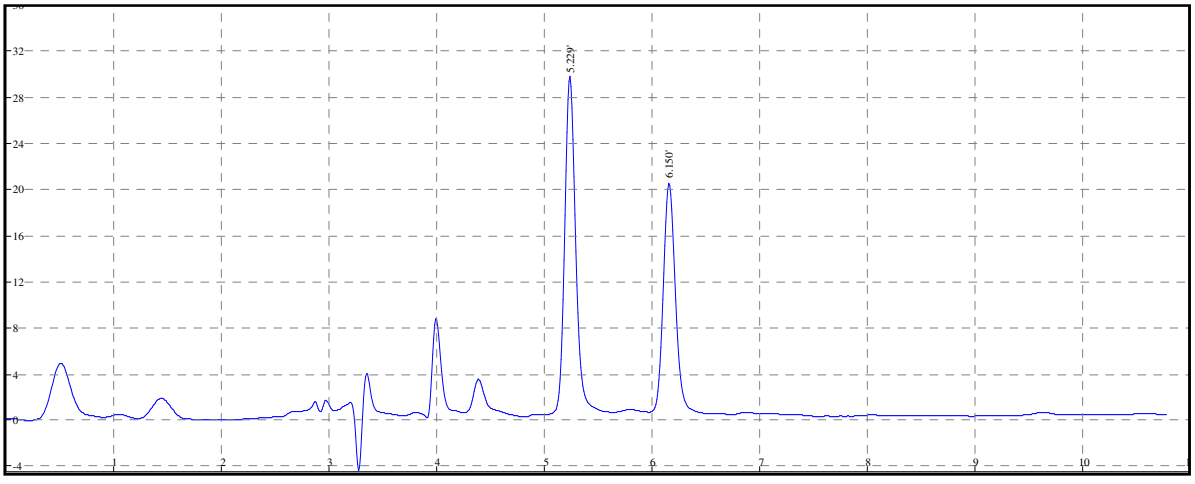


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.343	210670	0.00	16558	1.16
6.253	205759	0.00	55523	1.17

Sample Name: Dexamethasone Standard E

Run time: 10.78min

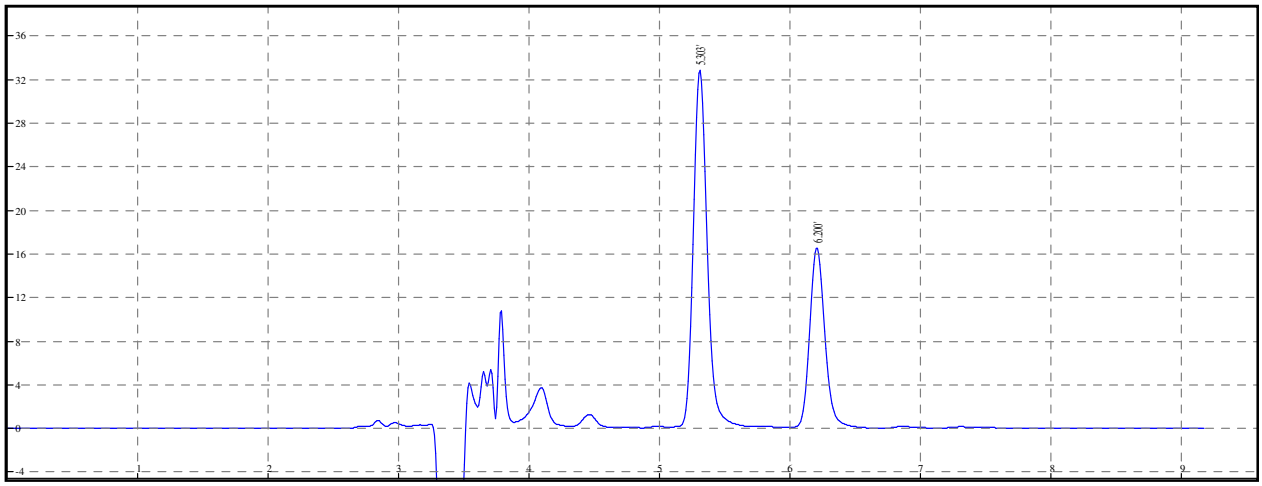


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.229	381615	2.07	5834	1.19
6.150	207289	0.00	7707	1.14

Sample Name: Dexamethasone Standard F

Run time: 9.17min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.303	520809	0.00	7683	1.18
6.200	208384	0.00	5308	1.17

Table No.6.4 Observation table for Linearity graph

Sample Name	Concentration ratio of sample and internal standard	Area ratio of sample and internal standard
Std-A	0.1458	0.1748
Std-B	0.1458	0.1748
Std-C	0.2916	0.3368
Std-D	0.875	1.023
Std-E	1.75	1.8369
Std-F	2.333	2.4992

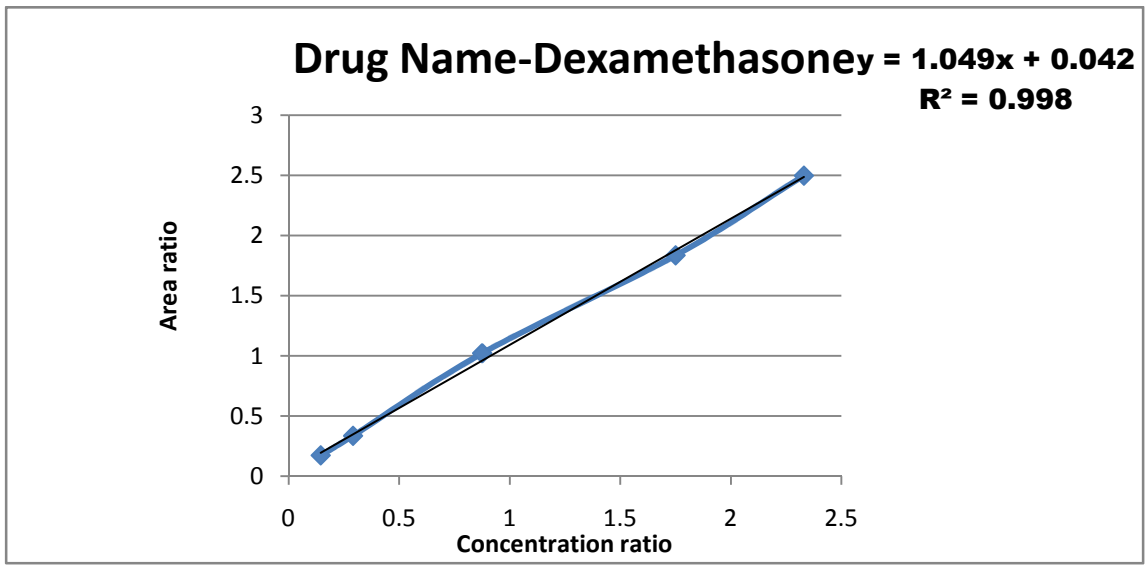


Fig No.6.6: Linearity graph

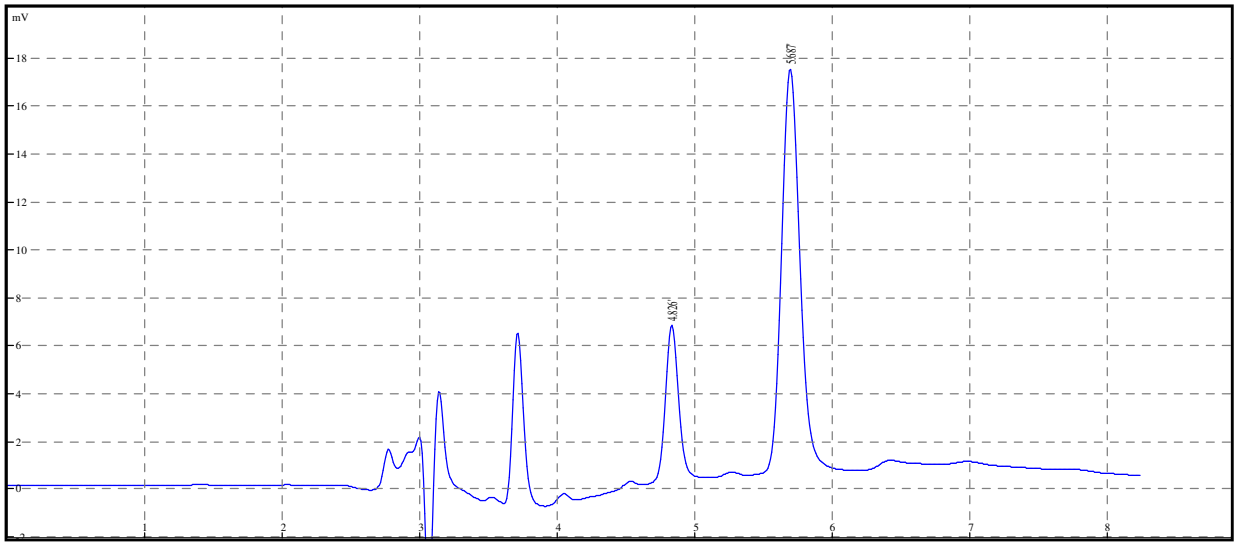
(Limit: The R^2 value should be near to 1)

Accuracy and Precision

Accuracy and Precision was evaluated by analyzing 5 bathches.Each batch consist of five replicates of LQC, MQC and HQC.

Sample Name: Dexamethasone LQC 01

Run time: 8.23min

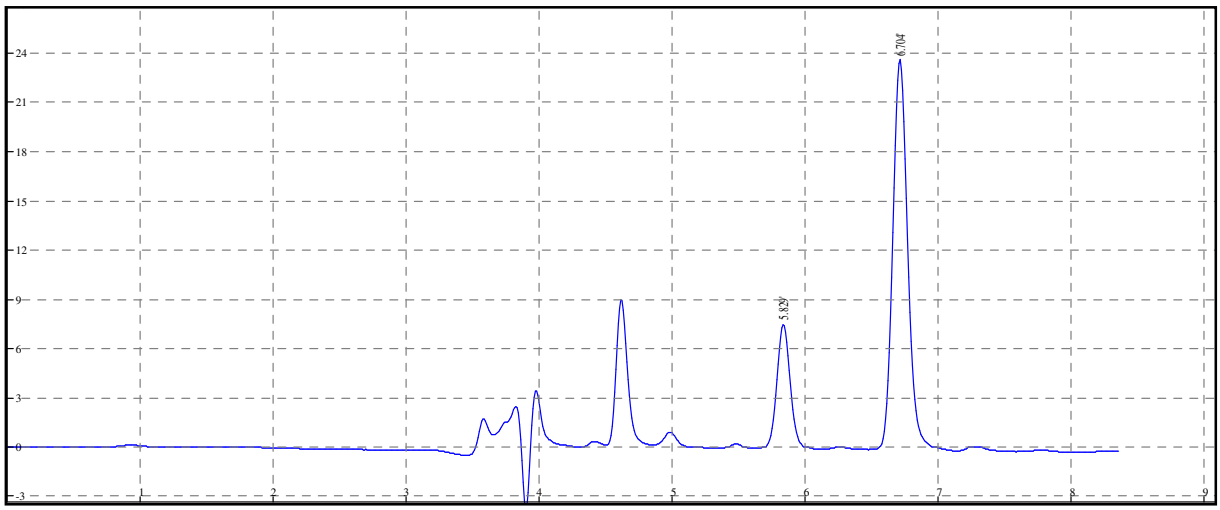


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.826	48074	2.23	6648	1.14
5.687	202076	0.00	6490	1.17

Sample Name: Dexamethasone LQC 02

Run time: 8.36min

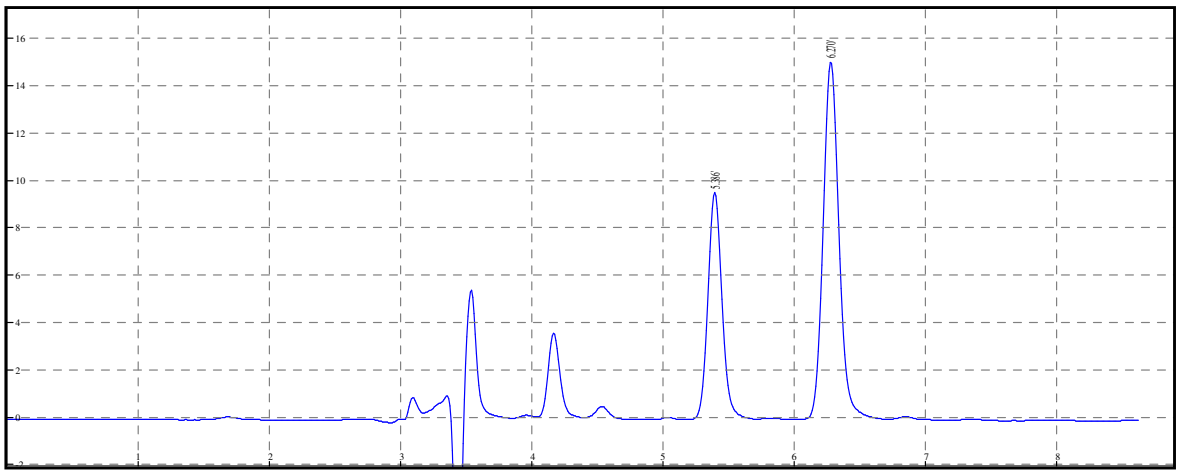


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.829	50576	3.05	5256	1.07
6.704	182915	0.00	12138	1.14

Sample Name: Dexamethasone LQC 03

Run time: 8.62min

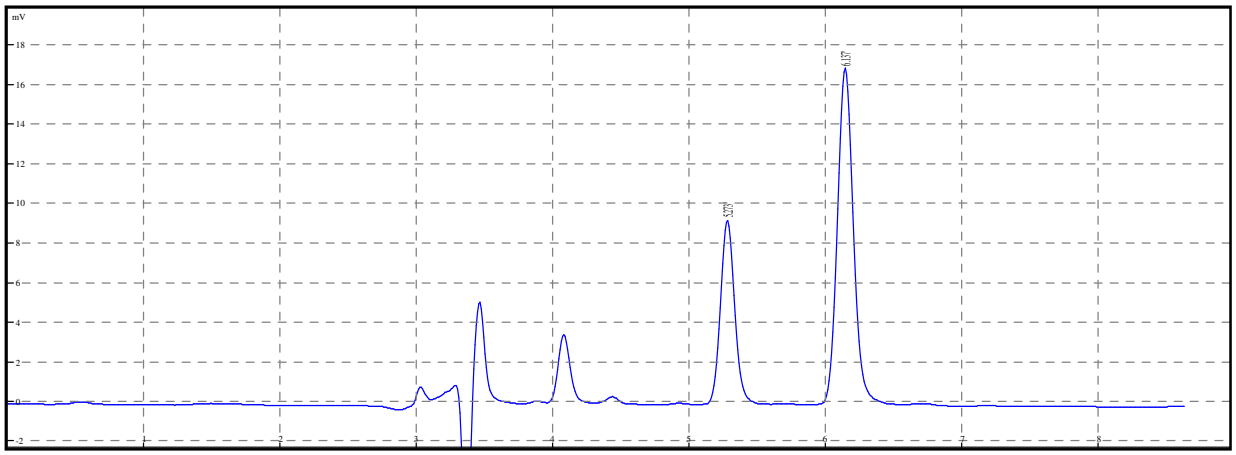


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.386	47909	2.13	7498	1.12
6.270	198957	0.00	6222	1.13

Sample Name: Dexamethasone LQC 04

Run time: 8.63min

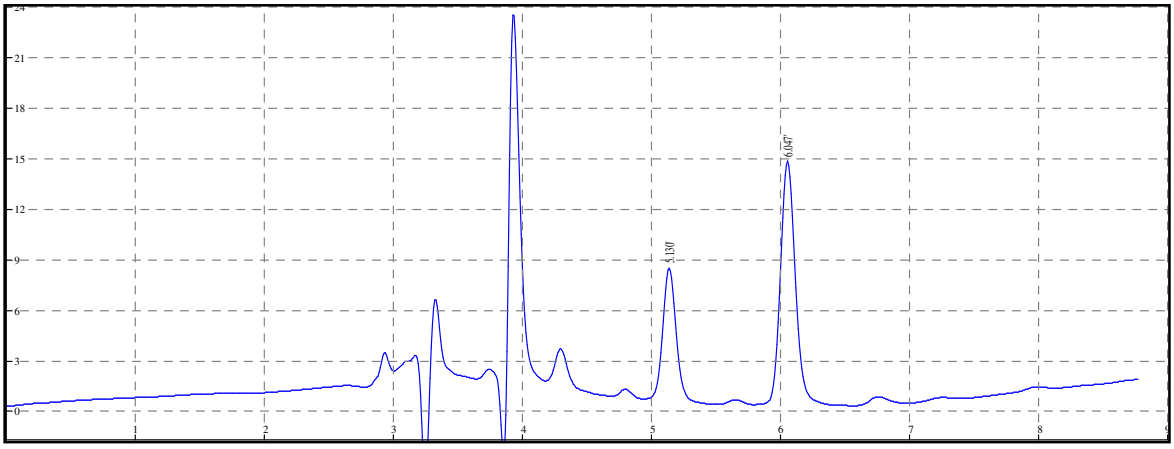


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.273	51829	2.15	6460	1.12
6.137	174802	0.00	7470	1.12

Sample Name: Dexamethasone LQC 05

Run time: 8.77min

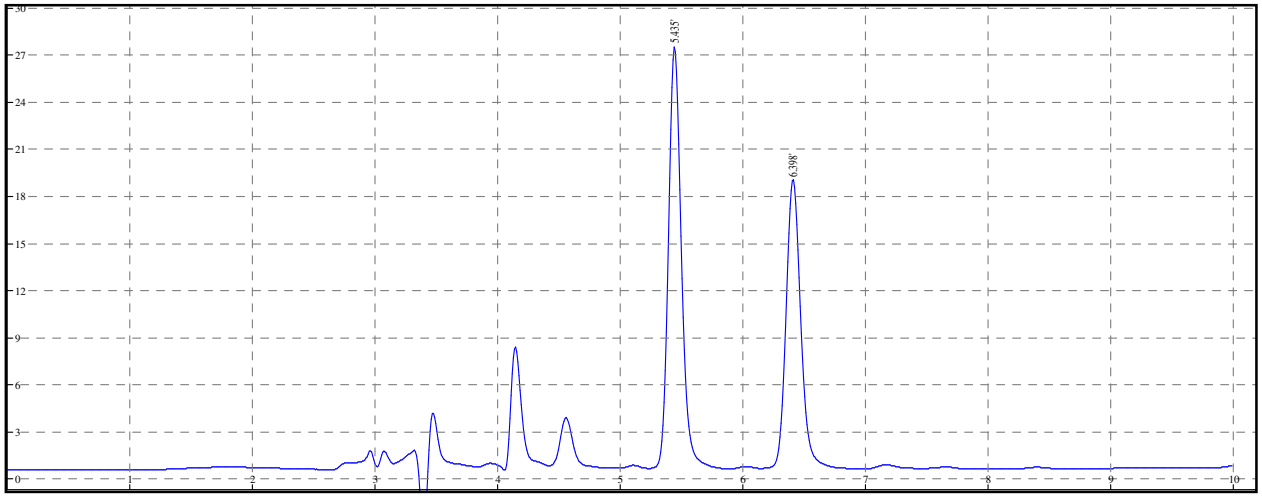


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.130	49975	2.65	9208	1.05
6.047	175782	0.00	8501	1.15

Sample Name: Dexamethasone MQC 01

Run time: 9.98min

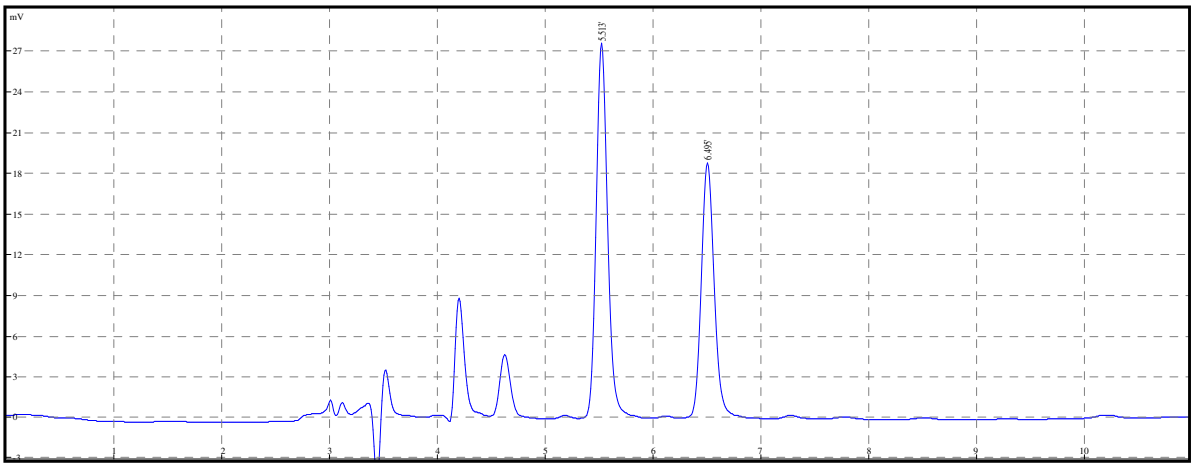


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.435	383080	3.07	7274	1.18
6.398	211576	0.00	6602	1.16

Sample Name: Dexamethasone MQC 02

Run time: 11.90min

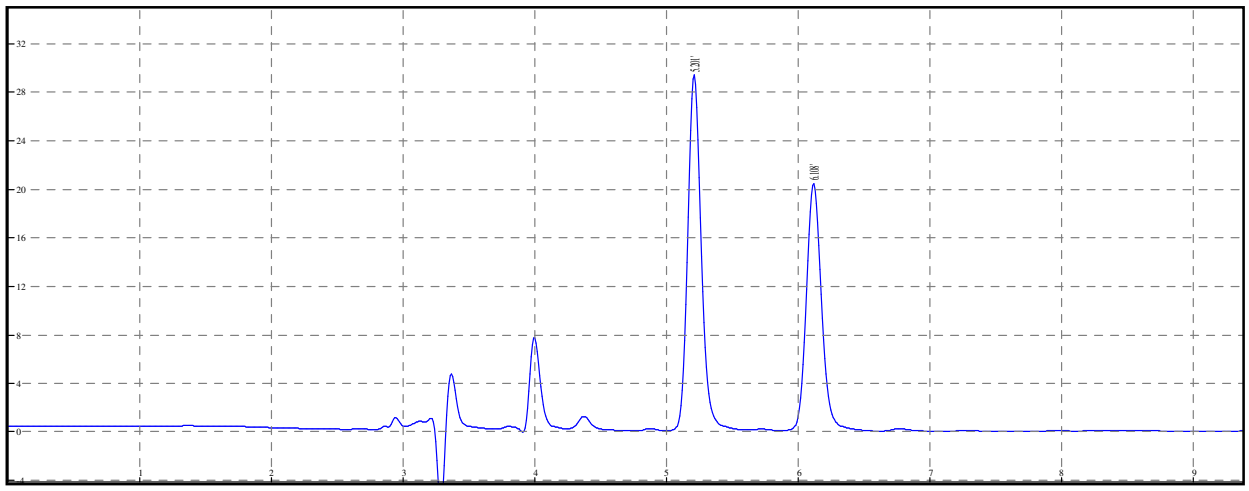


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.513	381373	3.25	6563	1.18
6.495	203236	0.00	6459	1.16

Sample Name: Dexamethasone MQC 03

Run time: 9.47min

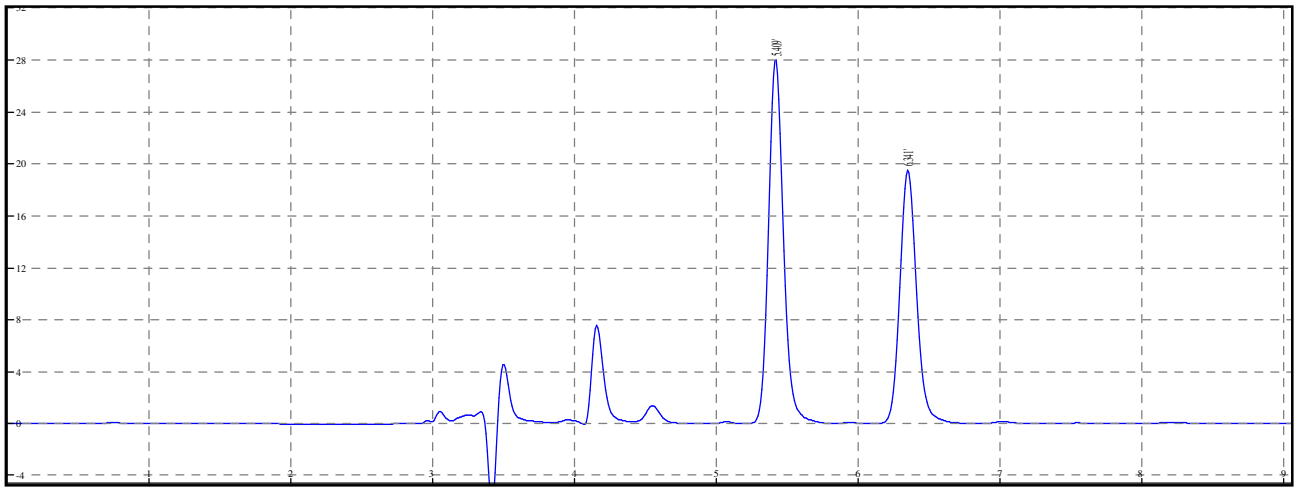


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.201	376290	2.84	5303	1.17
6.108	217244	0.00	5068	1.15

Sample Name: Dexamethasone MQC 04

Run time: 9.06min

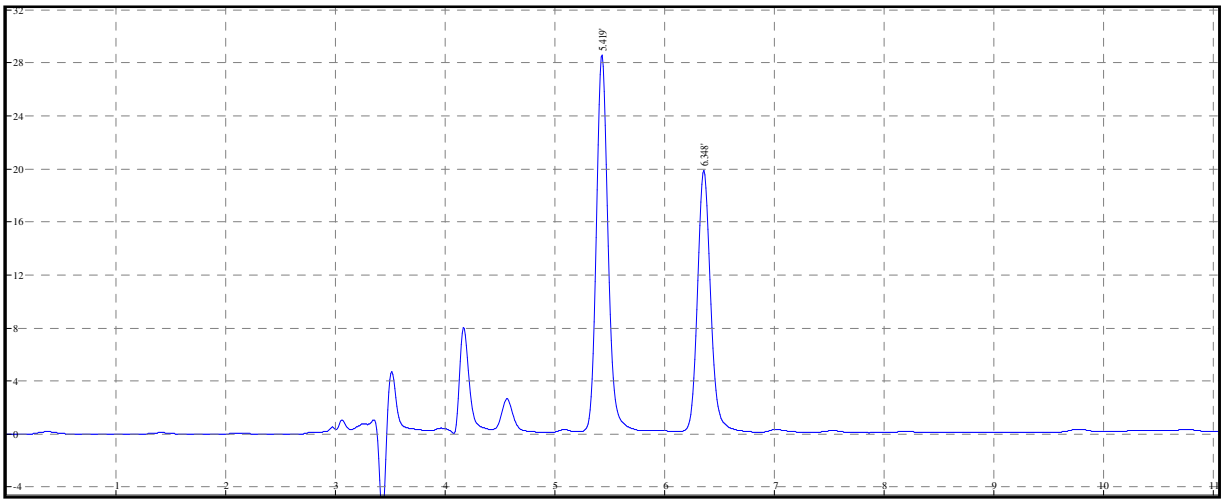


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.409	380874	2.89	5477	1.16
6.341	215097	0.00	5495	1.15

Sample Name: Dexamethasone MQC 05

Run time: 12.86min

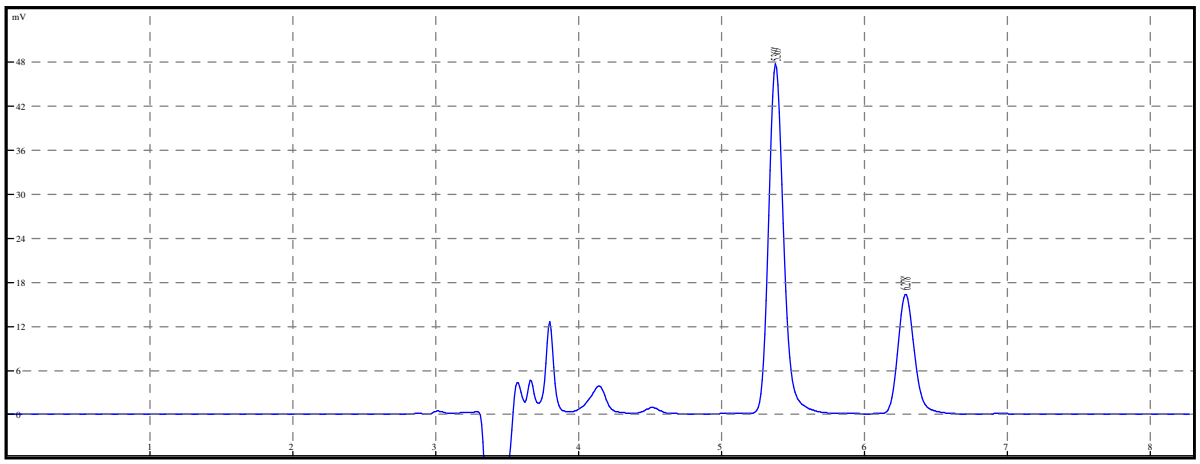


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.419	358074	2.63	4838	1.17
6.348	225033	0.00	4408	1.15

Sample Name: Dexamethasone HQC 01

Run time: 8.28min

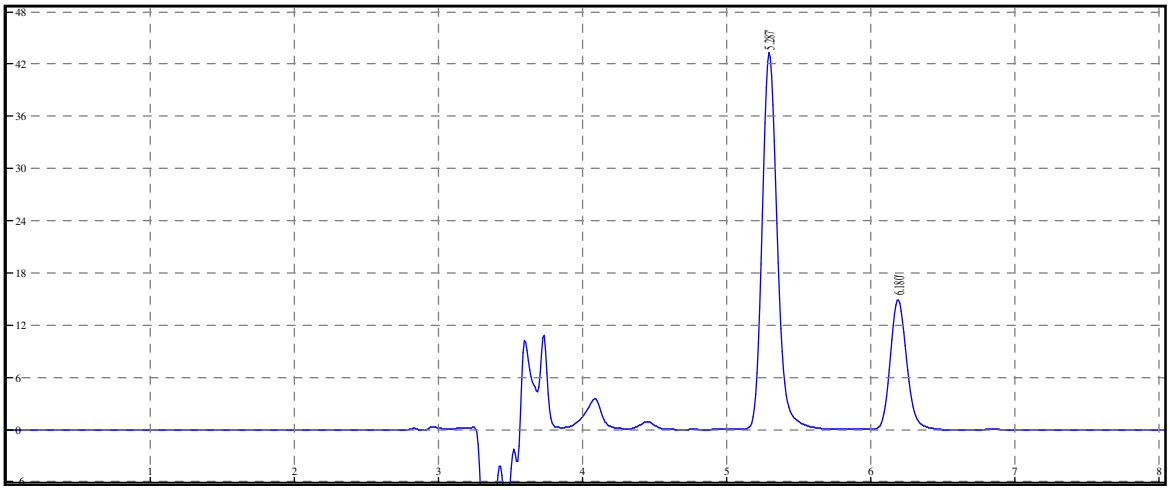


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.369	709820	3.10	4391	1.20
6.278	223255	0.00	6817	1.18

Sample Name: Dexamethasone HQC 02

Run time: 8.25min

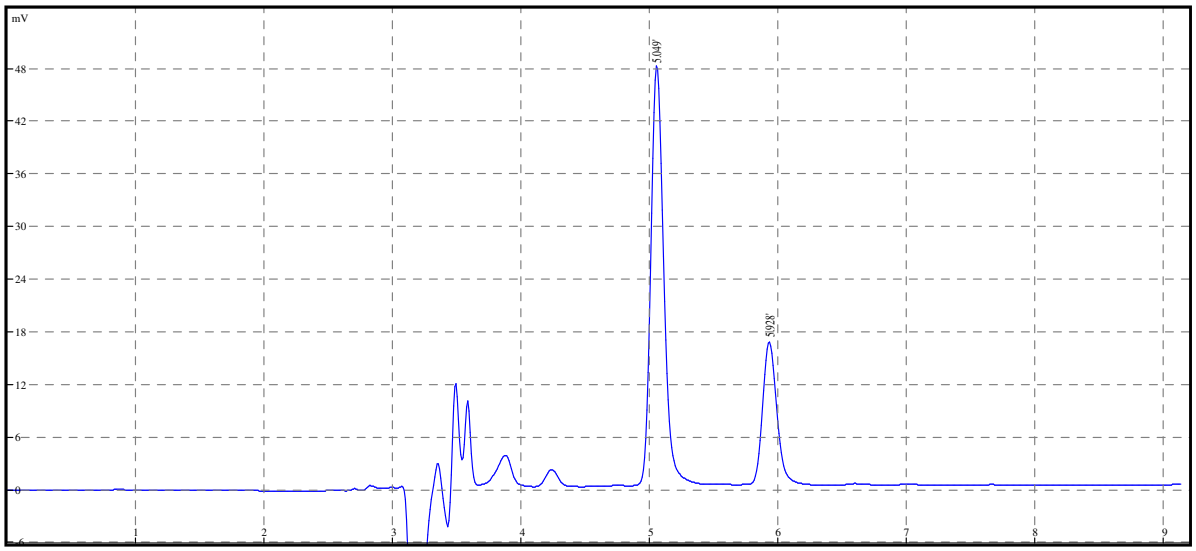


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.287	679182	3.43	6961	1.19
6.180	219189	0.00	6551	1.16

Sample Name: Dexamethasone HQC 03

Run time: 9.13min

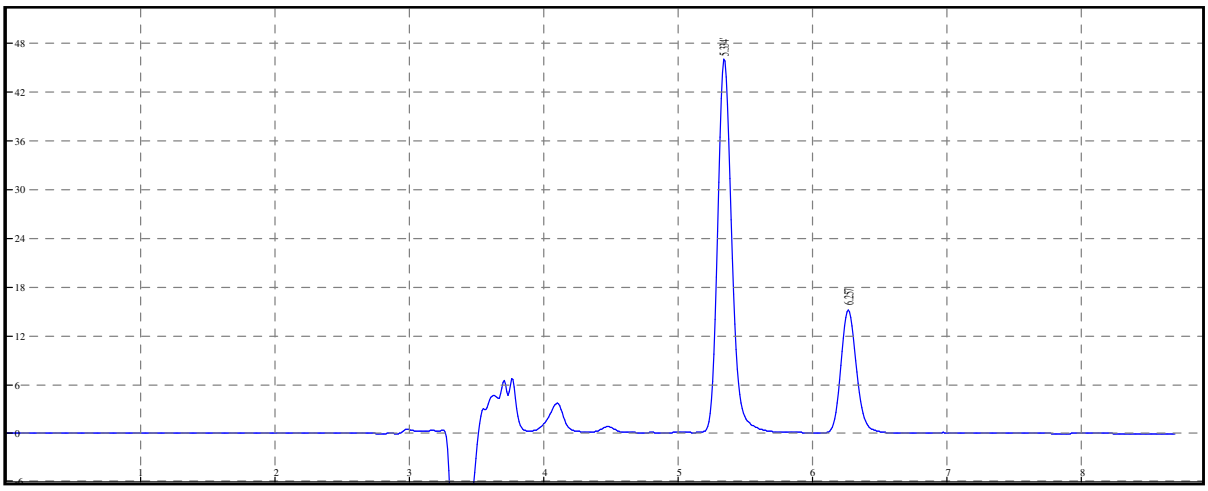


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.049	672768	3.08	6145	1.22
5.928	195959	0.00	5834	1.20

Sample Name: Dexamethasone HQC 04

Run time: 8.70min

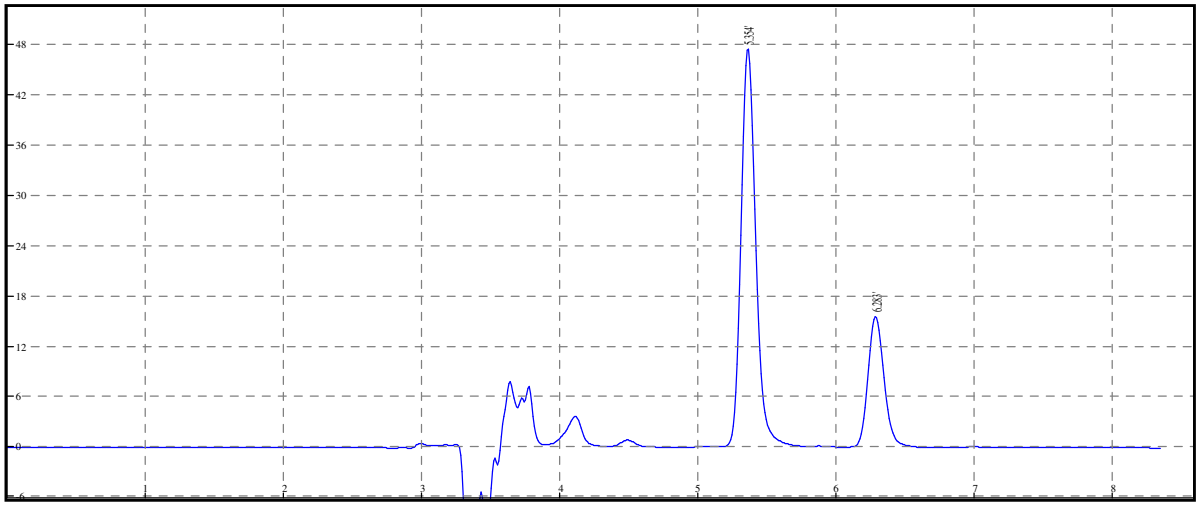


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.334	684456	2.16	6092	1.20
6.257	199363	0.00	7343	1.17

Sample Name: Dexamethasone HQC 05

Run time: 8.35min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.354	682247	0.00	4181	1.19
6.283	229134	0.00	7903	1.17

The precision and accuracy of the method for each concentration levels are represented in following Table.

Table No.6.5 Observation table for Accuracy & Precision

			Standard Deviation		Accuracy	Precision
Conc.	**Conc.**	**Area**	**Mean**	**SD**	**%SD**	**%RSD**
LQC	0.218	0.2379	0.2672	0.02642366	9.889096	9.889096
	0.218	0.2765				
	0.218	0.2408				
	0.218	0.2965				
	0.218	0.2843				
MQC	1.75	1.8106	1.756224	0.10659097	6.069327	6.069327
	1.75	1.8765				
	1.75	1.7321				
	1.75	1.7707				
	1.75	1.5912				
HQC	2.916	3.1794	3.18508	0.16943189	5.319549	5.319549

	2.916	3.0986				
	2.916	3.4332				
	2.916	3.2367				
	2.916	2.9775				

(Standard Limit: %SD & % RSD should be less than 15%)

Table No.6.6 Observation table for Coefficient varience (CV)

Sr.No	Concentration	Concentration Ratio	Coefficient Varience
1	LQC	0.2187	0.162553
2	MQC	1.75	0.326482
3	HQC	2.916	0.411621

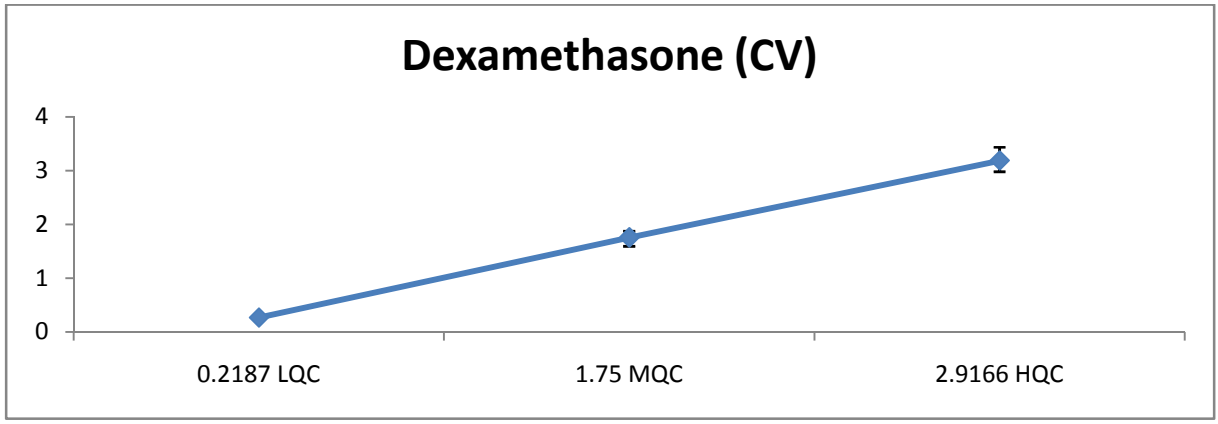


Fig No.6.7: coefficient variance graph

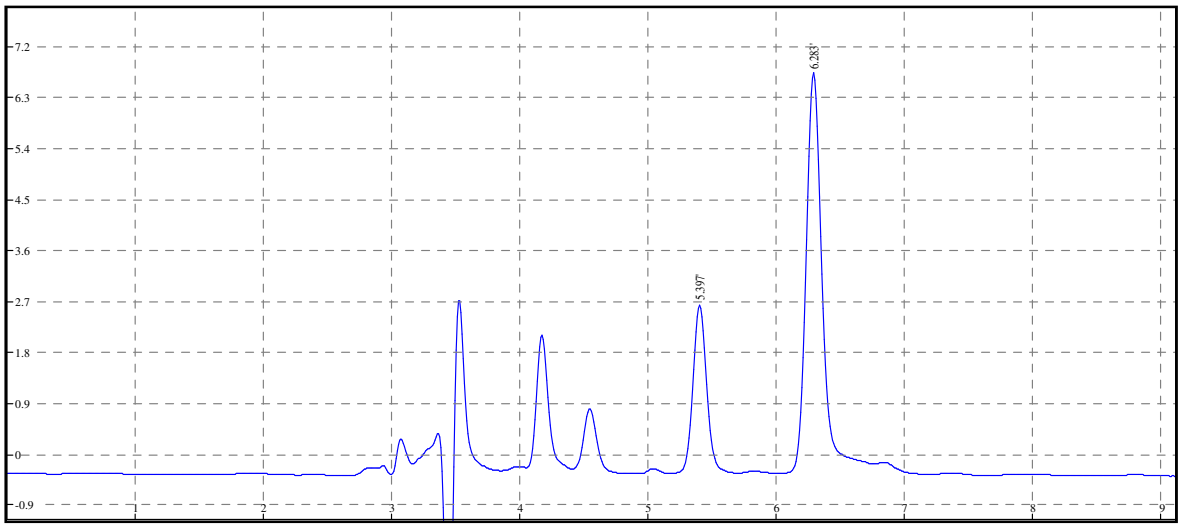
Recovery

The recovery was evaluated by comparing response of extracted and unextracted samples.The average recovery for Dexamethasone in plasma was found to around 75.2%.

Unextracted sample

Sample Name: Dexamethasone LQC

Run time: 9.13min



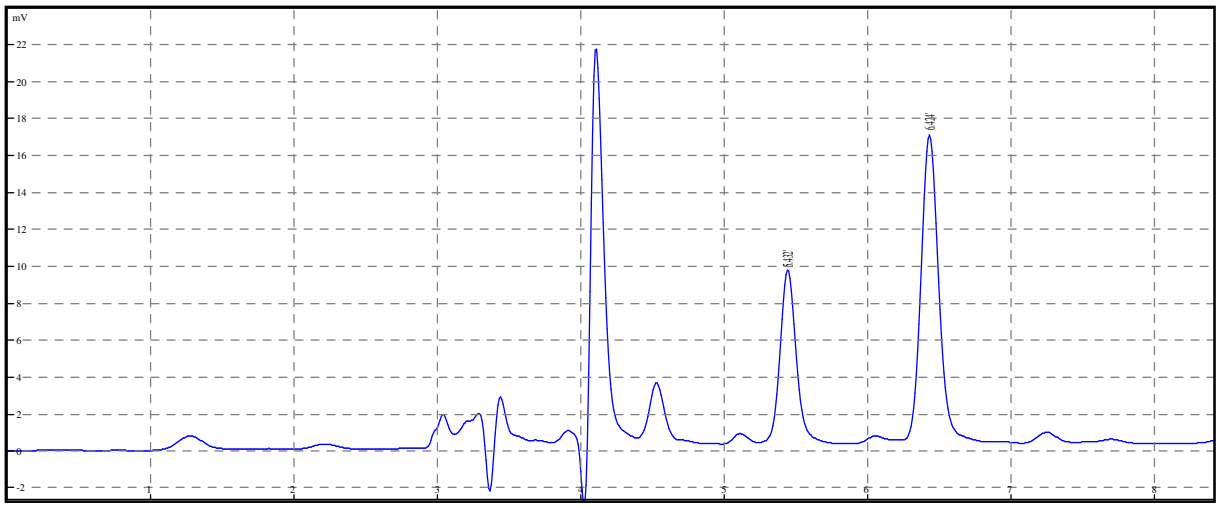
Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.397	102667	1.85	4909	1.09
6.283	259437	0.00	3105	1.17

Extracted sample

Sample Name: Dexamethasone LQC

Run time: 8.43min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.432	49441	2.67	24800	1.13
6.424	204813	0.00	20030	1.13

For stability study

Stability studies were performed to evaluate the stability of Dexamethasone both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include bench top stability, freeze thaw stability, long term and short term stock stability. Dexamethasone was found to be stable for three freeze and thaw cycles.

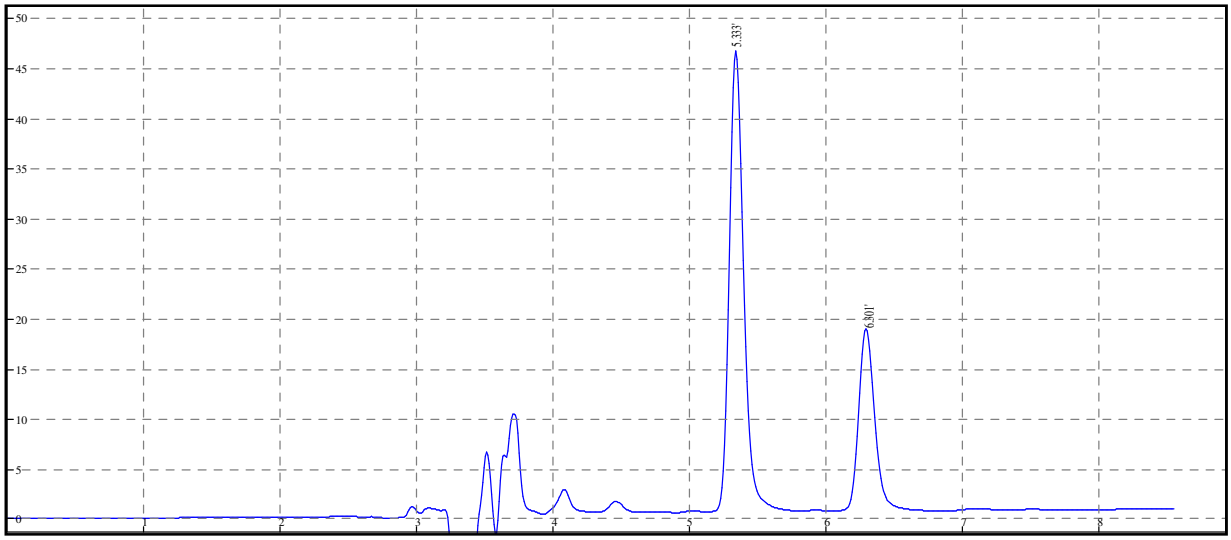
1. Bench top Stability:

A. Short Term Analysis: 2, 12 and 24hrs.

B. Long Tem Analysis: 10, 20 and 30days

Sample Name: Dexamethasone HQC 2hrs

Run time: 8.55min

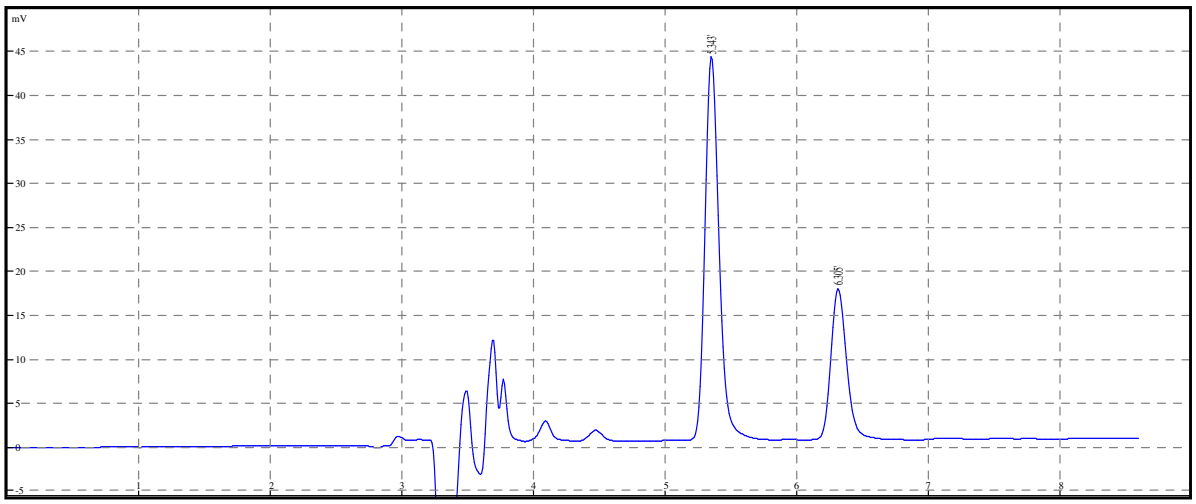


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.333	194966	0.00	8017	1.25
6.301	20332	0.00	8058	1.23

Sample Name: Dexamethasone HQC 12hrs

Run time: 8.58 min

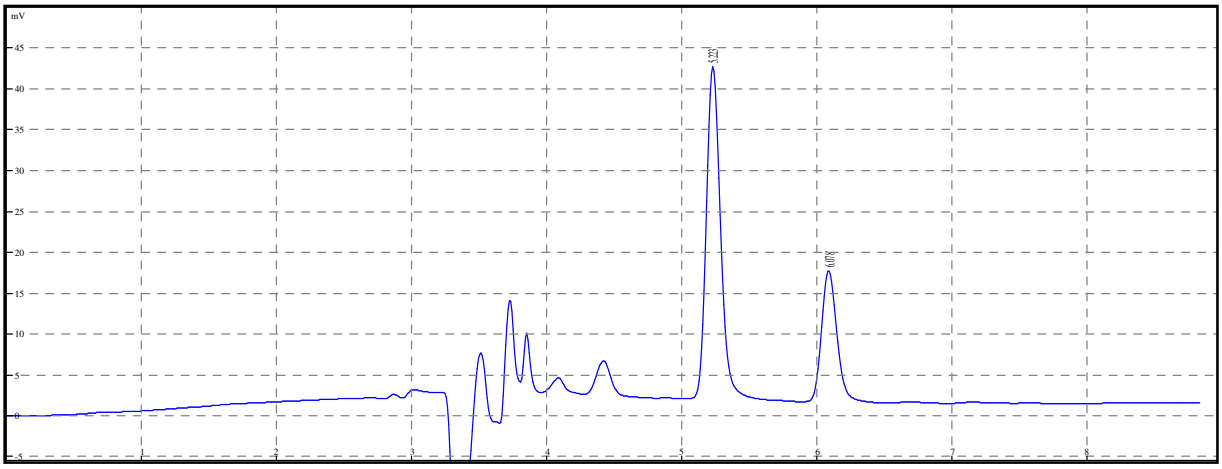


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.343	746005	2.17	3522	1.23
6.305	478837	0.00	2413	1.22

Sample Name: Dexamethasone HQC 24 hrs

Run time: 8.84 min

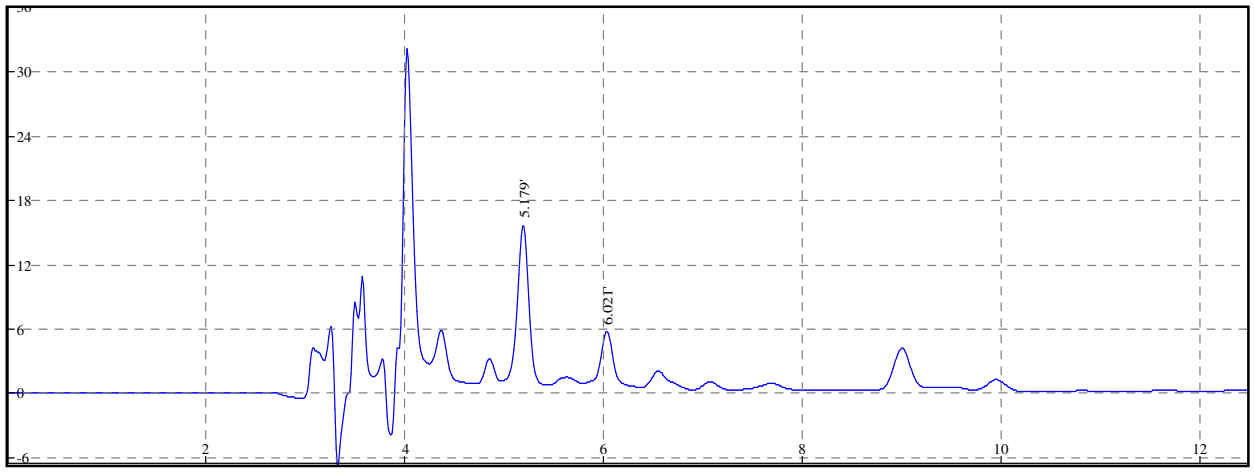


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.223	846232	1.91	2379	1.20
6.078	417558	0.00	2857	1.18

Sample Name: Dexamethasone HQC 10 days

Run time: 12.64 min

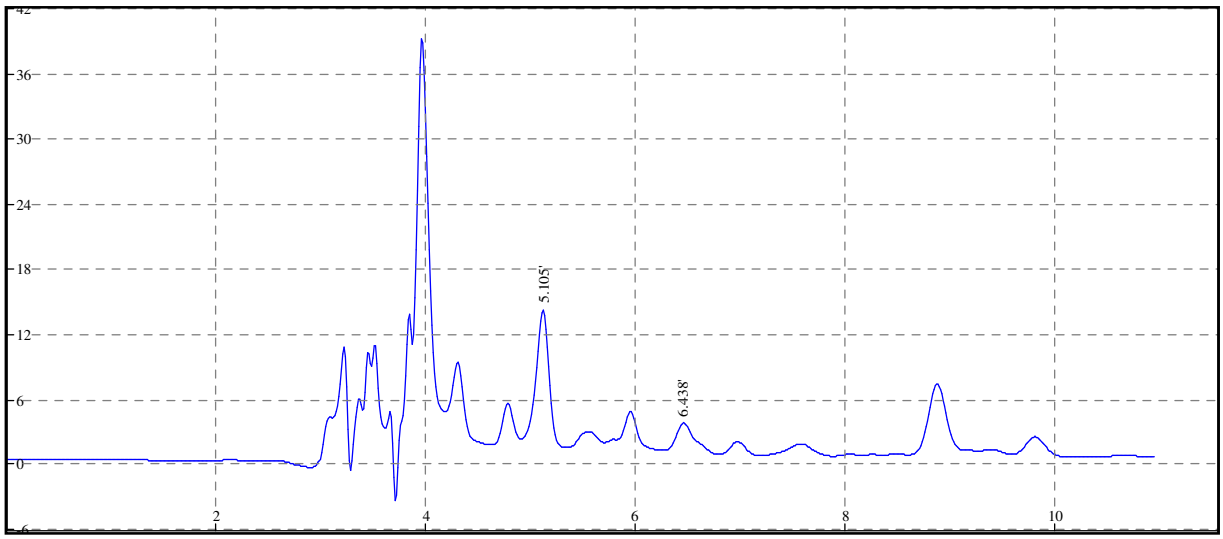


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.179	68669	0.00	17233	0.95
6.021	25125	0.00	20823	0.89

Sample Name: Dexamethasone HQC 20 days

Run time: 10.95 min

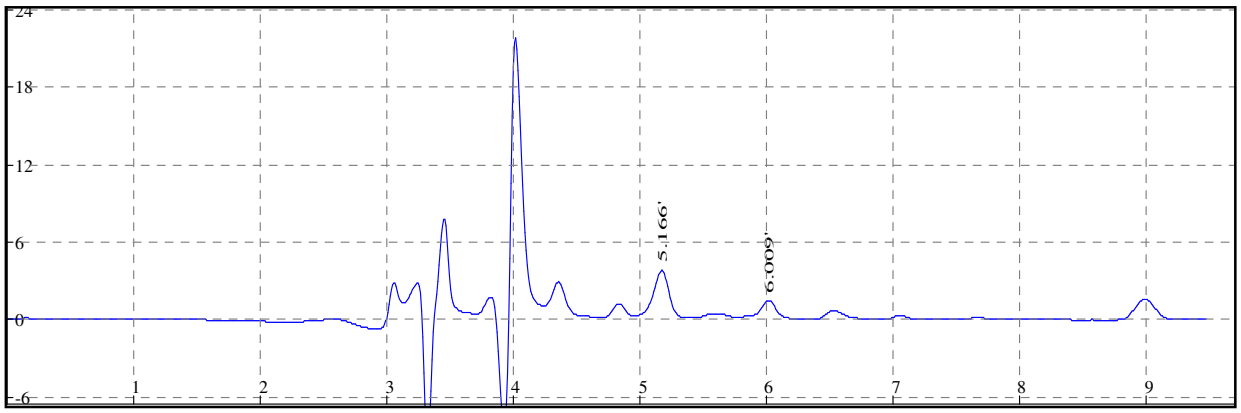


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.105	73748	0.00	13119	0.87
6.438	4511	0.00	43340	1.30

Sample Name: Dexamethasone HQC 30 days

Run time: 9.47 min

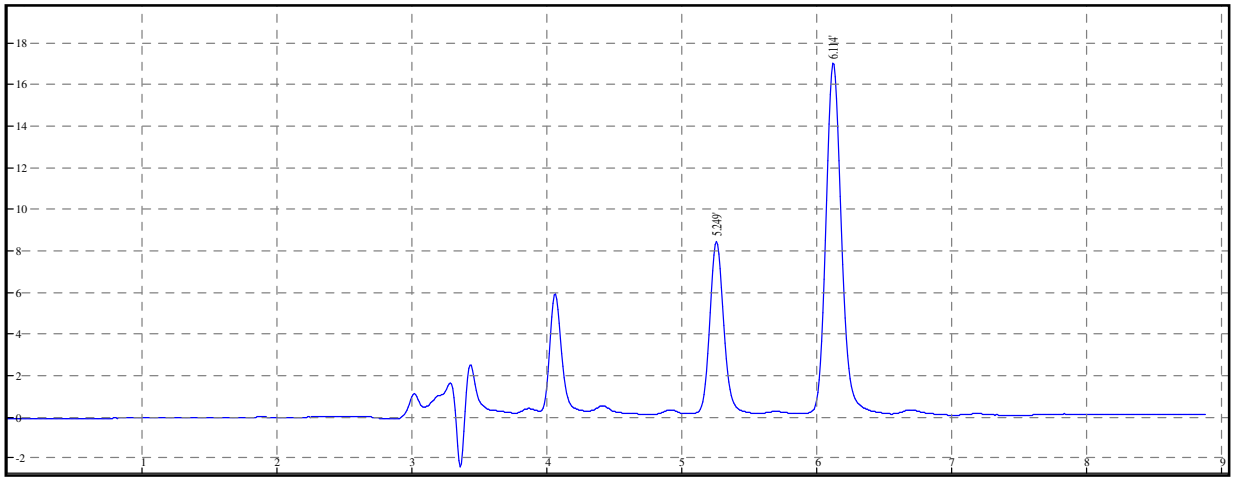


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.166	225947	2.26	1437	0.87
6.009	212093	0.00	954	0.78

Sample Name: Dexamethasone LQC 2hrs

Run time: 8.88 min

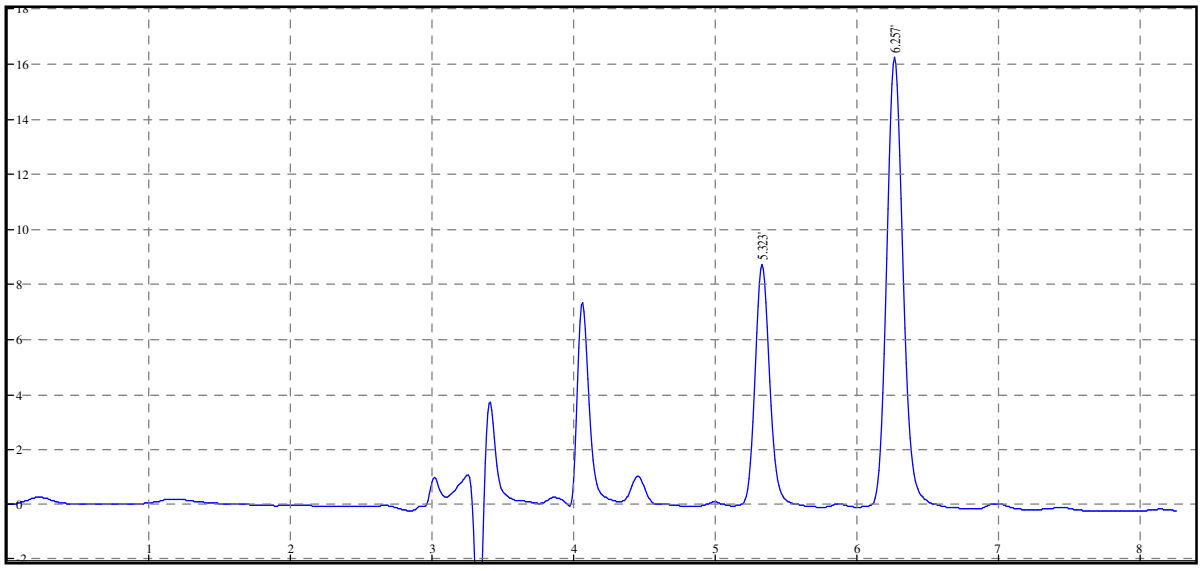


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.249	49355	4.25	15869	1.14
6.114	137475	0.00	13267	1.16

Sample Name: Dexamethasone LQC 12 hrs

Run time: 8.25 min

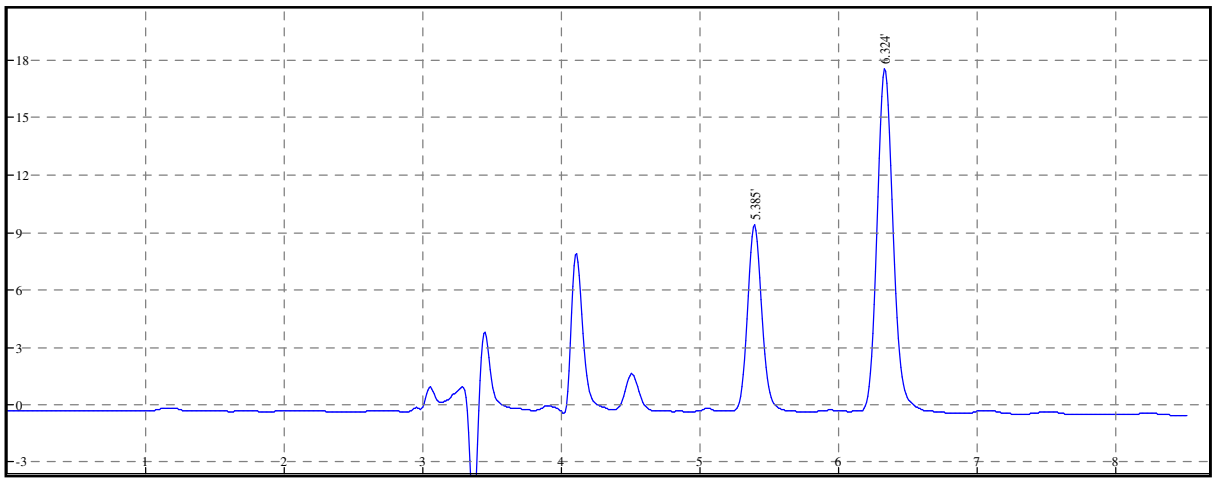


Observation Table

Time	**Area**	**Resolution**	**T.Plate no.**	**Asymmetry**
5.323	168765	2.68	3197	1.15
6.257	216742	0.00	6498	1.15

Sample Name: Dexamethasone LQC 24 hrs

Run time: 8.51 min

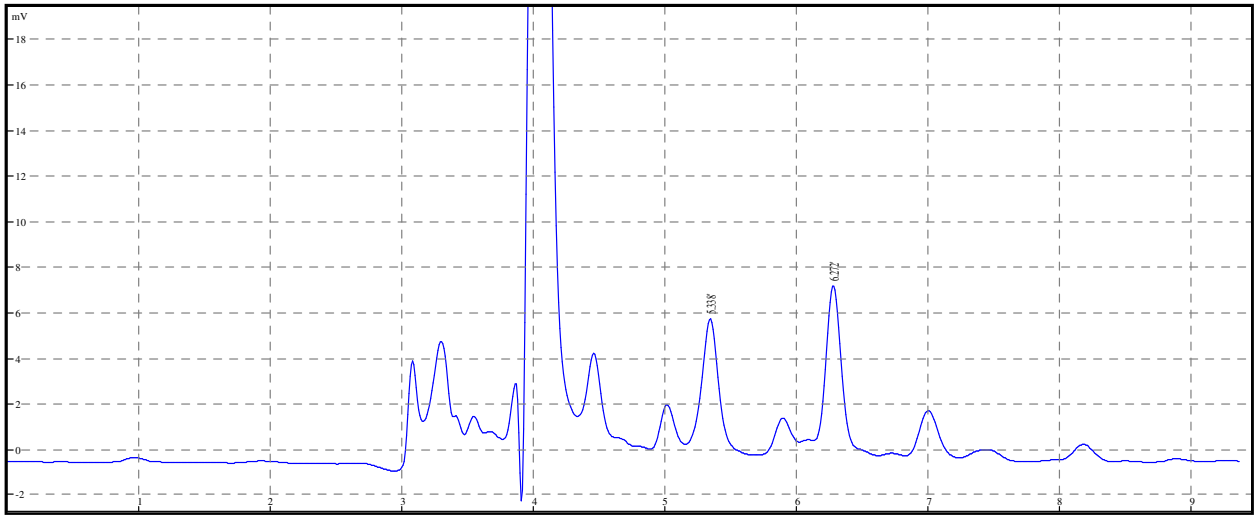


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.385	62690	4.10	15094	1.13
6.324	148765	0.00	13725	1.15

Sample Name: Dexamethasone LQC 10 days

Run time: 9.36 min

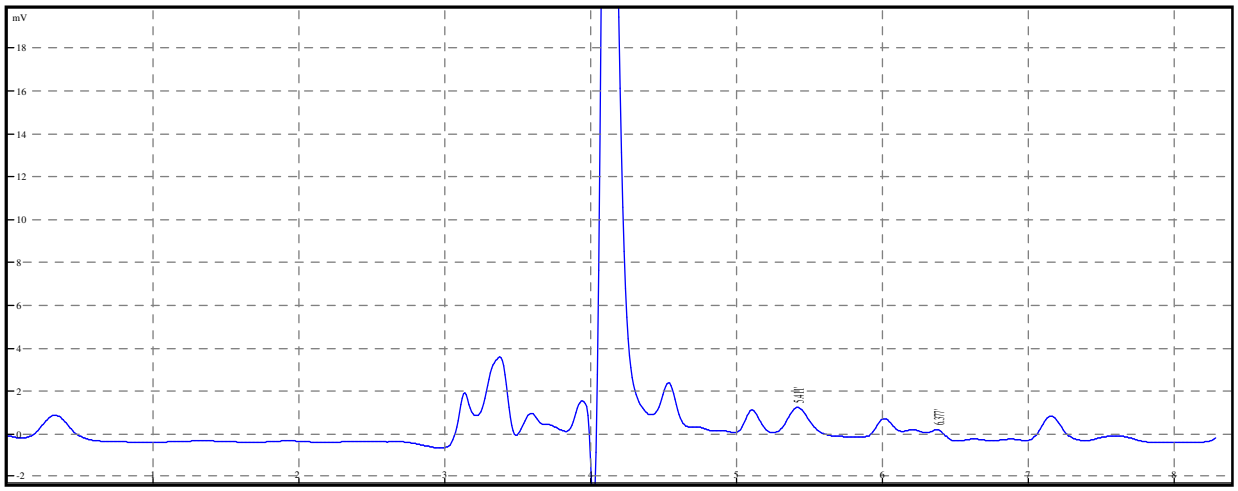


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.338	108073	2.74	3337	0.96
6.272	100842	0.00	6955	1.04

Sample Name: Dexamethasone LQC 20 days

Run time: 8.28 min

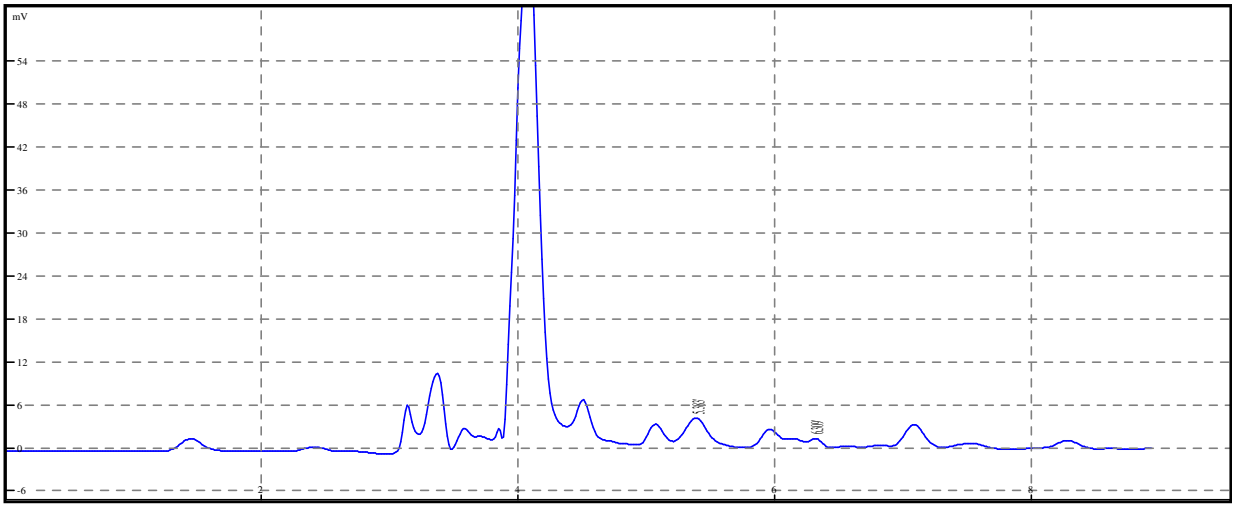


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.411	8348	0.00	10179	1.05
6.377	1508	0.00	36817	0.79

Sample Name: Dexamethasone LQC 30 days

Run time: 8.93 min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.383	15462	0.00	12557	0.89
6.309	3166	0.00	44506	0.82

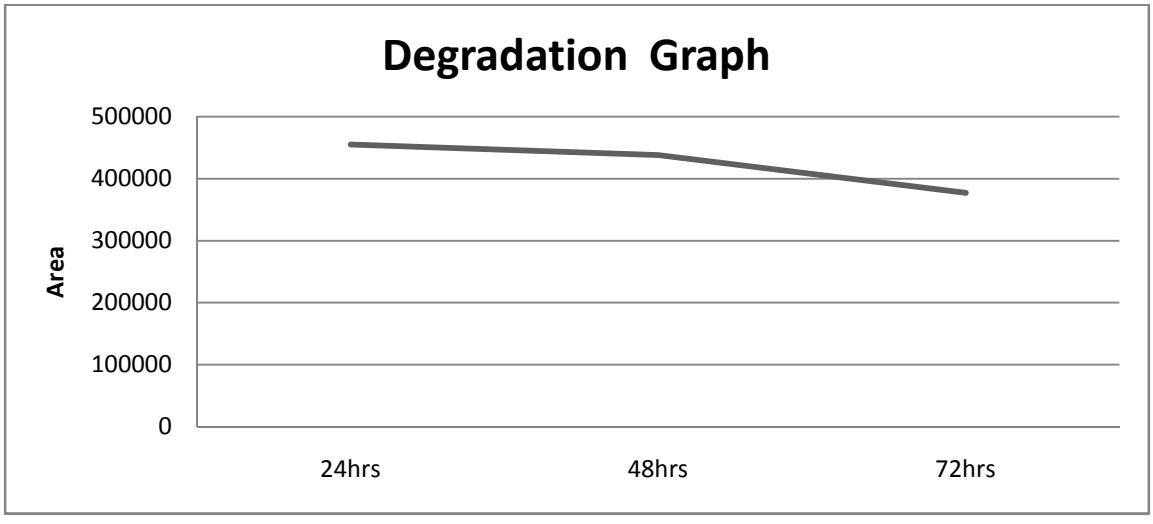
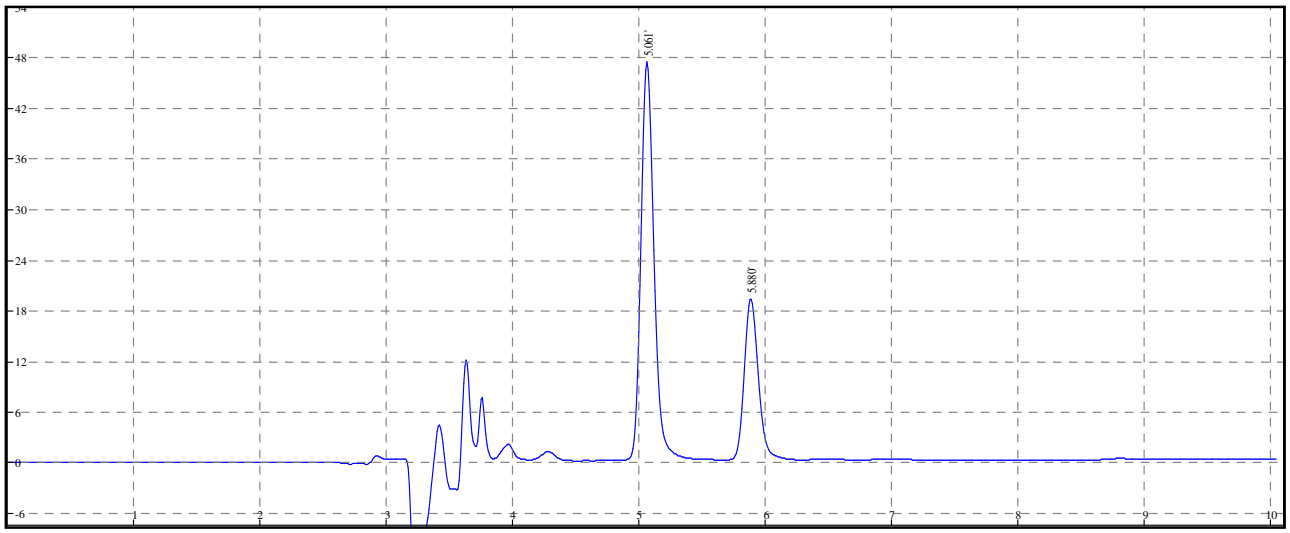


Fig No.6.8: Degradation graph for Bench top stabilty

2. Freeze thaw Cycles Stability: Each Cycle at 12, 24 and 48hrs.

Sample Name: Dexamethasone HQC 12hrs

Run time: 10.04min

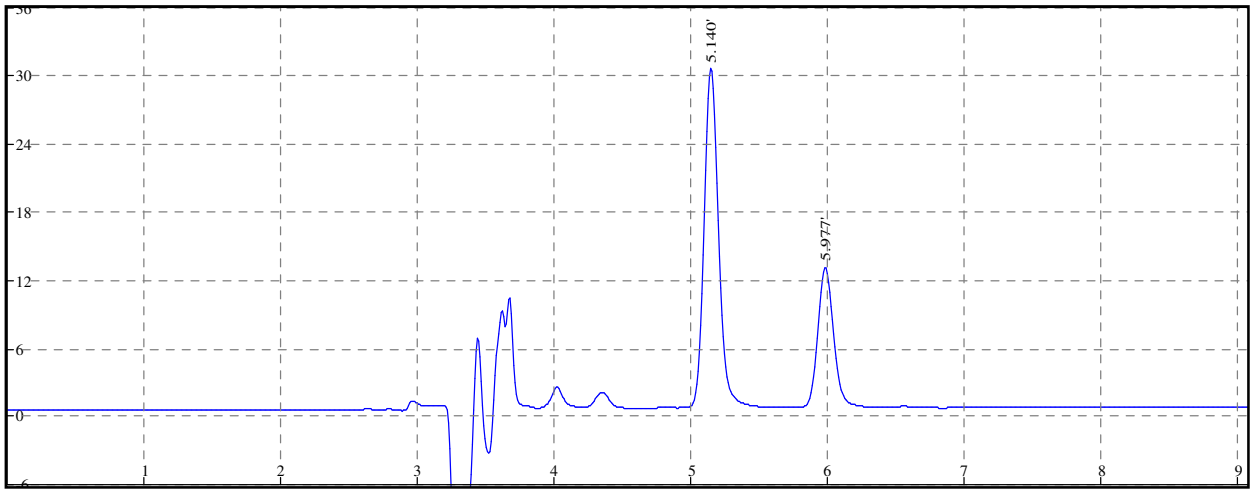


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.061	445723	2.87	8146	1.22
5.880	176126	0.00	7328	1.20

Sample Name: Dexamethasone HQC 24hrs

Run time: 9.13min

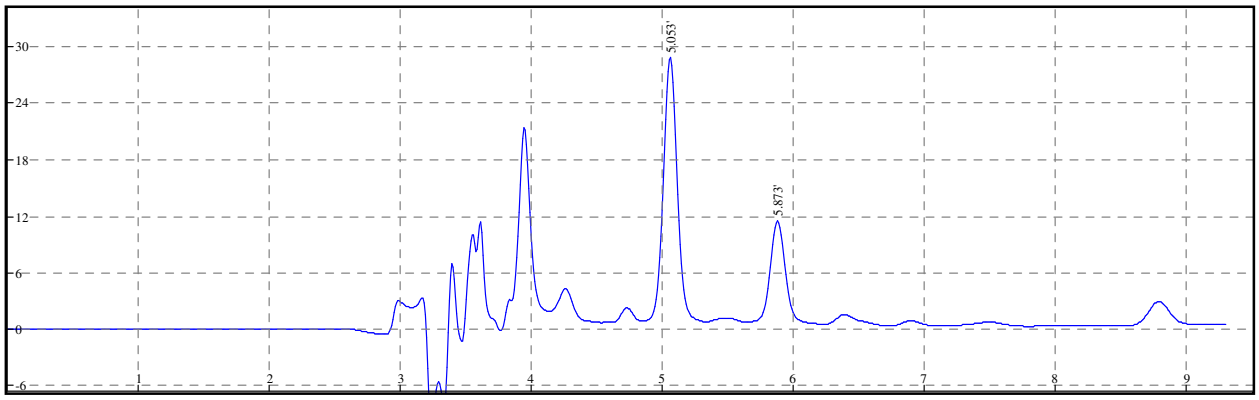


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.140	415491	2.63	4803	1.17
5.977	129790	0.00	6075	1.15

Sample Name: Dexamethasone HQC 48hrs

Run time: 9.30min

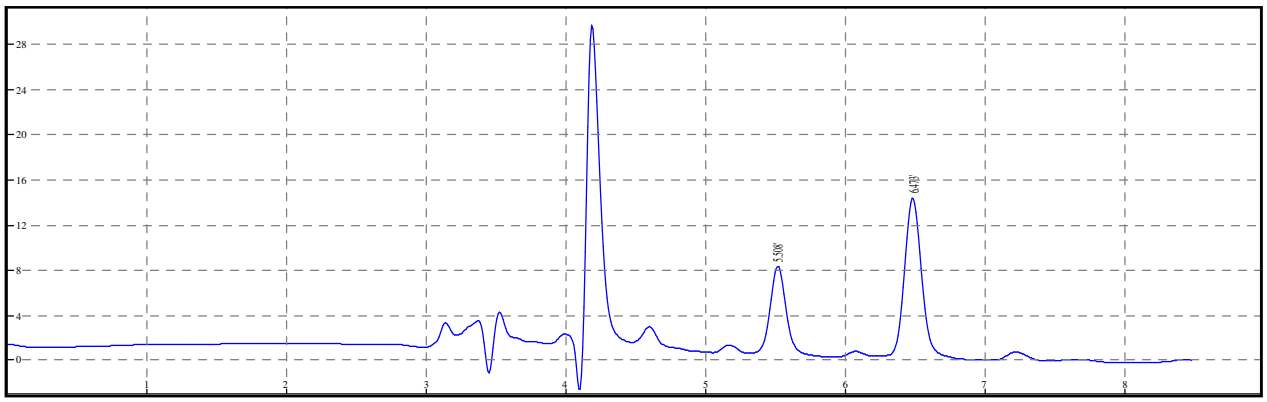


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.053	343934	2.05	3955	1.05
5.873	109710	0.00	2547	1.06

Sample Name: Dexamethasone LQC 12hrs

Run time: 8.47min

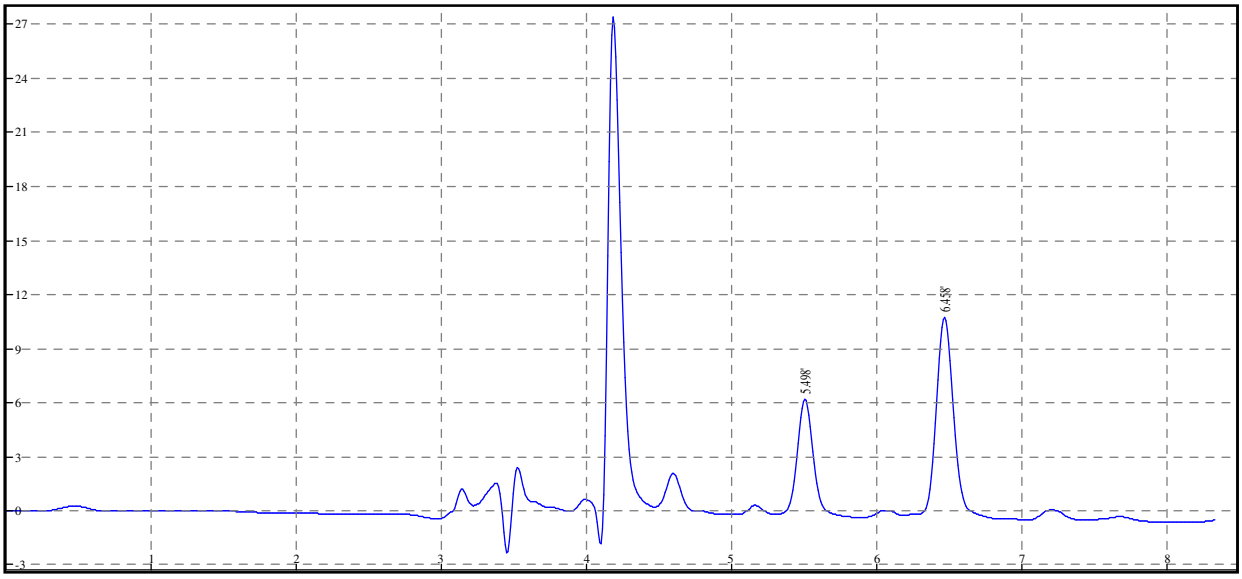


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.508	46109	2.97	4948	1.05
6.473	201594	0.00	5021	1.09

Sample Name: Dexamethasone LQC 24hrs

Run time: 8.33min

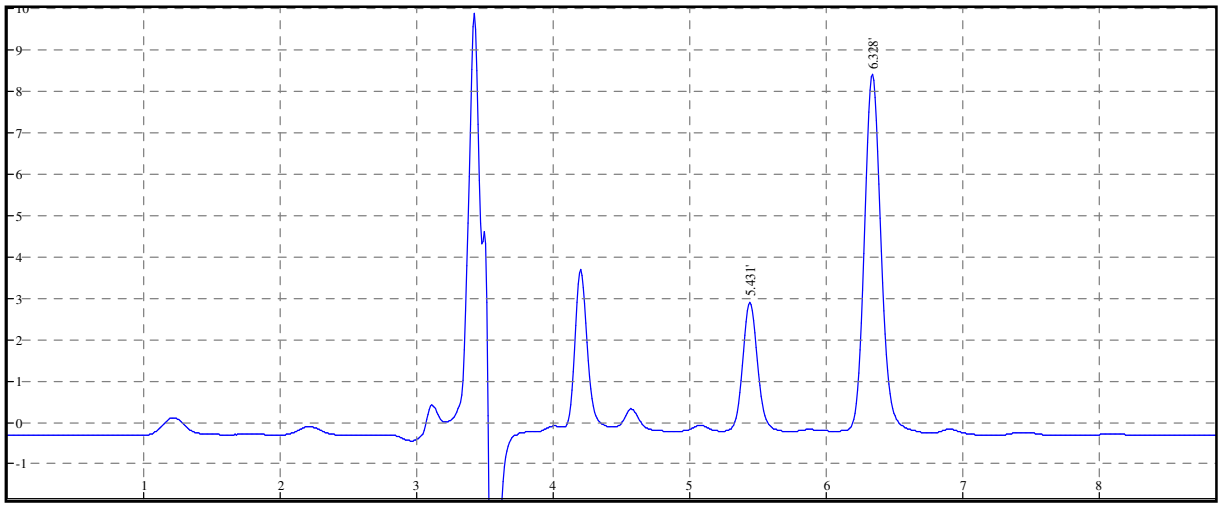


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.498	44512	3.50	5540	1.07
6.458	190566	0.00	5747	1.12

Sample Name: Dexamethasone LQC 48hrs

Run time: 8.99min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.431	17237	1.90	6084	1.08
6.328	123268	0.00	3847	1.13

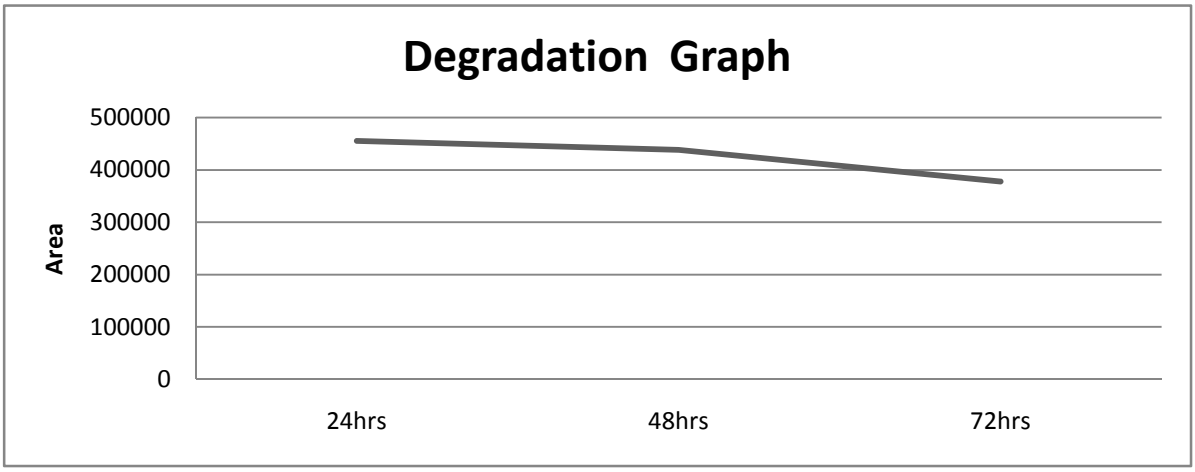
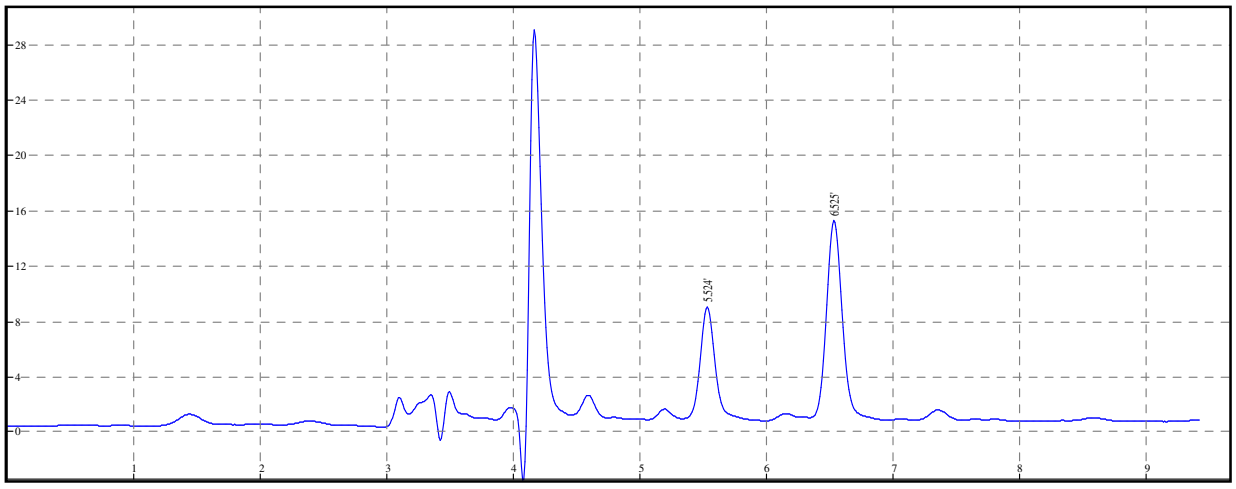


Fig No.6.9: Degradation graph for freeze thaw stability

3. Plane Solution Stability: 24, 48 and 72hrs.

Sample Name: Dexamethasone LQC Day 1

Run time: 9.42min

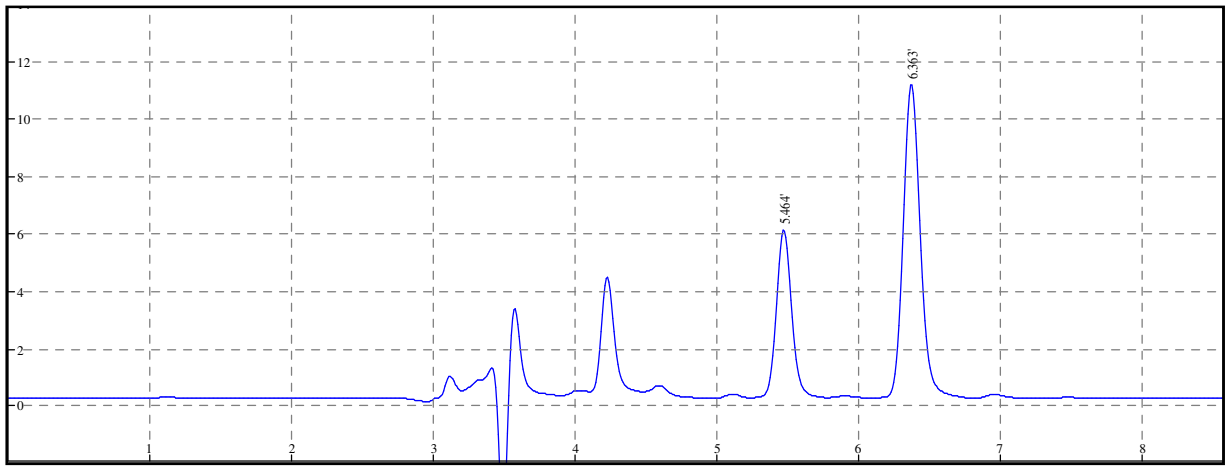


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.524	155631	2.58	4574	1.14
6.525	290156	0.00	6350	1.11

Sample Name: Dexamethasone LQC Day 2

Run time: 8.66min

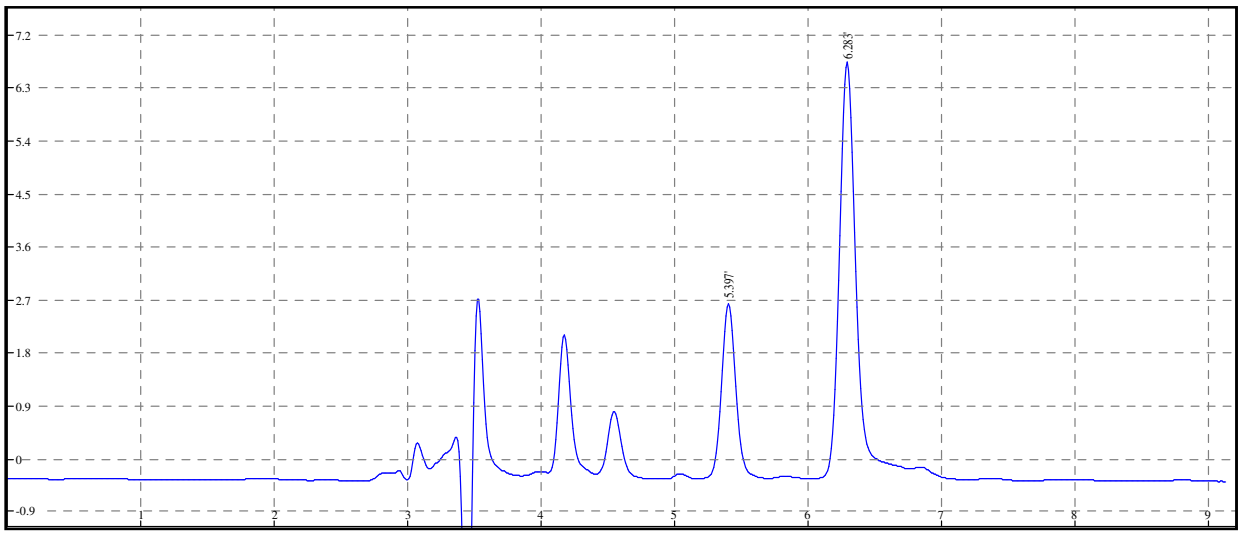


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.464	133670	2.37	3025	1.12
6.363	276223	0.00	5315	1.14

Sample Name: Dexamethasone LQC Day 3

Run time: 9.13min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
5.397	102667	1.85	4909	1.09
6.283	259437	0.00	3105	1.17

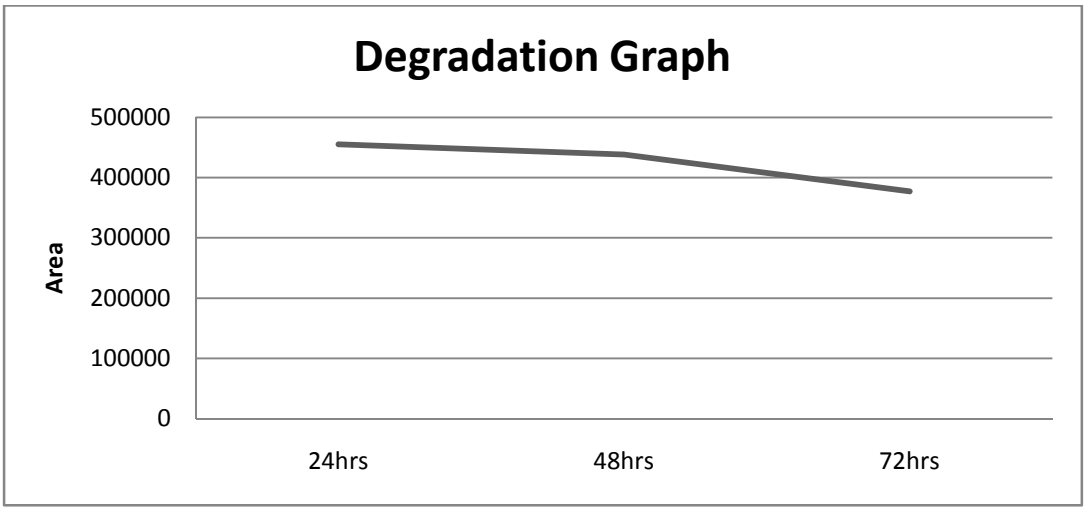


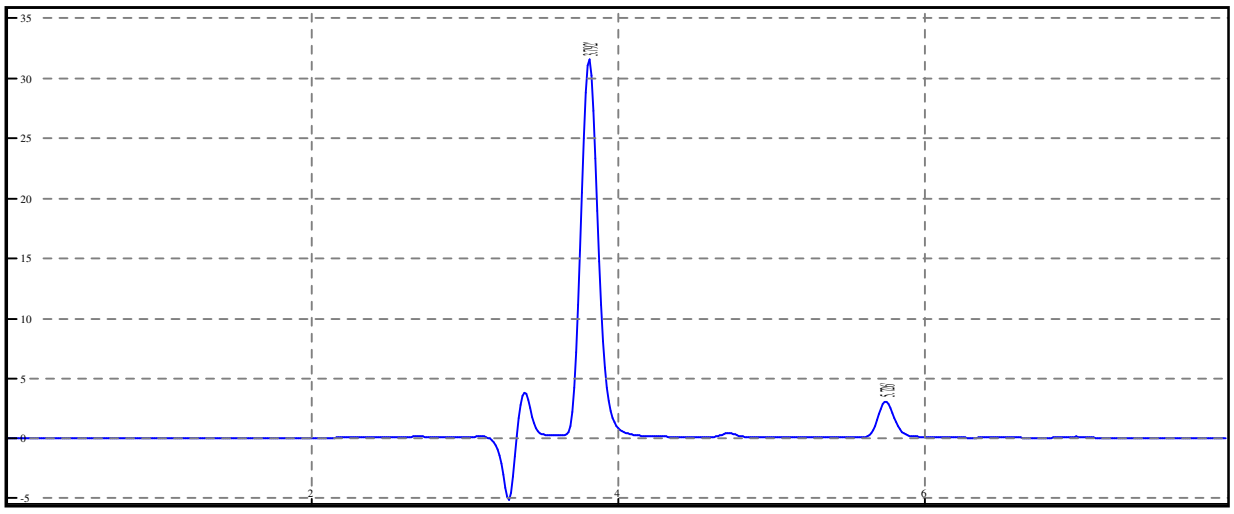
Fig No.6.10: Degradation graph for plane solution stability

Name of Drug: Prednisolone

Trial graphs for Prednisolone

Sample name: Prednisolone+IS Trial

Run time: 7.96min

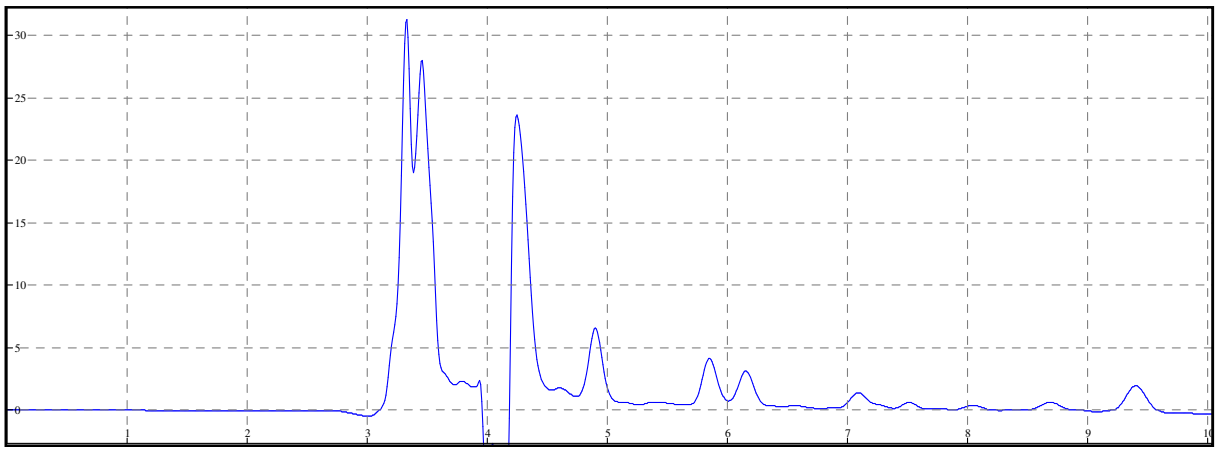


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.792	426124	2.87	5325	1.17
5.726	15389	0.00	6464	1.20

Sample name: Blank Plasma Trial

Run time: 11.23min



Selectivity

The desired method used RP-HPLC method for separation of Prednisolone from Metformin (IS) as well as plasma sample and was shown to be selective for the analyte and its IS(retention times for prednisolone and metformin were 5.24 and 6.11 minutes respectively).No interfering peaks were observed with the same retention time of the analyte, when different plasma samples were analysed. The chromatogram of blank plasma with IS is given below.

Chromatographic conditions

Sample Name: Blank Plasma+ Metformin

Wavelength: 238nm

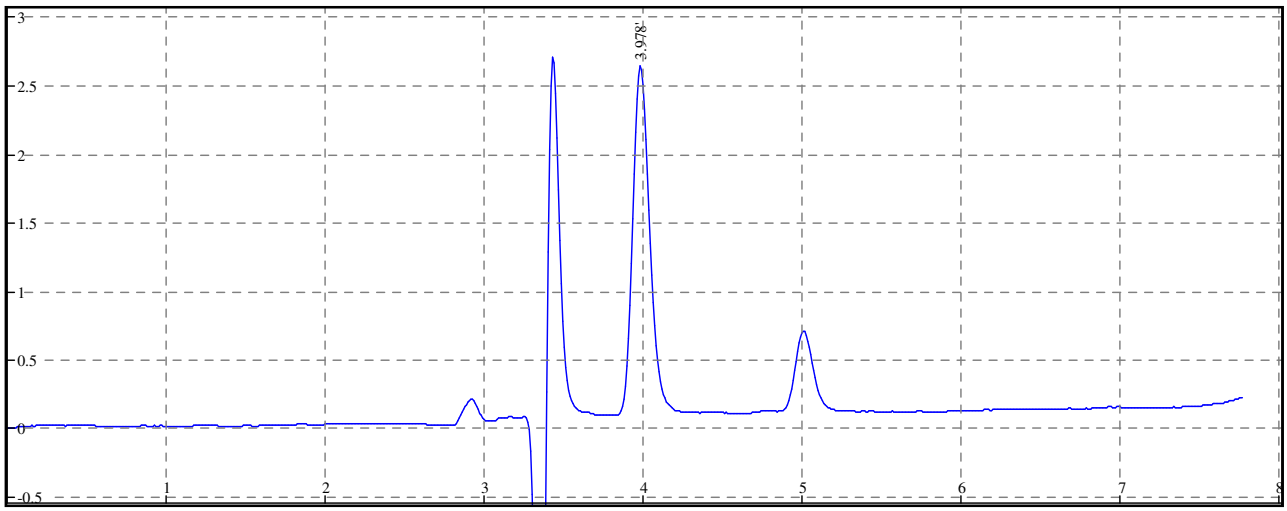
Mobile Phase: Methanol:Water (70:30)

Sample volume: 100µl

Flow rate: 0.9 ml/min

Pressure: 10-11MPa

Run time: 7.77min



Observation Table

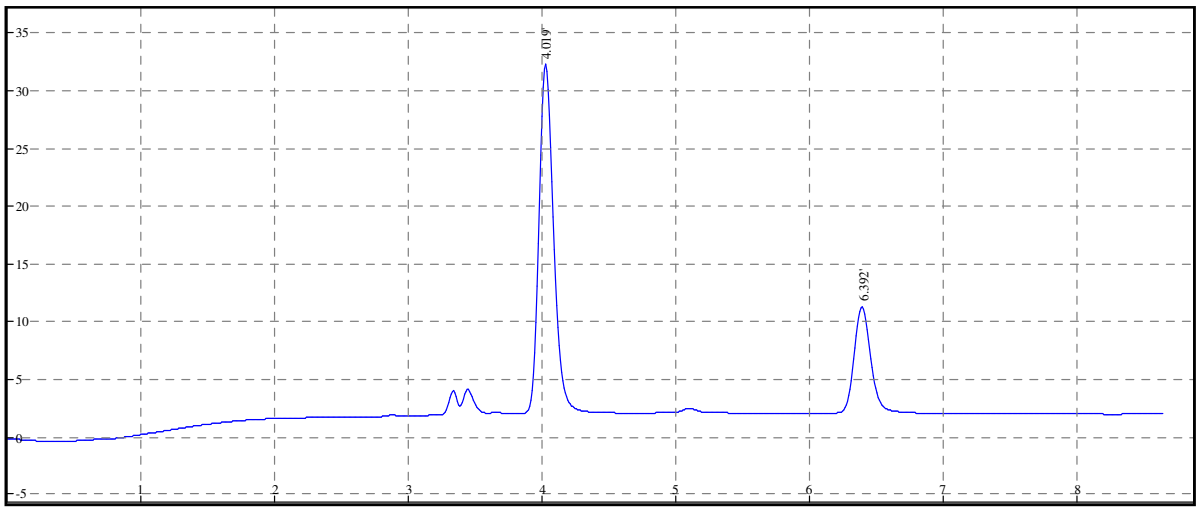
Time	Area	Resolution	T.Plate no.	Asymmetry
3.978	9129	0	13603	1.22

Linearity

Linearity was demonstrated by taking sample concentrations ranges from 100-1600 ng/ml. Fig. given below shows calibration curve of prednisolone.The calibration curve includes 6 calibration standards which are distributed 0.999 with goodness of fit.

Prednisolone Std A

Run time: 8.64min

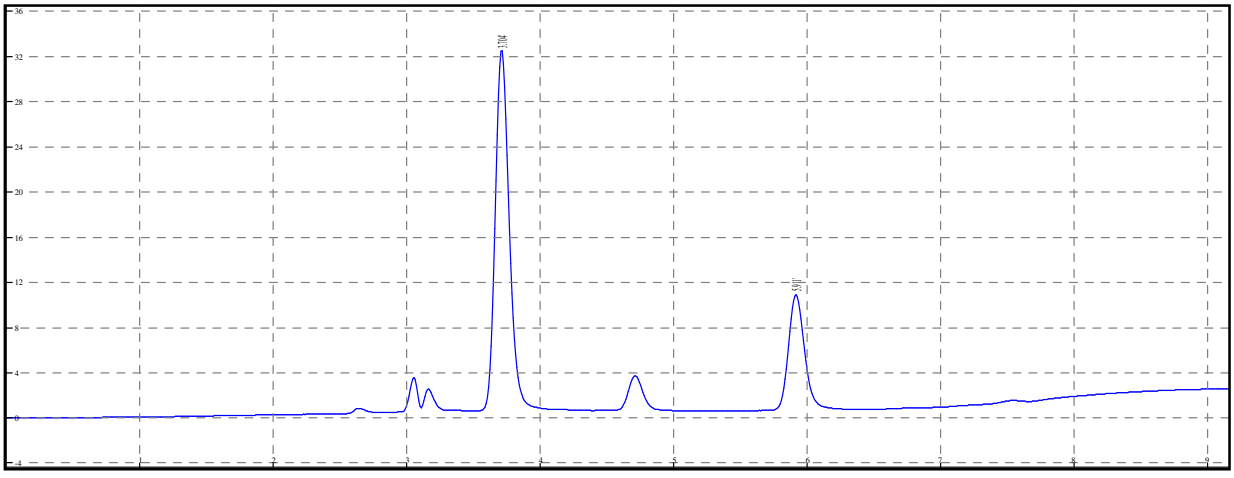


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.019	380427	2.69	5352	1.24
6.392	71931	0.00	36513	1.20

Prednisolone Std B

Run time: 9.27min

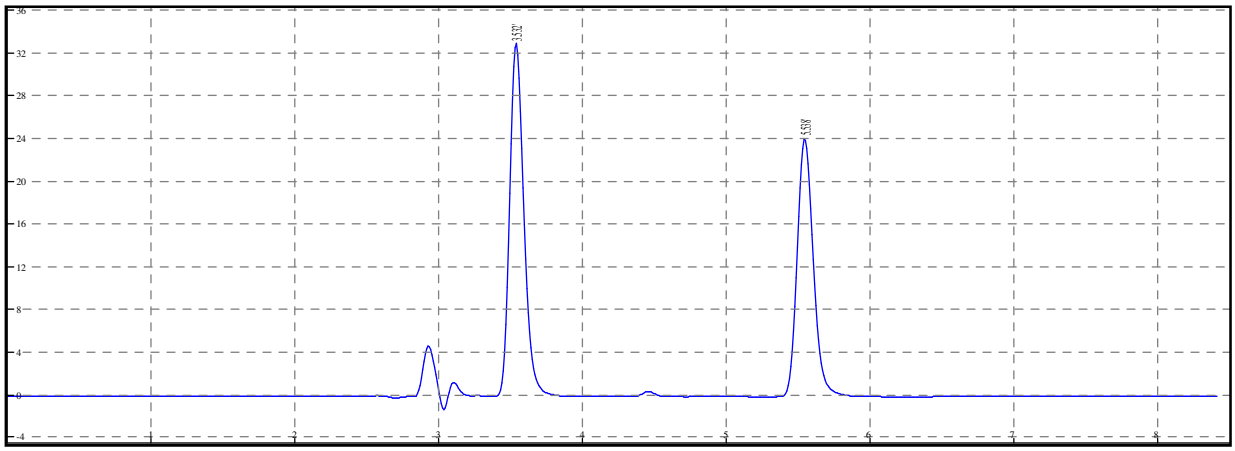


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.704	377156	4.36	5860	1.23
5.911	72917	0.00	18624	1.19

Prednisolone Std C

Run time: 8.41min

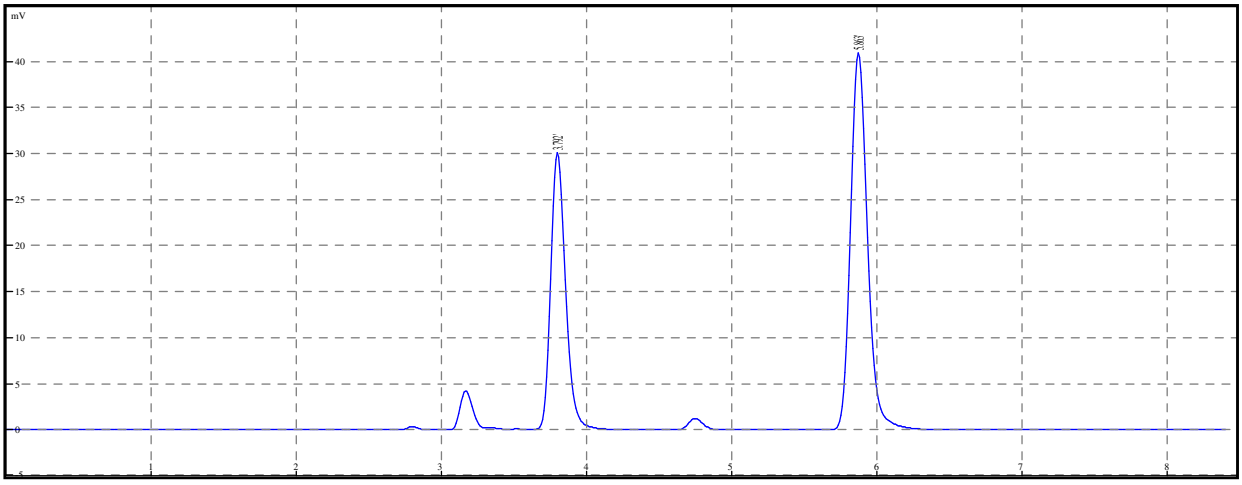


Observation Table

Time	**Area**	**Resolution**	**T.Plate no.**	**Asymmetry**
3.532	379578	9.36	4343	1.24
5.538	119288	0.00	11392	1.19

Prednisolone Std D

Run time: 8.40min

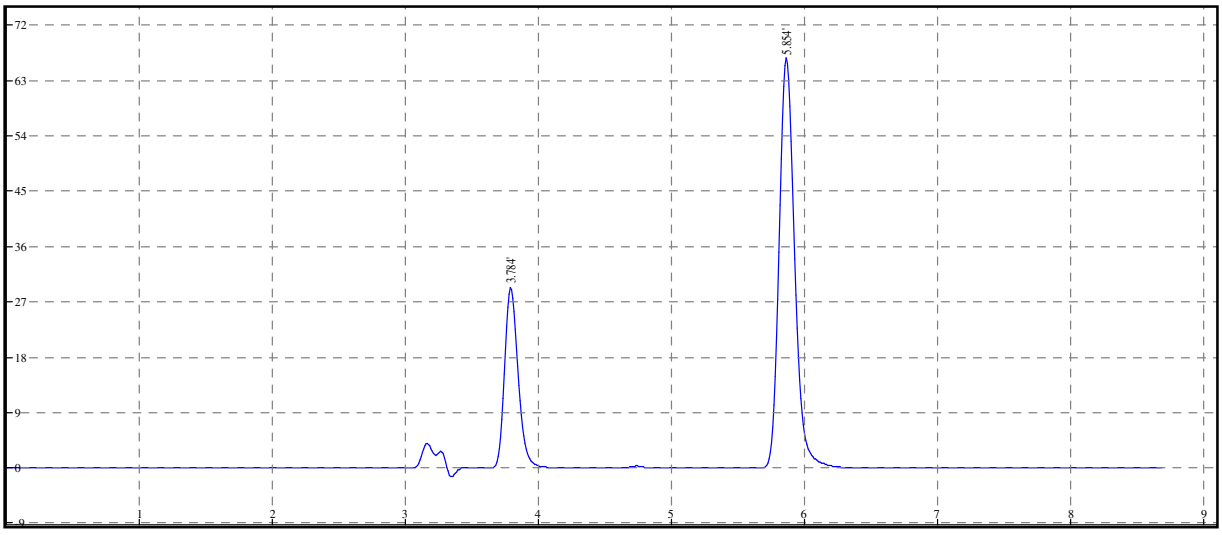


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.792	385671	10.11	6351	1.25
5.863	363909	0.00	12118	1.21

Prednisolone Std E

Run time: 8.68min

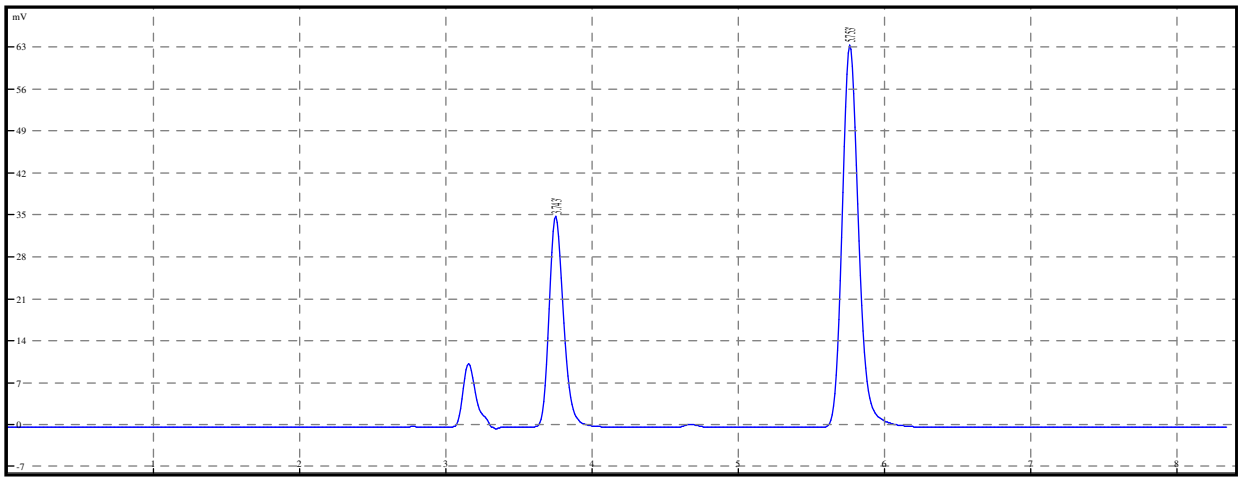


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.784	378107	9.70	5353	1.25
5.854	724731	0.00	12035	1.21

Prednisolone Std F

Run time: 8.34min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.743	373594	9.86	5943	1.24
5.753	965731	0.00	12329	1.20

Table No.6.7 Observation table for Linearity graph

Sample Name	Concentration ratio of sample and internal standard	Area ratio of sample and internal standard
Std-A	0.125	0.1891
Std-B	0.125	0.1891
Std-C	0.25	0.3379
Std-D	0.75	0.939
Std-E	1.5	1.9581
Std-F	2.0	2.5849

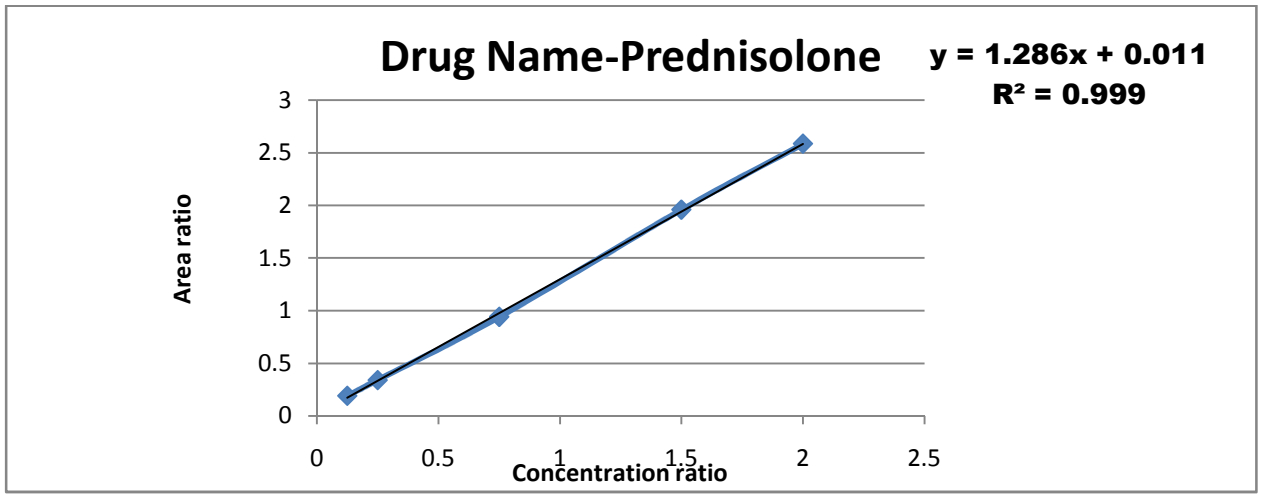


Fig No.6.11: Linearity graph

(Limit: The R^2 value should be near to 1)

LLOQ (Lower limit of quantification)

Chromatographic conditions

Sample Name: Prednisolone 50ng/ml + Metformin 800ng/ml

Wavelength: 238nm

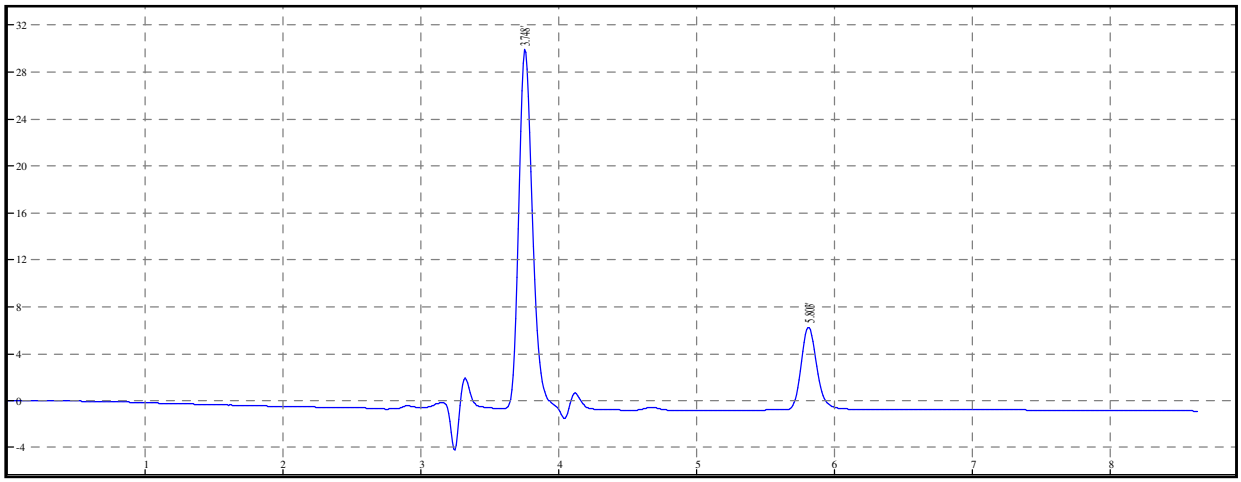
Mobile Phase: Methanol:Water (70:30)

Sample volume: 100µl

Flow rate: 0.9 ml/min

Pressure: 10-11MPa

Run time: 8.63min



Observation Table

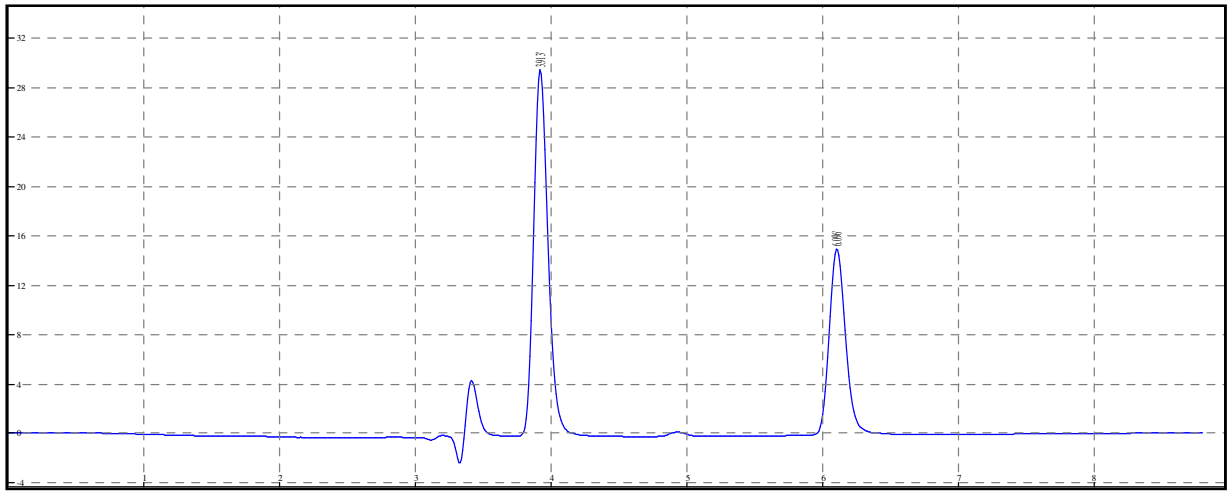
Time	Area	Resolution	T.Plate no.	Asymmetry
5.249	409355	4.25	15869	1.14
6.114	193475	0.00	13267	1.16

Accuracy and Precision

Accuracy and Precision was evaluated by analyzing 5 batches, each batch consist of five replicates of LQC, MQC and HQC.

Sample name: Prednisolone LQC 01

Run time: 8.79min

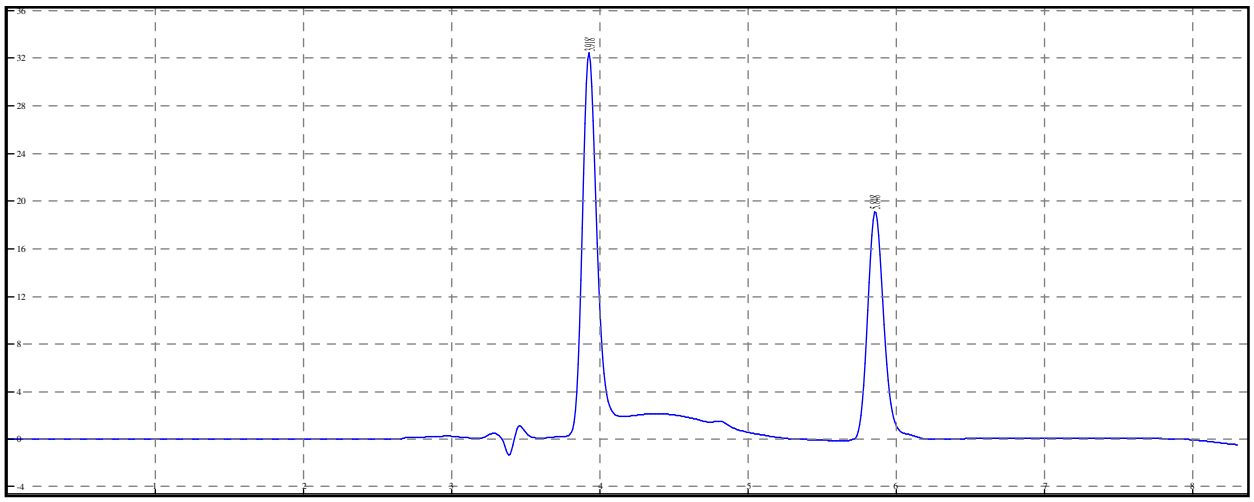


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.913	348898	9.87	5550	1.23
6.096	110496	0.00	11707	1.20

Sample name: Prednisolone LQC 02

Run time: 8.30min

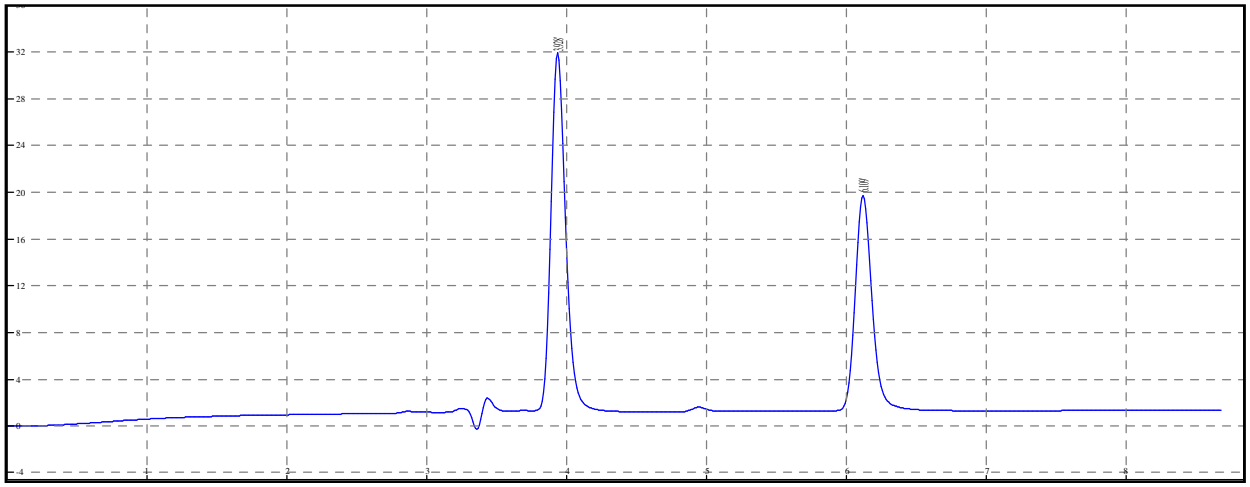


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.918	385116	6.62	6121	1.25
5.848	109219	0.00	9990	1.20

Sample name: Prednisolone LQC 03

Run time: 8.68min

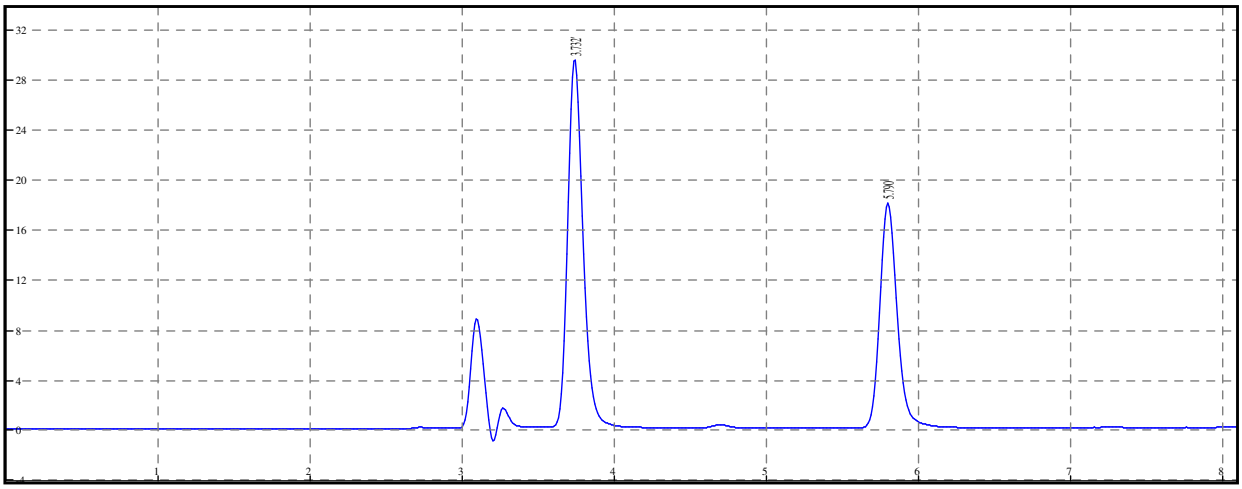


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.928	356704	9.65	5948	1.25
6.109	116464	0.00	12145	1.21

Sample name: Prednisolone LQC 04

Run time: 8.21min

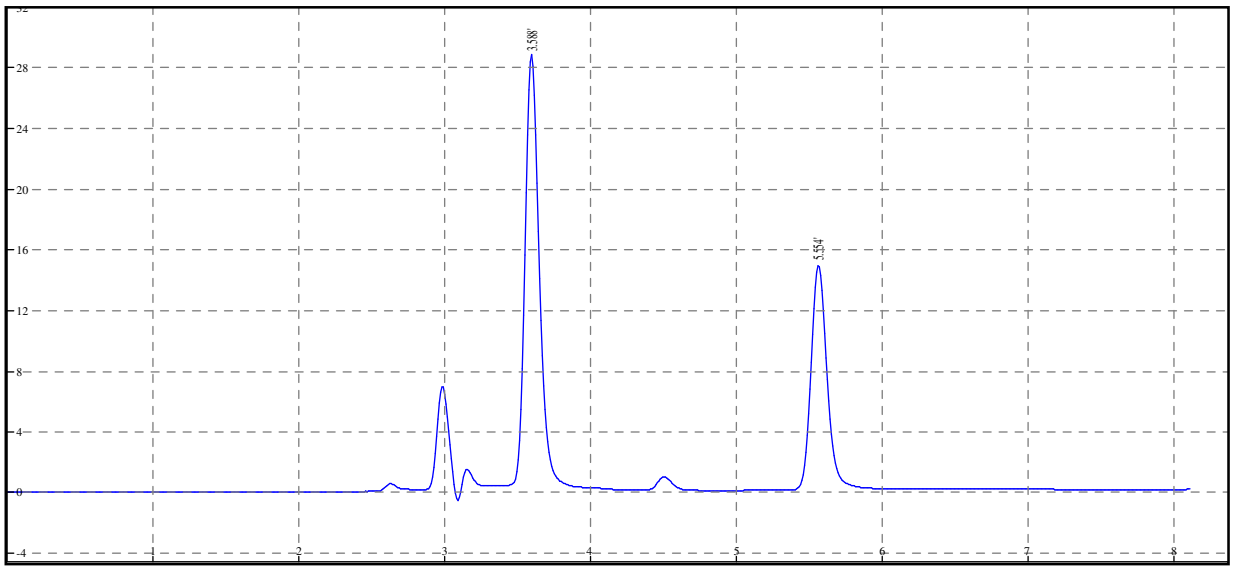


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.732	342955	9.40	4815	1.24
5.790	125316	0.00	11468	1.20

Sample name: Prednisolone LQC 05

Run time: 8.11min

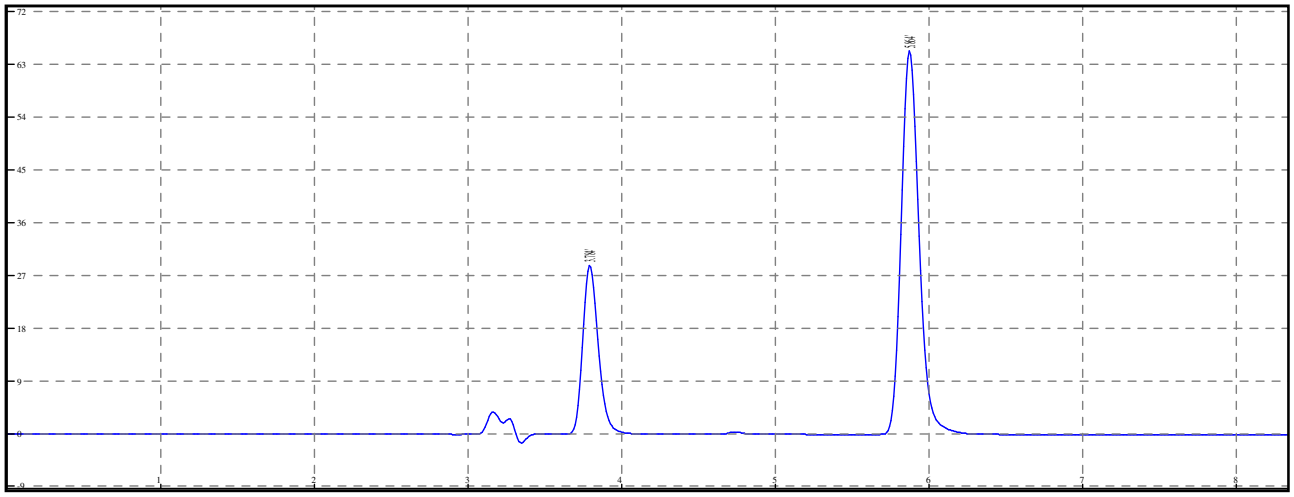


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.588	332534	9.59	4989	1.23
5.554	113361	0.00	12308	1.20

Sample name: Prednisolone MQC 01

Run time: 8.56min

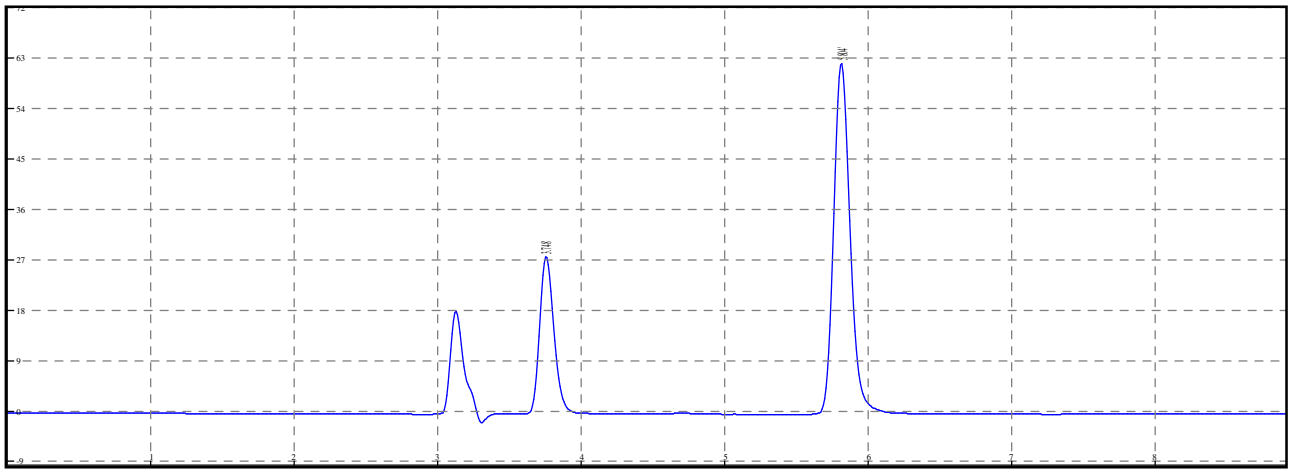


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.784	384887	8.98	3908	1.25
5.864	726783	0.00	12004	1.20

Sample name: Prednisolone MQC 02

Run time: 9.00min

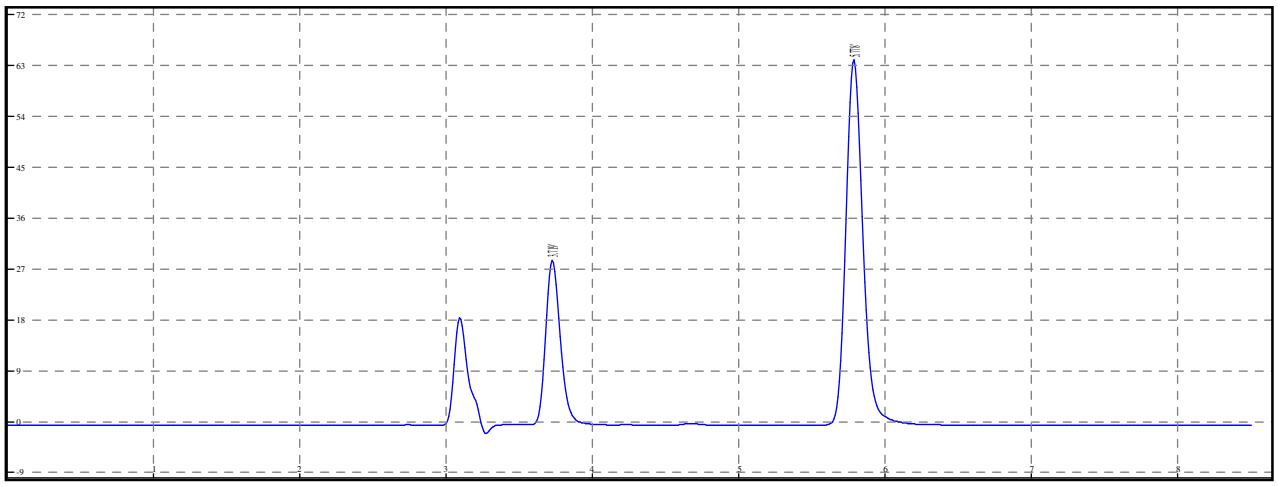


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.748	375206	7.79	2683	1.25
5.804	730001	0.00	10158	1.20

Sample name: Prednisolone MQC 03

Run time: 8.50min

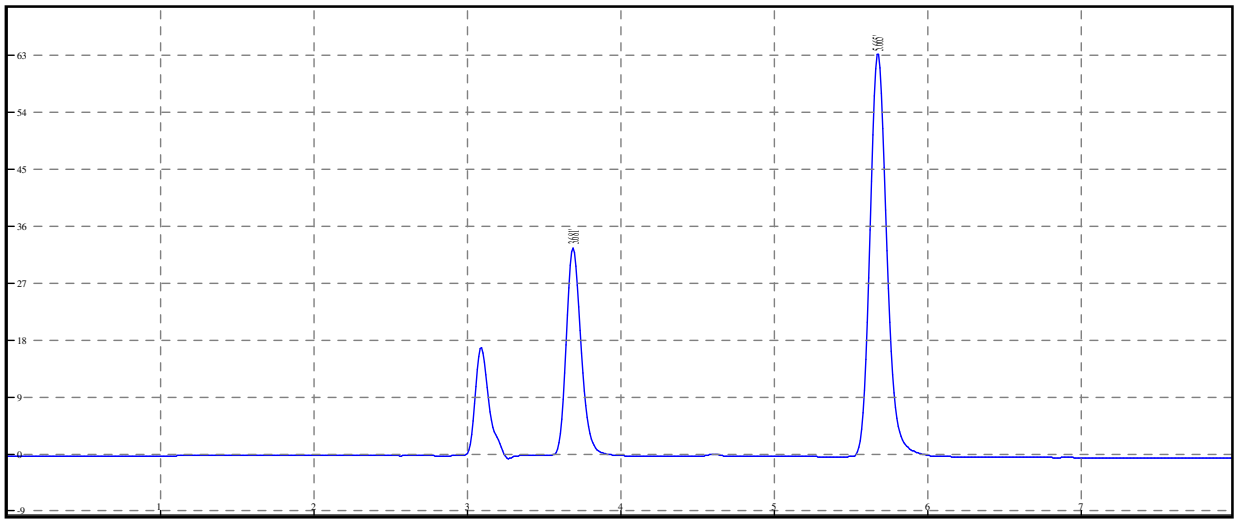


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.719	400394	9.70	5266	1.24
5.778	715705	0.00	11779	1.20

Sample name: Prednisolone MQC 04

Run time: 8.33min

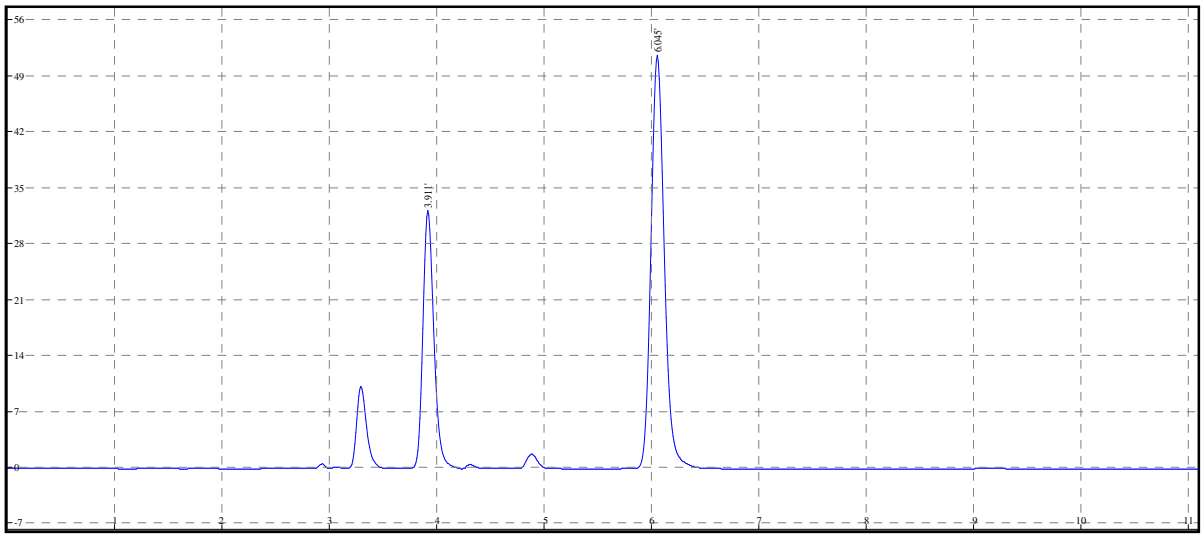


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.681	395676	9.49	5025	1.23
5.665	720409	0.00	12314	1.20

Sample name: Prednisolone MQC 05

Run time: 11.99min

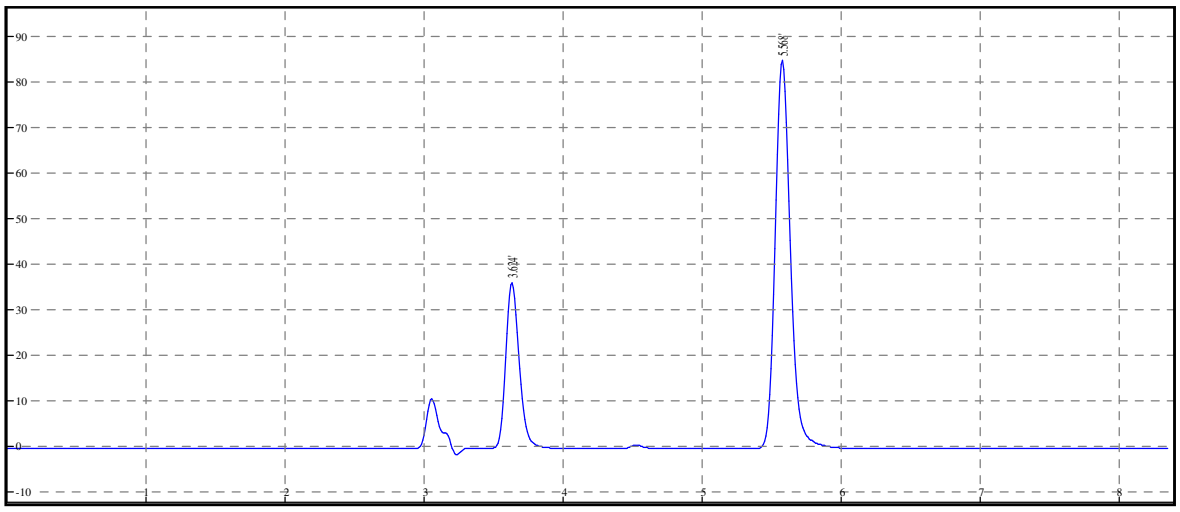


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.911	471747	10.09	6947	1.24
6.045	703480	0.00	11158	1.18

Sample name: Prednisolone HQC 01

Run time: 8.35min

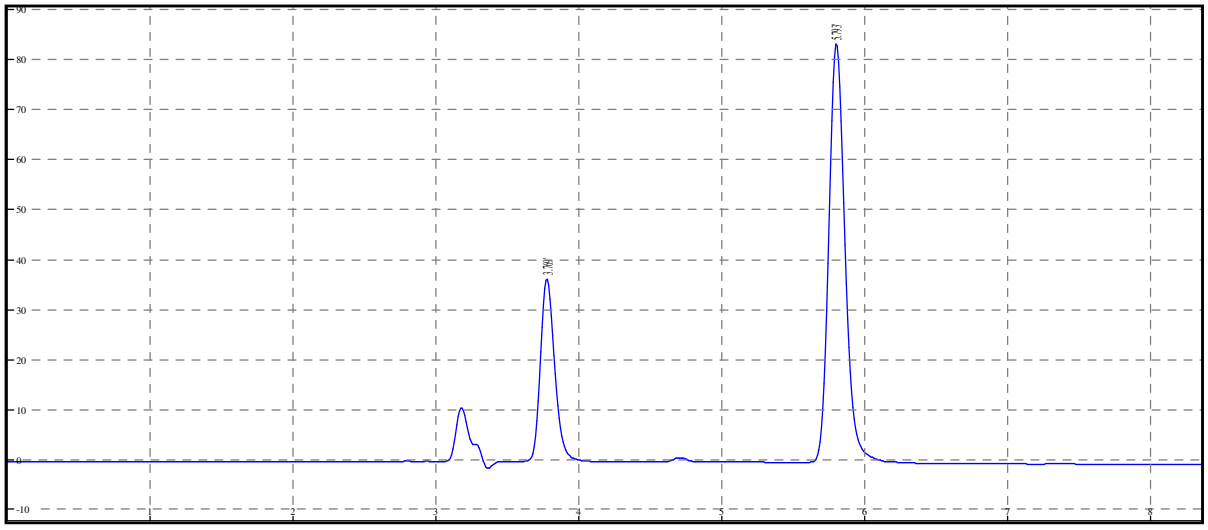


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.624	391930	7.98	3009	1.24
5.568	1208046	0.00	10702	1.20

Sample name: Prednisolone HQC 02

Run time: 8.35min

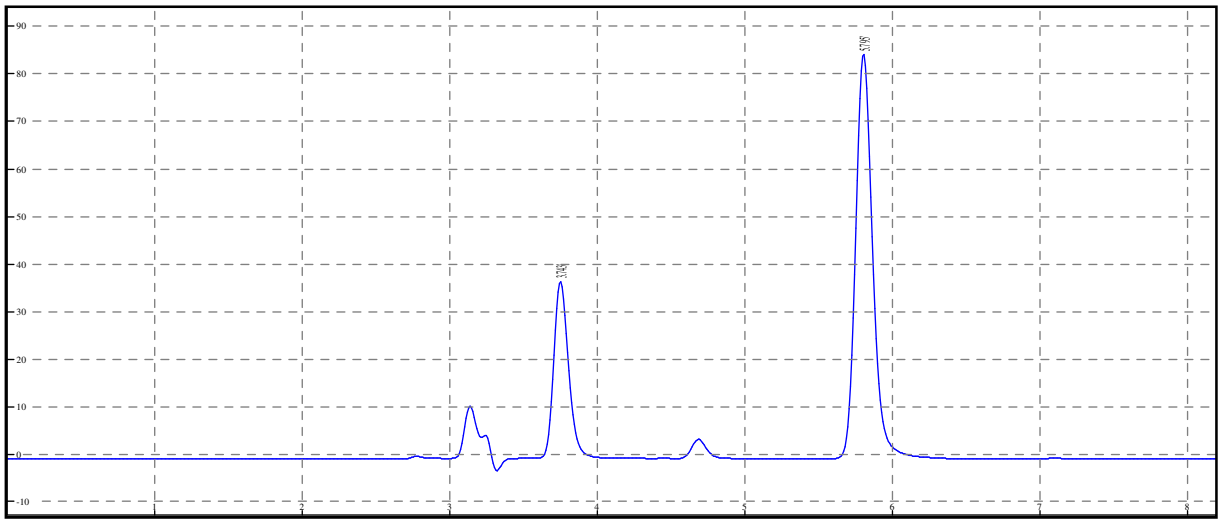


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.769	442159	8.13	3113	1.24
5.793	1213110	0.00	11105	1.19

Sample name: Prednisolone HQC 03

Run time: 8.34min

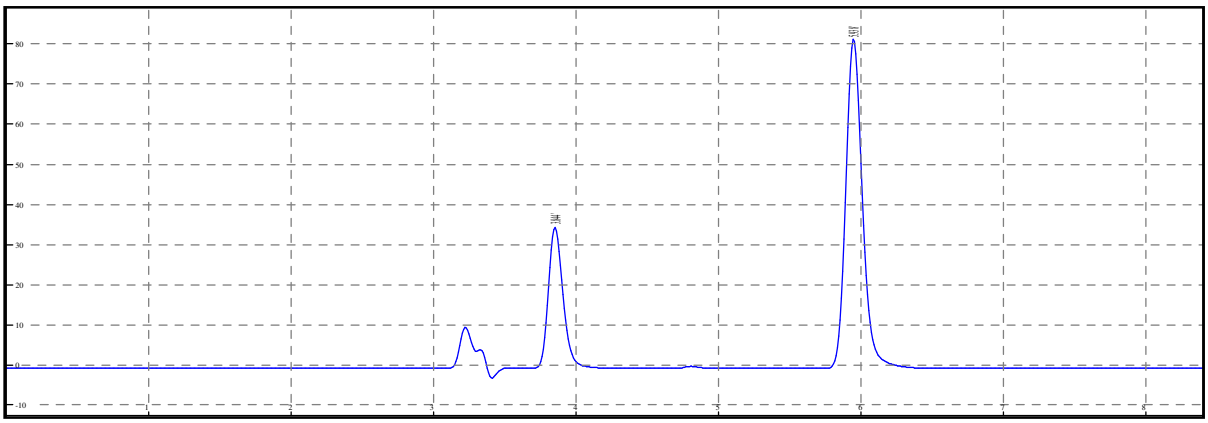


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.743	341324	9.28	4789	1.24
5.795	1218084	0.00	11205	1.19

Sample name: Prednisolone HQC 04

Run time: 8.40min

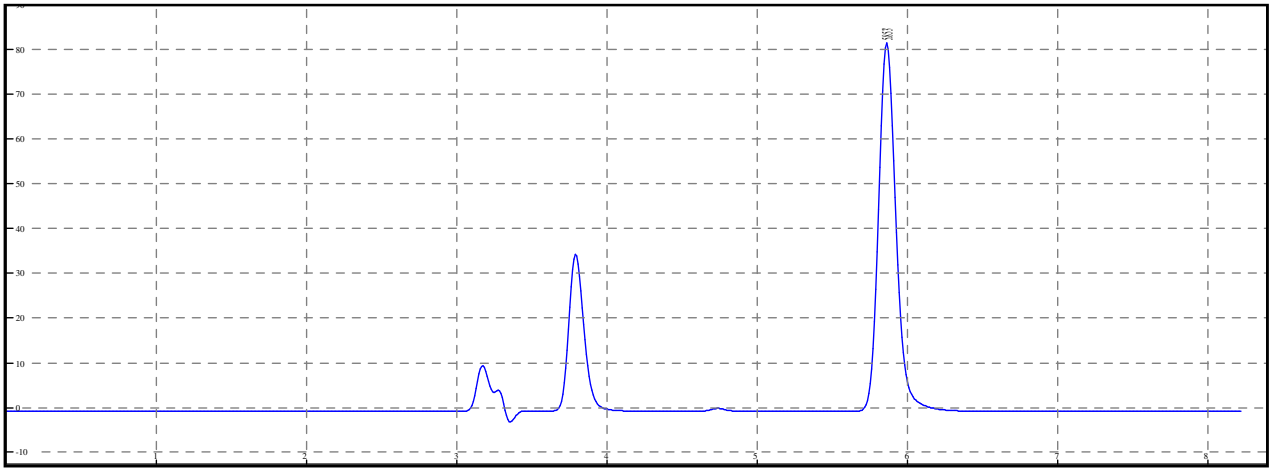


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.844	432692	7.44	6297	1.25
5.938	1244250	0.00	10328	1.20

Sample name: Prednisolone HQC 05

Run time: 8.22min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.874	407266	4.21	6785	1.16
5.853	1184250	0.00	9464	1.19

The precision and accuracy of the method for each concentration levels are represented in following Table.

Table No.6.8 Observation Table for Accuracy & Precision

			Standard Deviation		Accuracy	Precision
Conc.	**Conc.**	**Area**	**Mean**	**SD**	**%SD**	**%RSD**
LQC	0.187	0.3167	0.32662	0.0302363	9.257349	9.257349
	0.187	0.2836				
	0.187	0.3265				
	0.187	0.3654				
	0.187	0.3409				
MQC	1.5	1.8883	1.78666	0.176094	9.85604	9.85604
	1.5	1.9456				
	1.5	1.7875				
	1.5	1.8207				
	1.5	1.49122				
HQC	2.5	3.0823				

	2.5	2.7436	3.0356	0.321548	10.5925	10.5925
	2.5	3.5687				
	2.5	2.8756				
	2.5	2.9078				

(Standard Limit-%SD & % RSD should be less than 15%)

Table No.6.9 Observation table for Coefficient variance (CV)

Sr.No	Concentration	Concentration ratio	Coefficient Varience
1	LQC	0.1875	0.173886
2	MQC	1.5	0.419635
3	HQC	2.5	0.567052

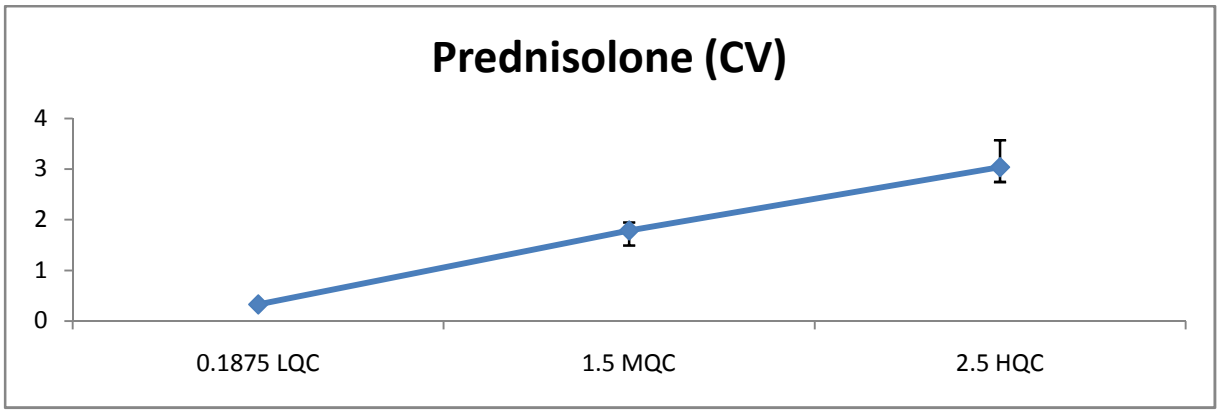


Fig No.6.12: coefficient variance

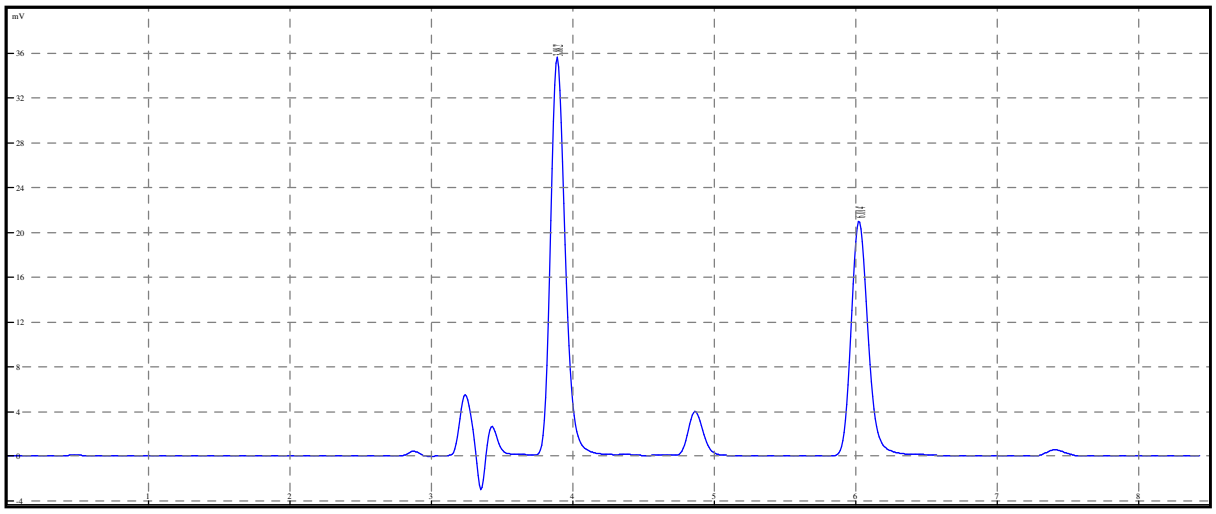
Recovery

The recovery was evaluated by comparing response of extracted and unextracted samples.The average recovery for Prednisolone in plasma was found to around 74.2%.

Unextracted sample

Sample name: Prednisolone LQC

Run time: 8.43min



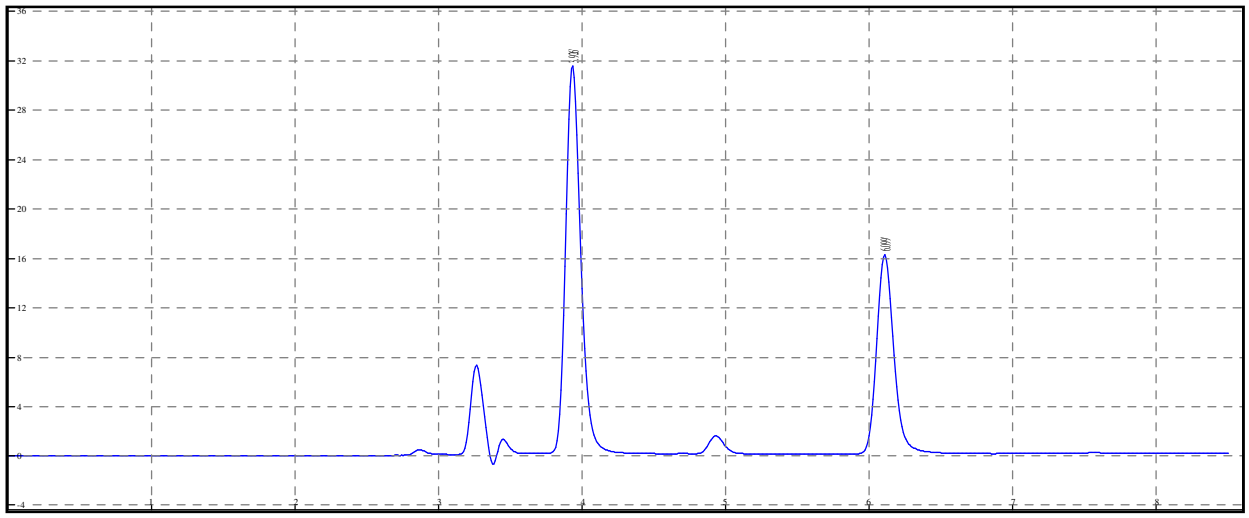
Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.882	454439	8.99	4028	1.24
6.014	178571	0.00	11698	1.20

Extracted sample

Sample name: Prednisolone LQC

Run time: 8.51min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.926	389472	10.28	6374	1.24
6.099	115389	0.00	12325	1.20

For stability study

Stability studies were performed to evaluate the stability of Prednisolone both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include bench top stability, freeze thaw stability, long term and short term stock stability. Prednisolone was found to be stable for three freeze and thaw cycles.

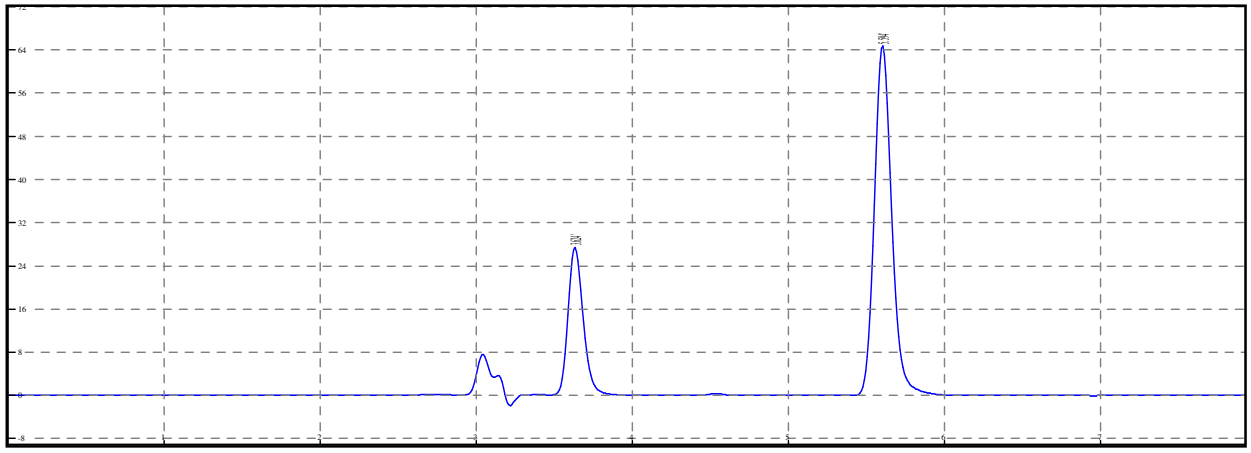
1. Bench top Stability:

A. Short Term Analysis: 2, 12 and 24hrs.

B. Long Tem Analysis: 10, 20 and 30days

Sample name: Prednisolone HQC 2hrs

Run time: 8.19min

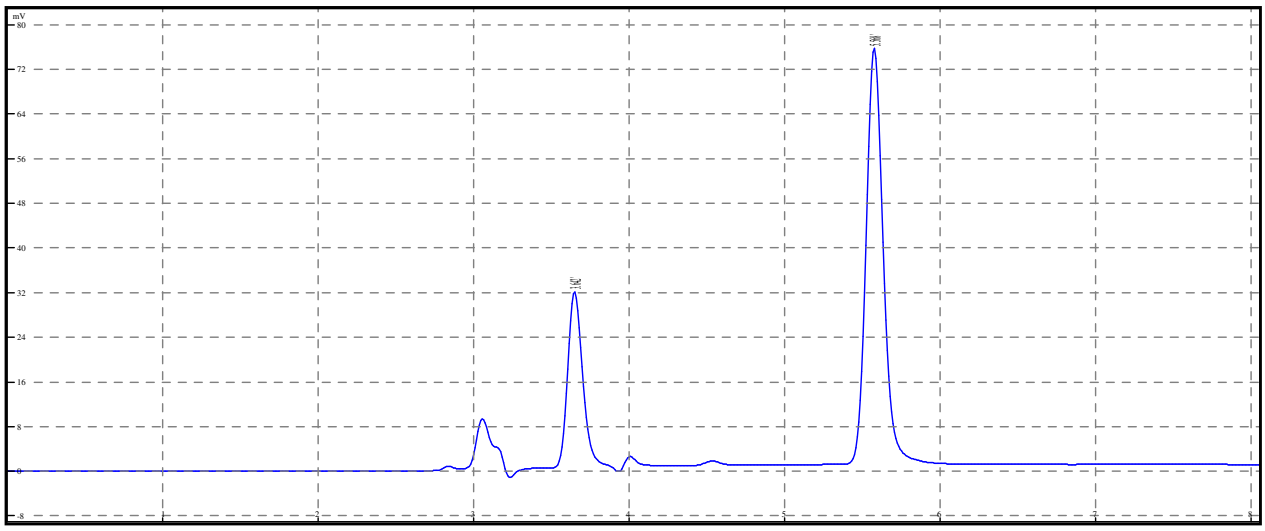


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.624	373538	9.50	5039	1.23
5.594	1189706	0.00	12028	1.17

Sample name: Prednisolone HQC 12hrs

Run time: 8.38min

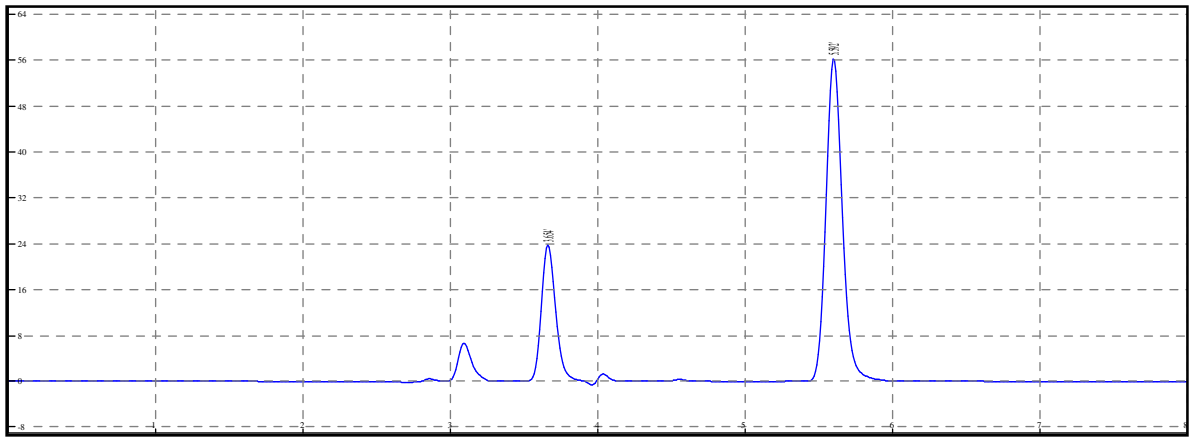


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.642	326700	8.77	4468	1.36
5.568	1121888	0.00	10765	1.16

Sample name: Prednisolone HQC 24hrs

Run time: 8.51min

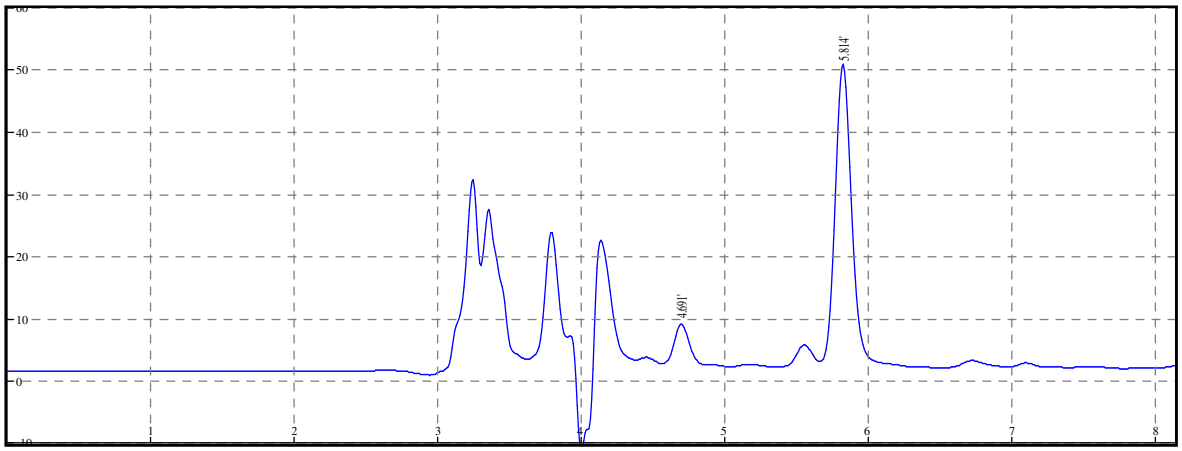


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.654	298069	9.66	5933	1.22
5.592	1089828	0.00	11870	1.17

Sample name: Prednisolone HQC 10days

Run time: 8.50min

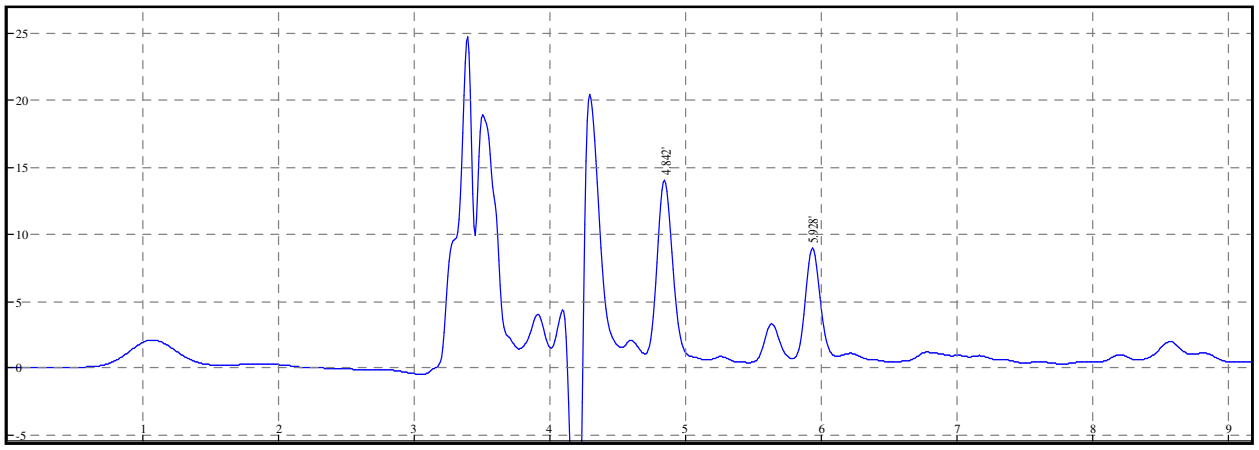


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.691	272559	2.91	4252	1.07
5.814	961342	0.00	4366	1.14

Sample name: Prednisolone HQC 20days

Run time: 10.22 min

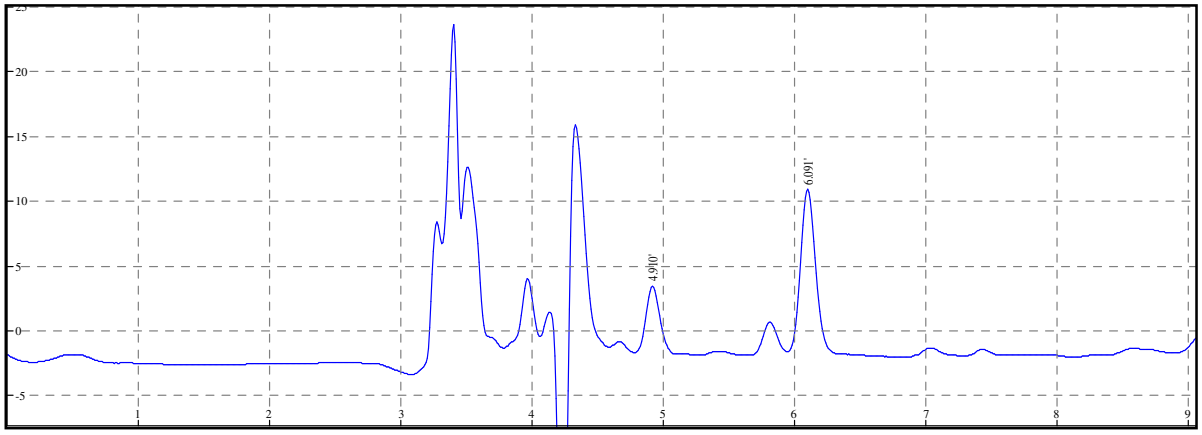


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.842	74481	2.05	6737	1.11
5.928	121900	0.00	7394	1.11

Sample name: Prednisolone HQC 30days

Run time: 9.49min

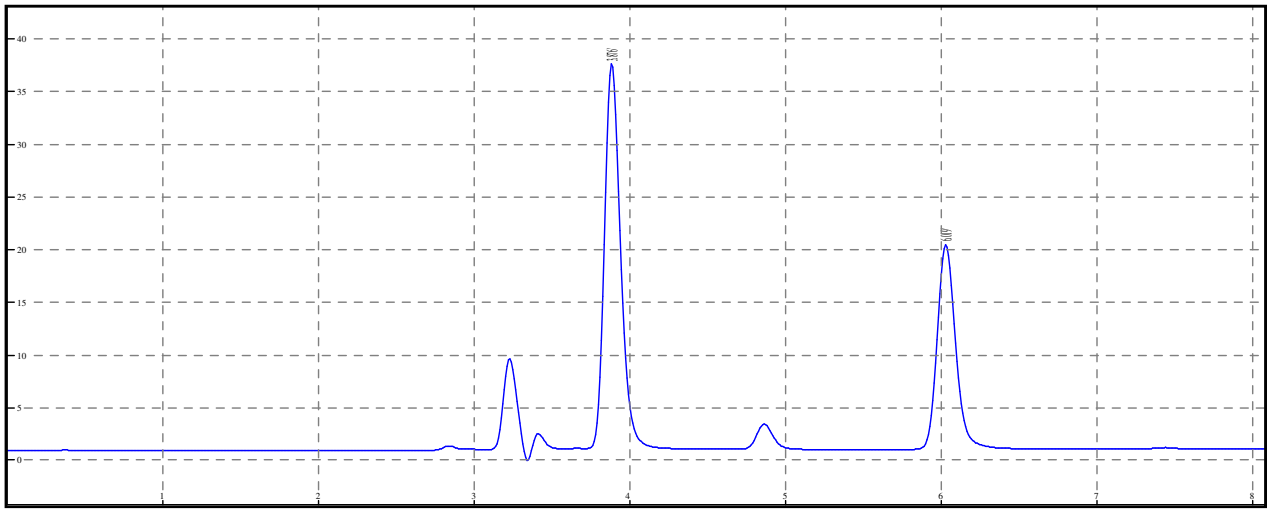


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.910	7006	2.83	3054	1.11
6.091	11777	0.00	4161	1.12

Sample name: Prednisolone LQC 2hrs

Run time: 8.34min

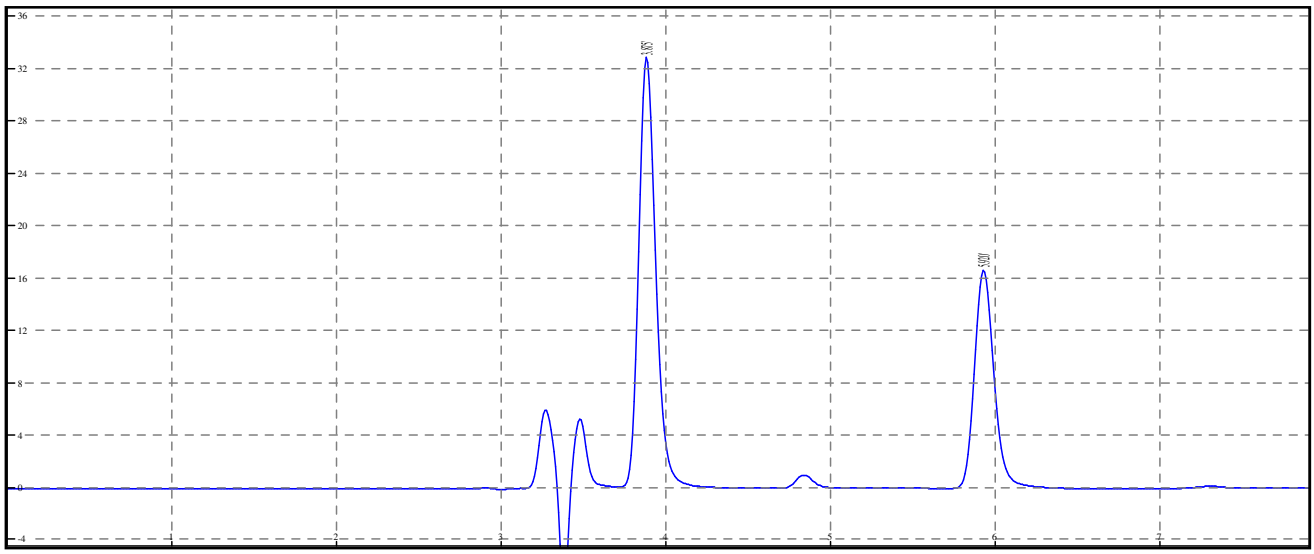


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.876	380350	9.25	6164	1.24
6.019	112361	0.00	10014	1.20

Sample name: Prednisolone LQC 12hrs

Run time: 8.36min

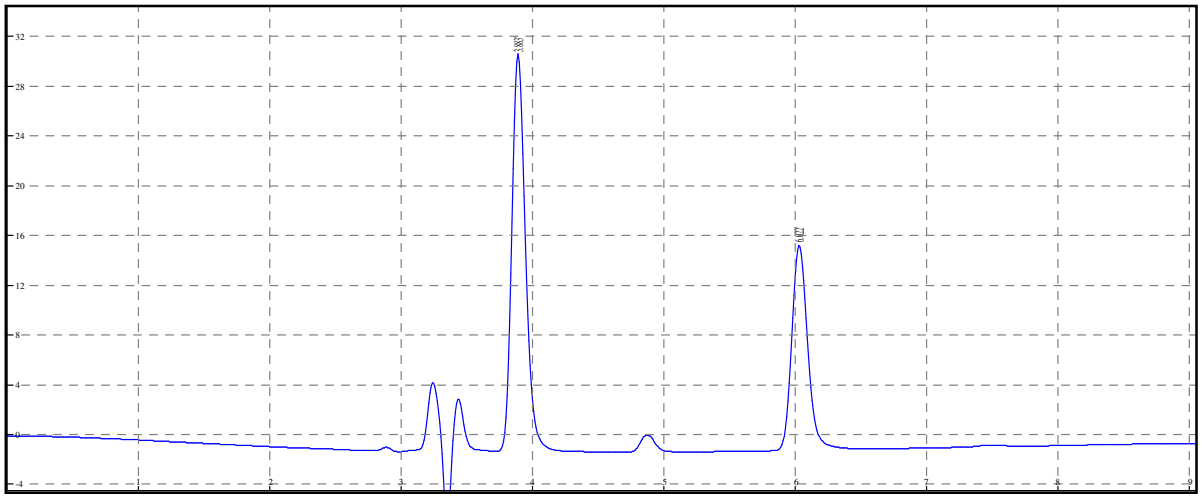


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.875	358827	2.19	5278	1.23
5.920	105470	0.00	4585	1.20

Sample name: Prednisolone LQC 24hrs

Run time: 9.31min

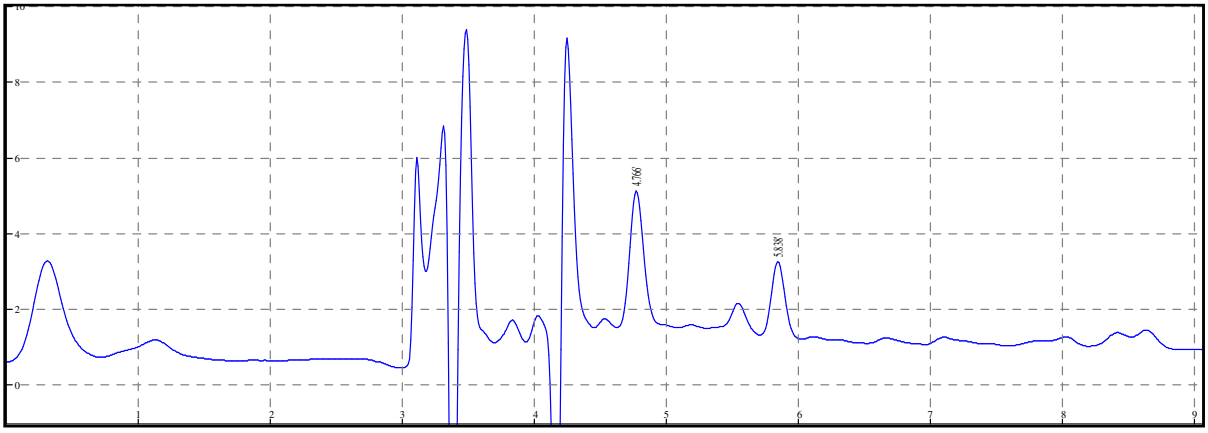


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.883	311170	2.37	6785	1.23
6.022	96105	0.00	5515	1.19

Sample name: Prednisolone LQC 10days

Run time: 14.15min

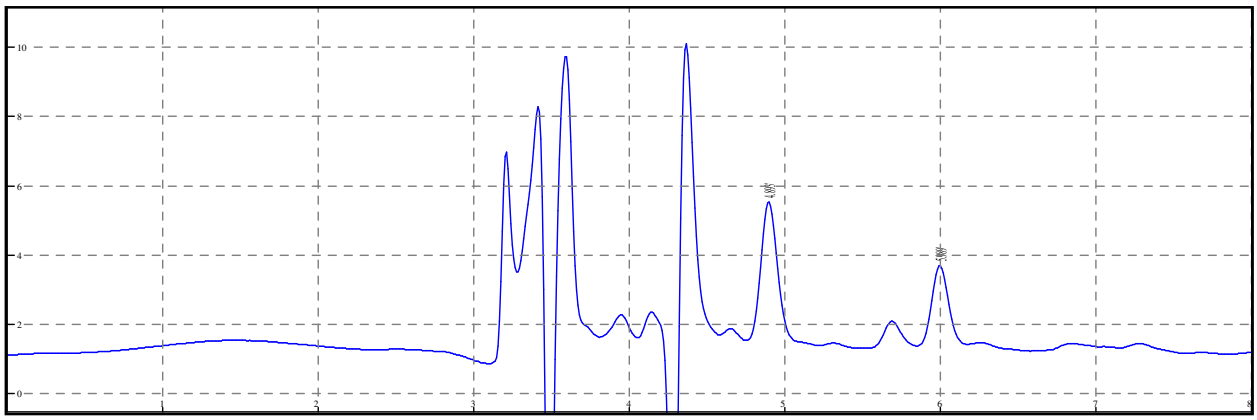


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.766	196949	0.00	2601	1.07
5.838	57932	0.00	4718	1.03

Sample name: Prednisolone LQC 20days

Run time: 8.55min

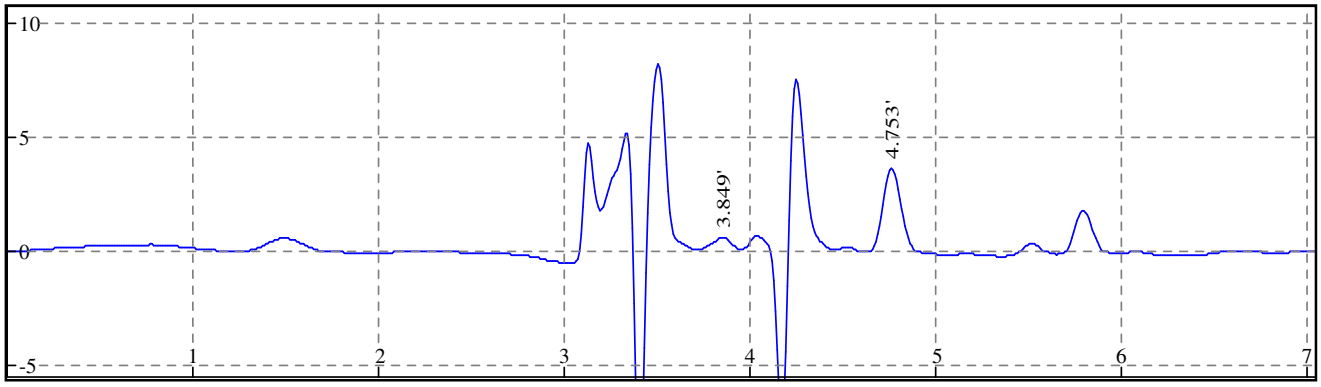


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
4.888	137740	2.01	2337	1.11
5.988	18921	0.00	3344	1.10

Sample name: Prednisolone LQC 30days

Run time: 10.28min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.849	2445	1.80	4977	1.05
4.753	7260	0.00	7859	1.08

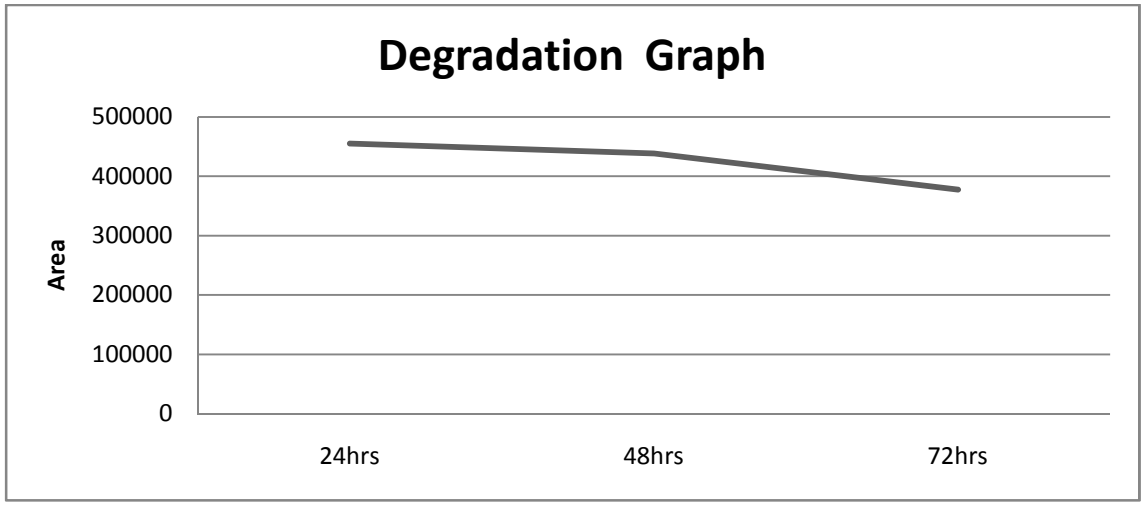
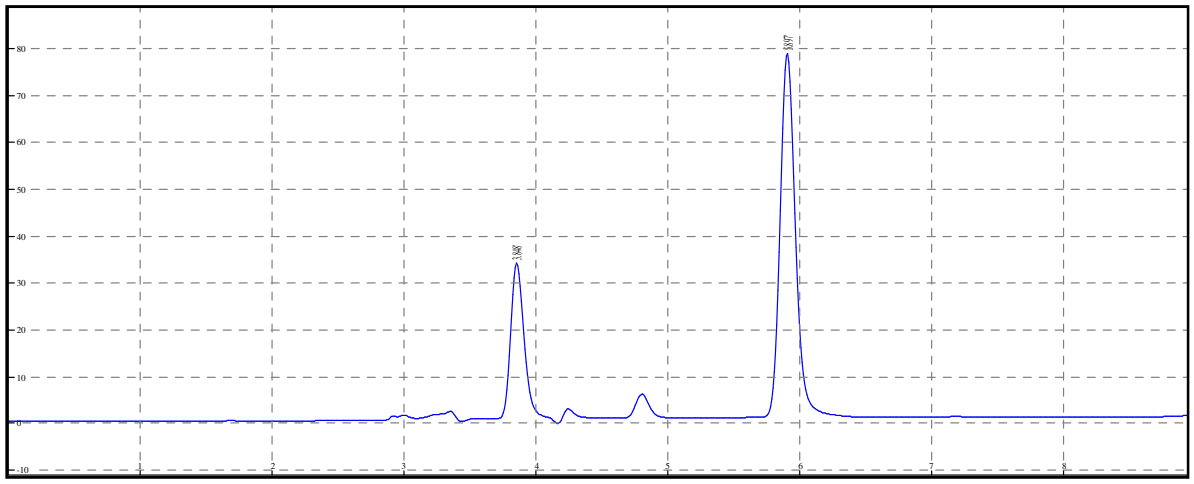


Fig No.6.13: Degradation graph for Bench top stability

2. Freeze thaw Cycles Stability: Each Cycle at 12, 24 and 48hrs.

Sample name: Prednisolone HQC 12hrs

Run time: 9.31min

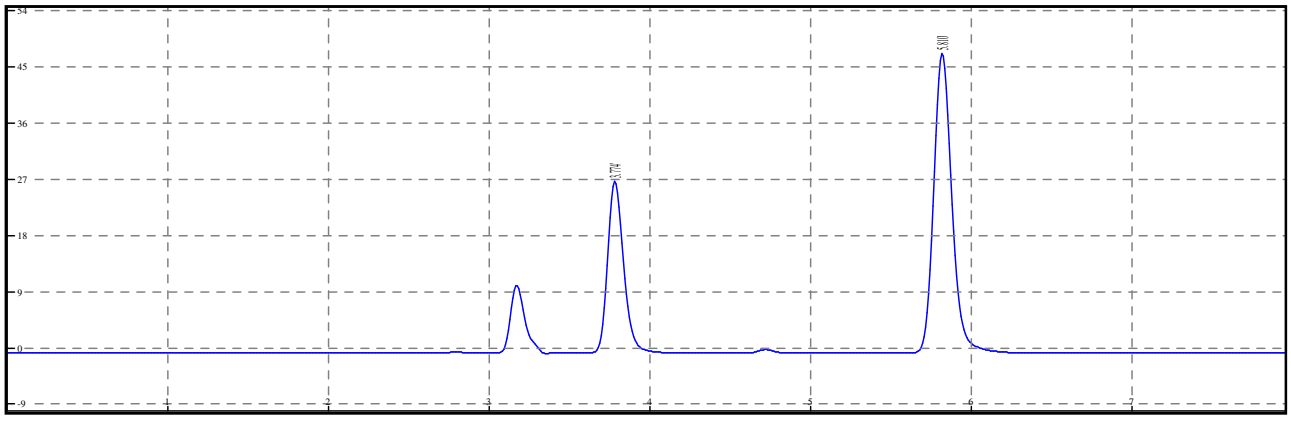


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.848	297237	9.43	5700	1.26
5.897	973673	0.00	11081	1.19

Sample name: Prednisolone HQC 24hrs

Run time: 8.20min

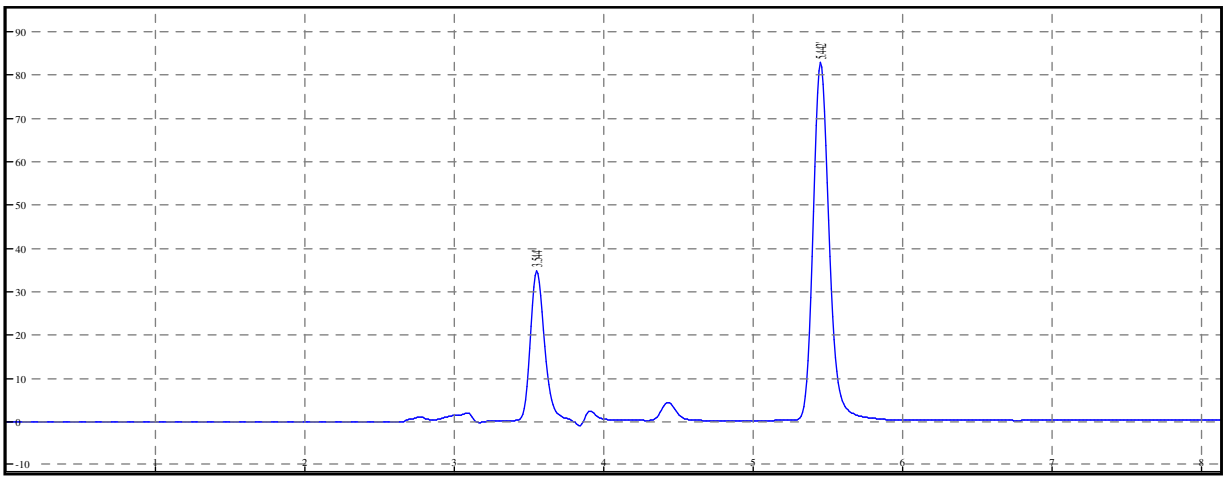


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.774	200150	10.12	6356	1.24
5.810	779465	0.00	12643	1.19

Sample name: Prednisolone HQC 48hrs

Run time: 8.13min

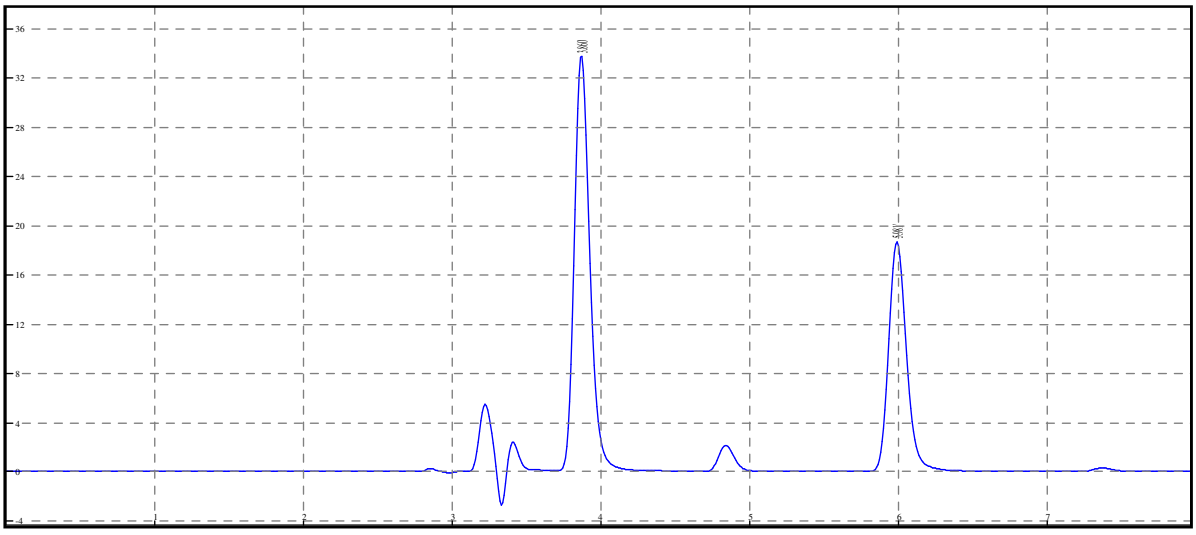


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.544	157623	9.30	5652	1.27
5.442	680002	0.00	10433	1.21

Sample name: Prednisolone LQC 12hrs

Run time: 8.35min

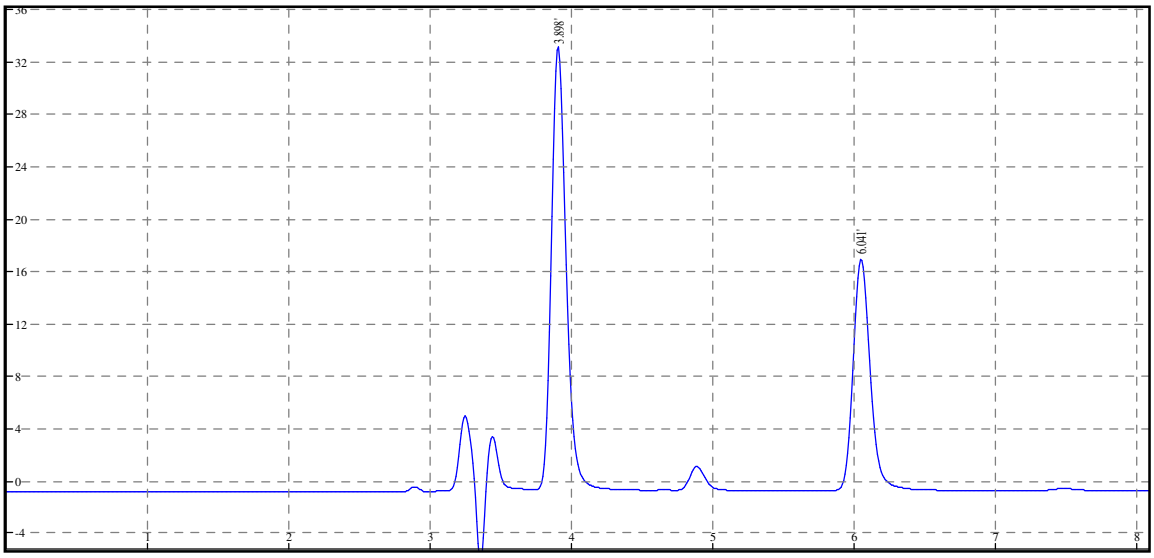


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.860	381671	6.37	3906	1.25
5.981	113762	0.00	6275	1.20

Sample name: Prednisolone LQC 24hrs

Run time: 8.34min

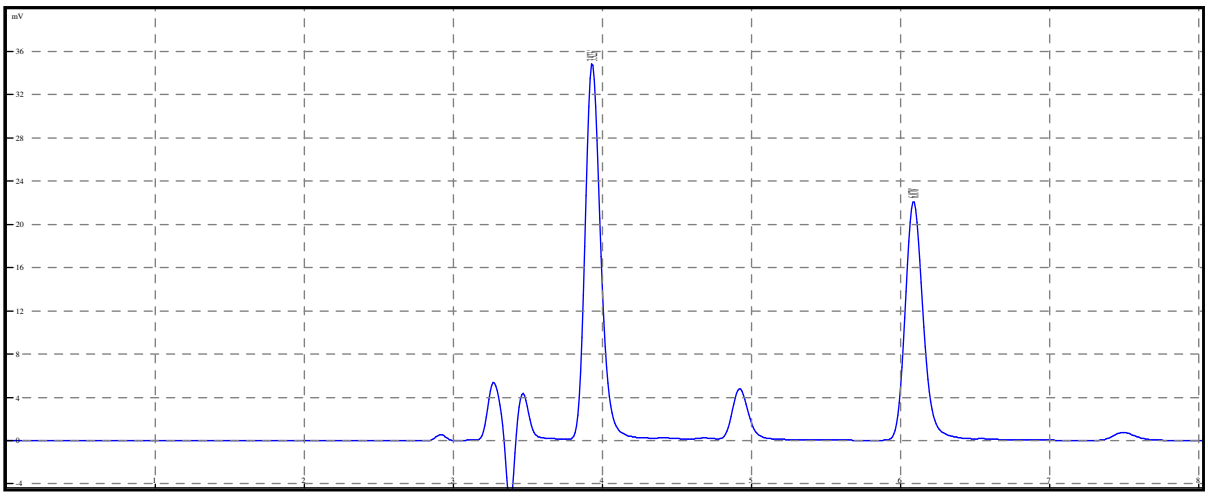


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.898	354126	8.69	3490	1.24
6.041	106935	0.00	11885	1.20

Sample name: Prednisolone LQC 48hrs

Run time: 8.67min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.923	302915	8.68	3458	1.24
6.078	100250	0.00	12034	1.19

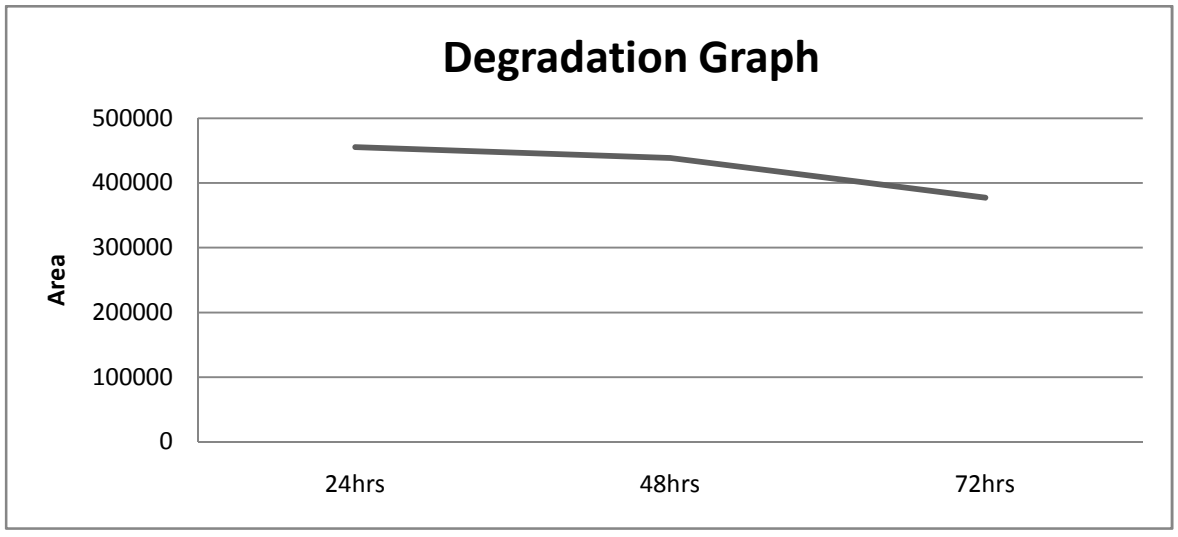
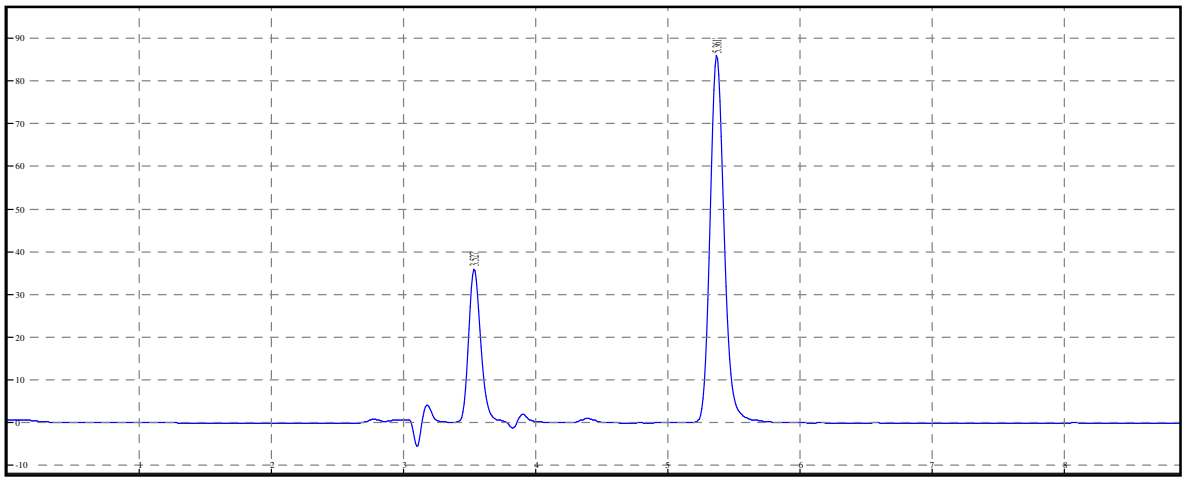


Fig No.6.14: Degradation graph for freeze thaw stability

3. Plane Solution Stability: 24, 48 and 72hrs.

Sample name: Prednisolone HQC Day 1

Run time: 10.24min

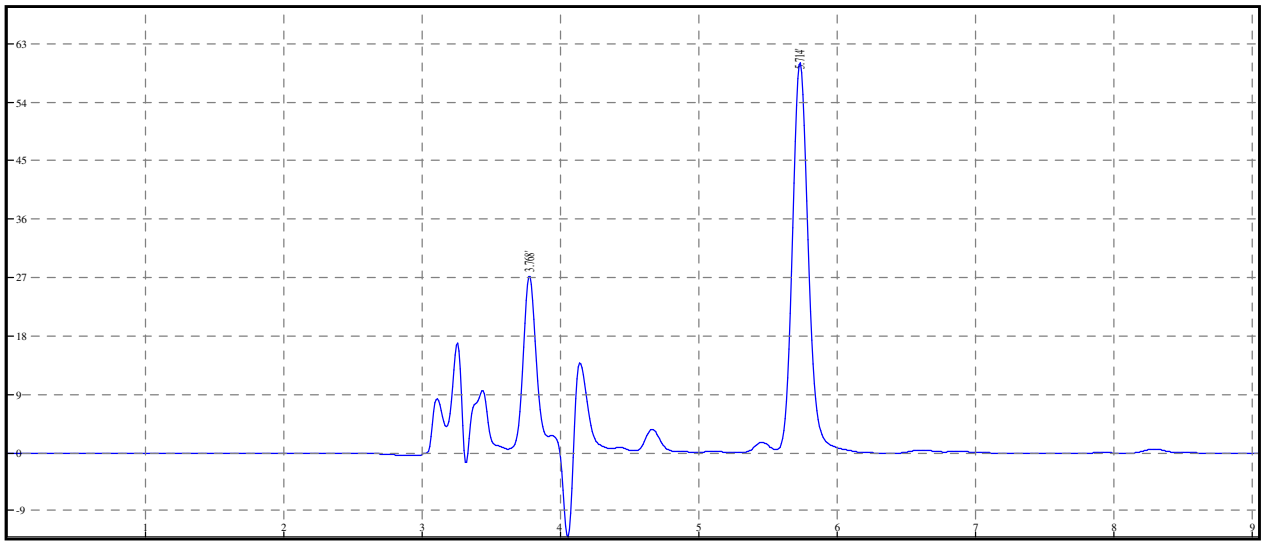


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.527	455179	2.97	3545	1.25
5.361	1498772	0.00	5883	1.18

Sample name: Prednisolone HQC Day 2

Run time: 12.16min

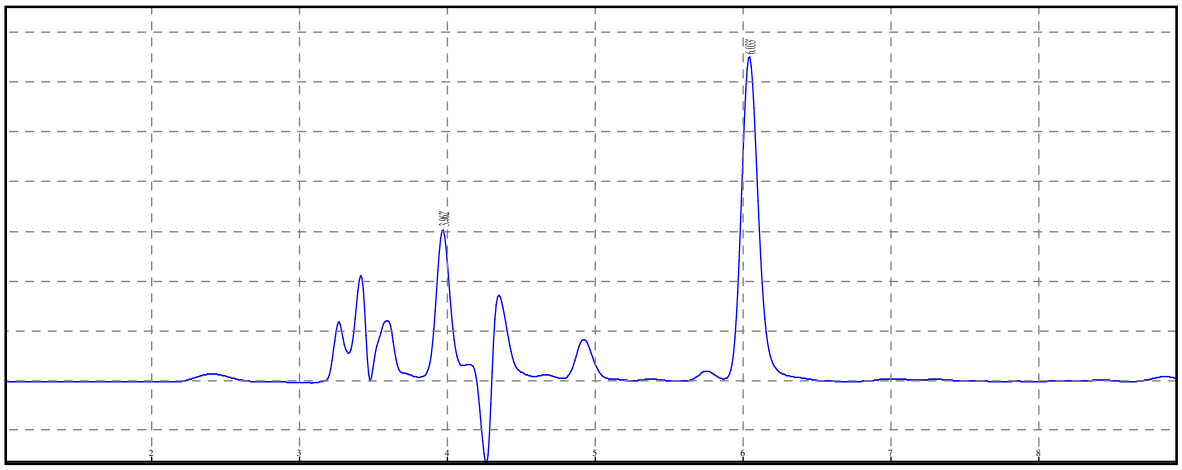


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.768	438181	2.40	5460	1.57
5.714	1475605	0.00	9801	1.15

Sample name: Prednisolone HQC Day3

Run time: 11.55min

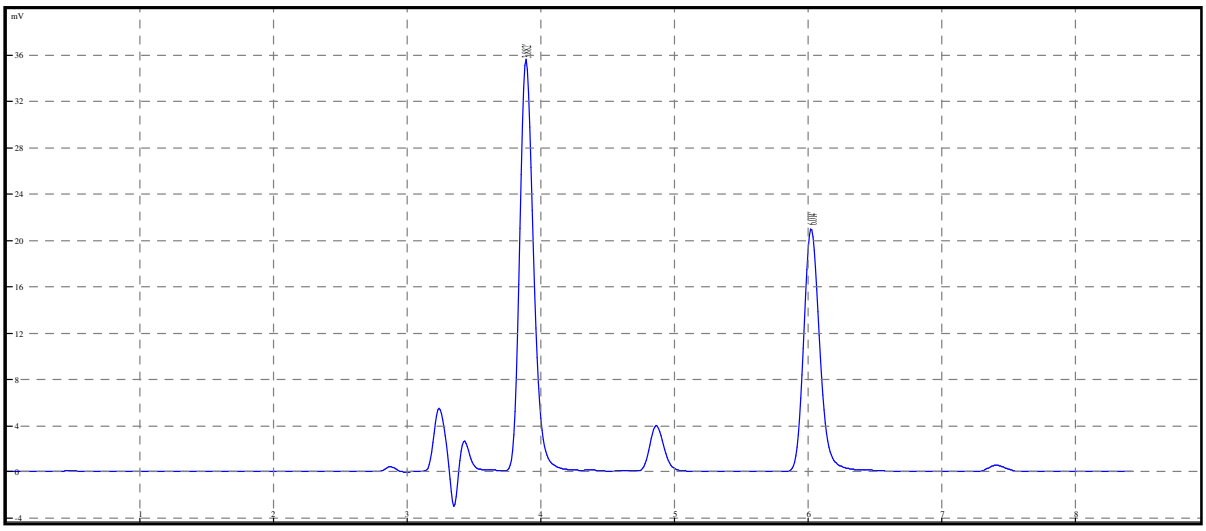


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.962	377242	5.58	3106	1.60
6.033	1336549	0.00	3902	1.19

Sample name: Prednisolone LQC Day1

Run time: 8.43min

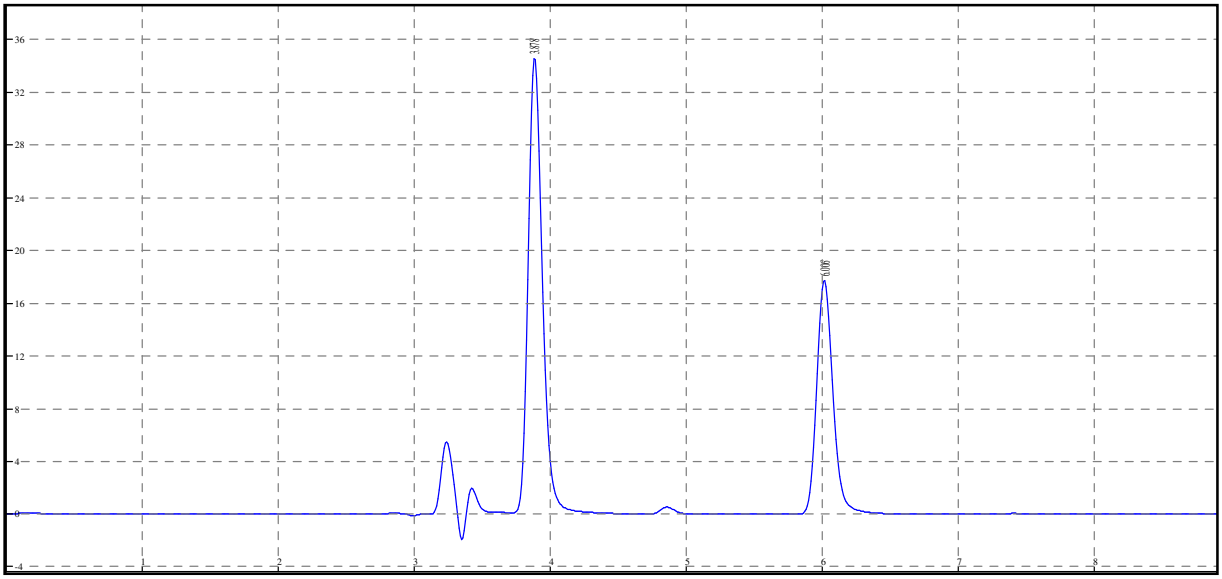


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.882	454439	8.99	4028	1.24
6.014	178571	0.00	11698	1.20

Sample name: Prednisolone LQC Day2

Run time: 8.96min

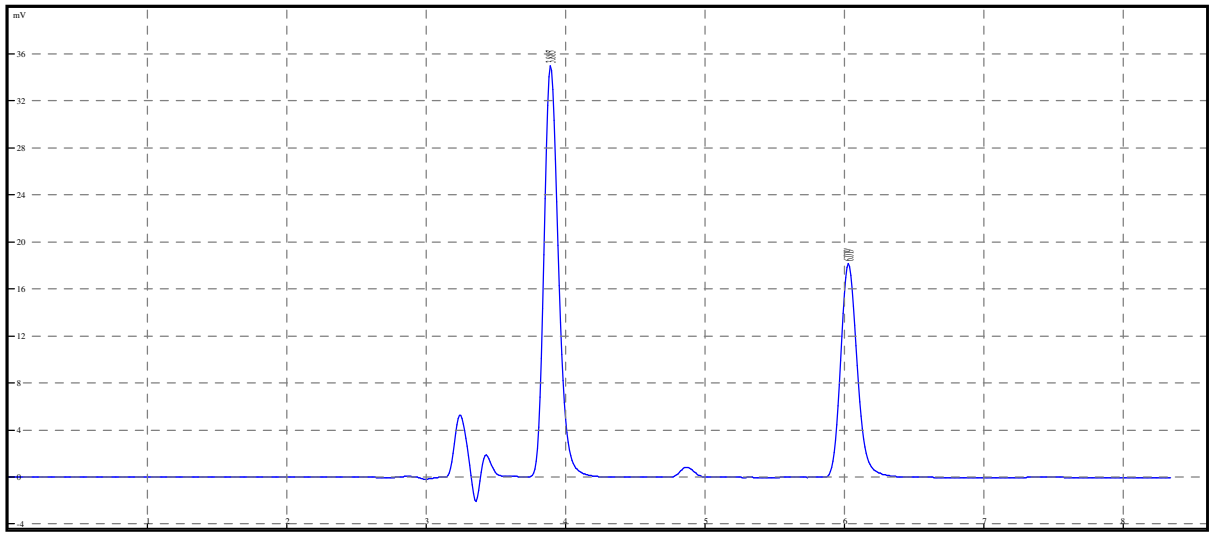


Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.878	433316	9.11	4134	1.24
6.006	148662	0.00	12041	1.20

Sample name: Prednisolone LQC Day 3

Run time: 8.34min



Observation Table

Time	Area	Resolution	T.Plate no.	Asymmetry
3.885	422145	9.63	5171	1.24
6.019	143373	0.00	11969	1.20

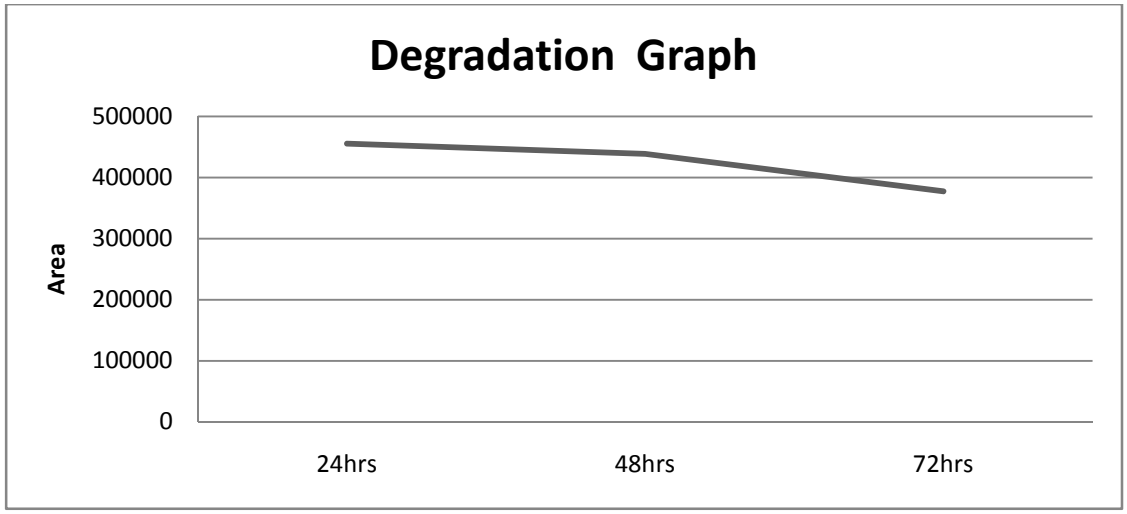


Fig No.6.15: Degradation graph for plane solution stability

8. SUMMARY AND CONCLUSION

A simple, rapid and sensitive bioanalytical method for quantitative estimation of corticosteroids such as Betamethasone, Dexamethasone and Prednisolone in rat plasma using RP-HPLC method has been developed and validated and results obtained are given in the following table.

Table No. 7.1 Summary Table for validation parameters

Sr.No	Parameters	Acceptance criteria	Results obtained
01	Selectivity	Should be no interference	Pass
02	System suitability	Resolution-should be greater than 1 Theoretical plates-should be greater than 2000 Tailing/Assymetry factor-should be less than 2	Pass
03	Accuracy & Precision	%SD & % RSD should be less than 15%	pass
04	Linearity	R^2 value should be near to 1	Betamethasone- $R^2 = 0.998$ Dexamethasone- $R^2 = 0.998$ Prednisolone- $R^2 = 0.999$
05	Recovery	-	Betamethasone- 73.2% Dexamethasone- 75.2% Prednisolone- 74.26%

06	Bench top stability	-	Short term stock stability-(2hrs,12hrs,24 hrs) Long term stock stability-(10days,20days,30 days)
07	Freeze thaw stability	-	Pass (3 cycles)
08	Plane solution stability	-	24hrs,48hrs,72hrs

- By analyzing these above selected drugs (Betamethasone, Dexamethasone and Prednisolone) and validating with the parameters we conclude that they showed excellent recovery and reproducibility in all aspects comparing with reported methods. Hence the developed method will be much useful to analyze plasma samples in rat/human, which will be more effective when compared with the reported methods.
- The current validated bioanalytical RP-HPLC method for Betamethasone, Dexamethasone & Prednisolone offers good accuracy and significant advantages in terms of linearity, stability & selectivity.
- The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peaks of above selected corticosteroids without any interference.
- By using current developed method we can get maximum absorptivity of selected drugs as compared to earlier reported methods.
- The developed method is most economical as compared to earlier methods due to use of Methanol as a solvent.
- In this method I try to maintain minimum retention time (RT) with consideration of plasma peaks.

CERTIFICATE

This is to certify that this dissertation work titled "**BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF SELECTED CORTICOSTEROIDS IN RAT PLASMA USING RP-HPLC METHOD"** of the candidate **Mr.** Nathe Kiran Ramesh, M.Pharm., with registration number **141540002** for the award of **Doctor of Philosophy** in the branch of **Pharmacy**. I personally verified that two publication of this dissertation has been published and the copy of published articles are attached.

SUPERVISOR AND GUIDE

Dr. W.D. SAM SOLOMON, M.Pharm., Ph.D.,

Place: Sulur

Date:

LIST OF PUBLICATIONS

1. **Kiran Nathe*,** Sam Solomon. Bioanalytical Method Development and Validation of Prednisolone in Rat Plasma Using RP-HPLC Method. International Journal of Pharmaceutical Science and Research. 2017; 2(6): 8-11.

2. **Kiran Nathe*,** Sam Solomon. Bioanalytical Method Development and Validation of Selected Corticosteroid in Rat Plasma Using RP-HPLC Method. International Journal of Pharmacy and Pharmaceutical Sciences. 2017; 2(5): 11-14.

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Bio analytical Method Development and Validation of Selected Corticosteroid in Rat Plasma Using RP-HPLC method

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Abstract
A simple, selective, rapid, precise and economical reverse phase HPLC method has been developed and validated for quantitative determination of Betamethasone in plasma. Paracetamol is used as an internal standard. The method was carried out with Analytical technologies Lts. Model no. 3000Series. The optimized chromatographic conditions were optimized using a mobile phase methanol: water in the ratio of 60:40 at flow rate 0.9ml/min. Stationary phase was used as Grace C18column (250mm x 4.6ID, partical size: 5 micron). Detection was carried out at 242nm.the method was developed and tested for linearity range of 10ng/ml to 160ng/ml. The developed method was validated in terms of selectivity, accuracy, precision, linearity and stability study. Proposed developed method can be used in bio analytical, bioequivalence & pharmacokinetic studies with desired precision and accuracy.

Keywords: HPLC, Vortex mixer, Betamethasone

Introduction
Methods of measuring drugs in biological media are increasingly important for the study of bioavailability and bioequivalence studies, new drug development study, clinical pharmacokinetics and therapeutic drug monitoring. Liquid-liquid extraction is probably the most widely used technique because the analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination and also the technique is simple, rapid, and has a relatively small cost factor per sample. Literature survey revealed that validated RP-HPLC method for the quantification of Betamethasone in rat plasma is not reported earlier. For estimation of the drugs present in biological fluid, HPLC method is considered to be more suitable. In this study we have developed compatible RP-HPLC method with liquid-liquid extraction process for determination of Betamethasone in plasma and the developed method is validated as per regulatory requirements.

Materials and Methods
Chemicals
API of Betamethasone was gifted by Zydus cadila, Ahmadabad. Solvents used are water for HPLC grade (Millie Q or equivalet), Ethyl acetate (HPLC grade), Diethyl ether, Chloroform and Dichloromethane.

Standard solution preparation
Standard solutions was prepared by using HPLC grade methanol and water in the ratio 1:1. Initially 10 mg of drug was weighted and transferred into the standard flask; the combined solvent (methanol and water) added and finally made the volume with the same up to 100ml to get 100ppm stock solution. The stock solution further seriely diluted was used for the analysis. The stock solution was maintained refrigerated at 8ºC.

Extraction method
In this process first take 1 ml of plasma from sample which is previously stored at 5-7^{0}C. In this add 0.0125 mililitre of 1ppm of drug (Betamethasone) which is prepared in methanol: water combination & 0.125 mililiter of 1ppm of internal standard. After this vortex the above prepared mixture for 3 mins. Also in this add 0.200 mililiter of 1% of hydrochloric acid to provide acidic nature to the plasma. Again vortex the above mixture for 3-5 mins. In this add ethyl acetate which act as a extracting solvent & again vortex the mixture for 3-5 mins. Now withdraw 2ml of ethyl acetate in which drug is extracted in fresh tube & finaly allow to evaporate the solvent which will leave dried drug in tube & dilute it with 0.500 mililiter of mobile phase.

Method validation
The method performance was evaluated for selectivity, accuracy, precision, linearity, stability at various conditions including bench top stability, freeze thaw stability and recovery.

Results and Discussion
Chromatographic optimization
The chromatogram was developed initially using separation condition such as mobile phase (methanol: water in the ratio of 10:90 increasing order). The system was used Anaytical technologies Lts. Model no. 3000Series. The optimized chromatographic conditions were optimized using a mobile phase methanol: water in the ratio of 60:40 at flow rate 0.9ml/min with the stationary phase was used as Grace

C18column (250mm x 4.6ID, partical size: 5 micron).
The chromatograms of Betamethasone with IS have been shown in fig.1.

Selectivity
The desired method used RP-HPLC method for separation of betamethasone from Paracetamol (IS) and was shown to be selective for the analyte and its IS (retention times for betamethasone and Paracetamol were 5.80 and 7.20 minutes respectively). No interfering peaks were observed with the same retention time of the analyte when different plasma samples were analysed.fig.2 and fig.3 represent the chromatograms of blank plasma and plasma sample spiked with drugs respectively.

Linearity
Linearity was demonstrated from 10.0-160 ng/ml.fig.4 shows calibration curve of betamethasone. The calibration curve includes 6 calibration standards which are distributed 0.758 with goodness of fit.

Accuracy and Precision
Accuracy and Precision was evaluated by analyzing 3 bathches. Each batch consist of three replicates of LQC, MQC and HQC. The interday and intraday precision and accuracy of the method for each concentration levels are represented in Table 1.

Table 1: Intraday and Inter day Precision and Accuracy of Betamethasone.

			Standard Deviation		Accuracy	Precision
Conc.	**Conc.**	**Area**	**Mean**	**SD**	**%SD**	**%RSD**
LQC	0.1875	3.0972	3.0692	0.03857	1.2568	1.2568
	0.1875	3.0252				
	0.1875	3.0852				
MQC	1.5	0.0662	0.066	0.00385	5.8345	5.8345
	1.5	0.0621				
	1.5	0.0698				
HQC	2.5	0.0325	0.036	0.003325	9.2266	9.2266
	2.5	0.0365				
	2.5	0.0391				

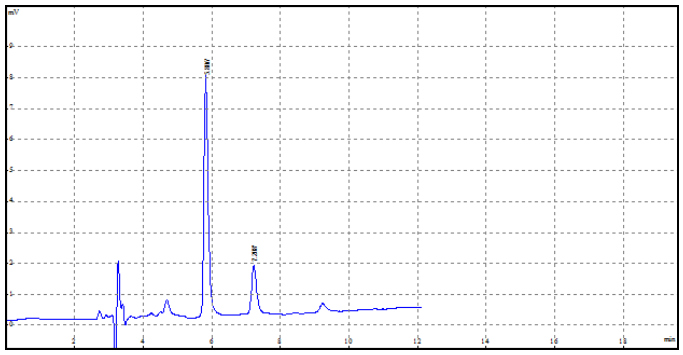

Fig 1: Typical chromatogram of Betamethasone with Paracetamol.

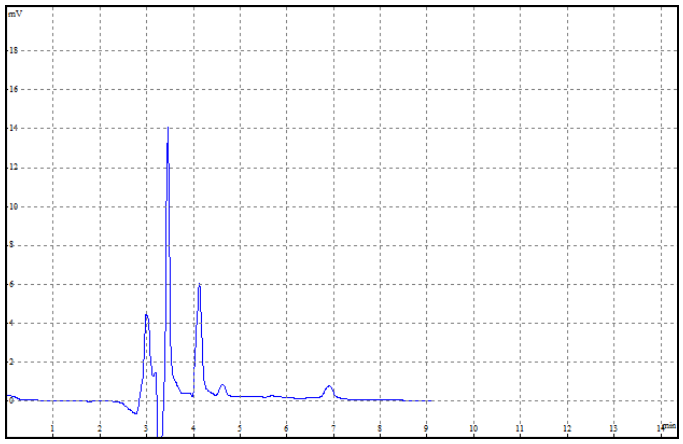

Fig 2: Typical chromatogram of blank plasma sample.

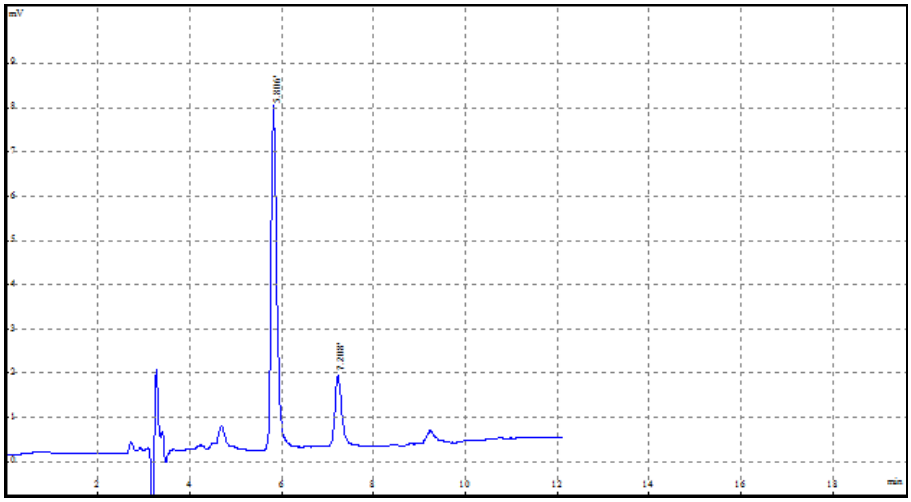

Fig 3: Typical chromatogram of plasma sample spiked with Betamethasone and Paracetamol.

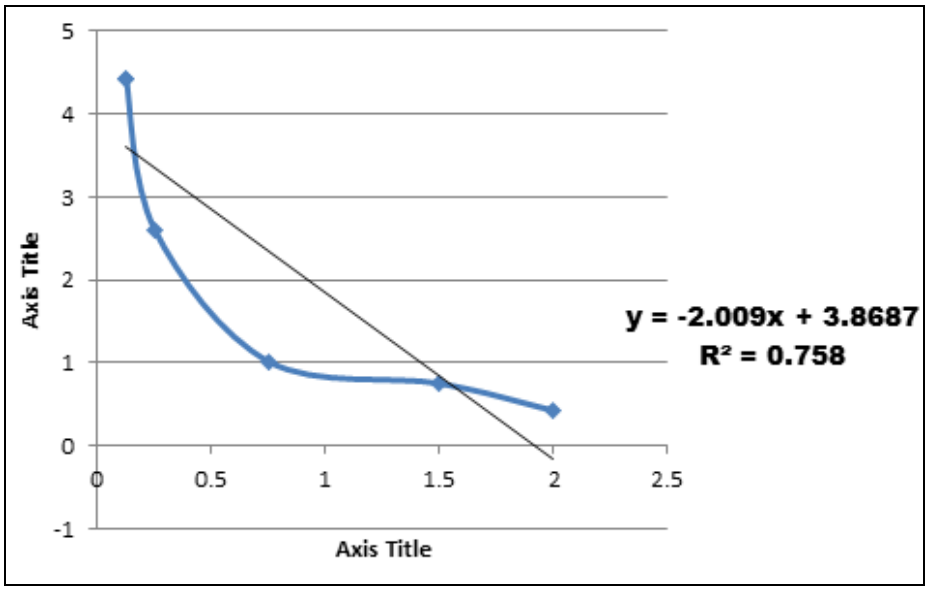

Fig 4: Calibration curve of Betamethasone.

Recovery

The recovery was evaluated by comparing response of extracted and unextracted samples. The average recovery for Betamethasone in plasma was ranged from 85.2 to 88.6% for the low, medium and high quality control samples with an average of 87.2%.

Stability Studies

Stability studies were performed to evaluate the stability of Betamethasone both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include bench top stability, freeze thaw stability, long term and short term stock stability. Betamethasone was found to be stable for three freeze and thaw cycles.

Table 2: Validation Parameters of Betamethasone by HPLC method.

Sr. No	Parameters	Results
01	Selectivity	Pass
02	System suitability	Pass
03	Accuracy & precision	Pass
04	Linearity	Betamethasone- $R^2 = 0.758$, Dexamethasone- $R^2 = 0.721$ Prdnisolone- $R^2 = 0.725$
05	Recovery	Pass
06	Bench top stability	Short term stock stability-(2hrs, 12hrs, 24 hrs) Long term stock stability-(10days, 20days, 30 days)
07	Freeze thaw stability	Pass (3 cycles)

Conclusion

The current validated bio analytical HPLC method for Betamethasone offers good accuracy and significant advantages in terms of linearity, stability & selectivity. The separation method developed produce acceptable values of recovery. The chromatograms developed has well resolved peaks of above selected corticosteroid without any interference. From the results we conclude that the developed method can be used in bio analytical, bioequivalence & pharmacokinetic studies with desired precision and accuracy.

Acknowledgement

Authors are thankful to Zydus Cadila Healthcare Ltd., for providing drug as gift sample.

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Bioanalytical method development and validation of prednisolone in rat plasma using RP-HPLC method

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Abstract
A simple, selective, rapid, precise and economical reverse phase HPLC method has been developed and validated for quantitative determination of Prednisolone in plasma. Metformin is used as an internal standard. The method was carried out with Anaytical technologies Lts. Model no. 3000Series. The optimized chromatographic conditions were optimized using a mobile phase methanol: water in the ratio of 70:30 at flow rate 0.9ml/min. Stationary phase was used as Grace C18 column (250mm x 4.6ID, partical size:5 micron). Detection was carried out at 238nm.The method was developed and tested for linearity range of 100ng/ml to 1600ng/ml. The developed method was validated in terms of selectivity, accuracy, precision, linearity and stability study. Proposed developed method can be used in bioanalytical, bioequivalence & pharmacokinetic studies with desired precision and accuracy.

Keywords: HPLC, vortex mixer, prednisolone

Introduction
Methods of measuring drugs in biological media are increasingly important for the study of bio availability and bioequivalence studies, new drug development study, clinical pharmacokinetics and therapeutic drug monitoring. Liquid-liquid extraction is probably the most widely used technique because the analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination and also the technique is simple, rapid, and has a relatively small cost factor per sample. Literature survey revealed that validated RP-HPLC method for the quantification of Prednisolone in rat plasma is not reported earlier. For estimation of the drugs present in biological fluid, HPLC method is considered to be more suitable. In this study we have developed compatible RP-HPLC method with liquid-liquid extraction process for determination of Prednisolone in plasma and the developed method is validated as per regulatory requirements.

Materials and Methods
Chemicals
API of Prednisolone was gifted by Zydus cadila, Ahmadabad. Solvents used are water for HPLC grade (Millie Q or equivalet), Ethyl acetate (HPLC grade), Diethyl ether, Chloroform and Dichloromethane.

Standard Solution Preparation
Standard solutions were prepared by using HPLC grade methanol and water in the ratio 1:1. Initially 10 mg of drug was weighted and transferred into the standard flask; the combined solvent (methanol and water) added and finally made the volume with the same up to 100ml to get 100ppm stock solution. The stock solution further seriely diluted was used for the analysis. The stock solution was maintained refrigerated at 8ºC.

Extraction Method
In this process first take 1 ml of plasma from sample which is previously stored at 5-7^{0}C. In this add 0.0125 mililitre of 1ppm of drug (prednisolone) which is prepared in methanol: water combination & 0.125 mililiter of 1ppm of internal standard. After this vortex the above prepared mixture for 3 mins. also in this add 0.200 mililiter of 1% of hydrochloric acid to provide acidic nature to the plasma. Again vortex the above mixture for 3-5 mins. In this add ethyl acetate which act as a extracting solvent & again vortex the mixture for 3-5 mins. Now withdraw 2ml of ethyl acetate in which drug is extracted in fresh tube & finaly allow to evaporate the solvent which will leave dried drug in tube & dilute it with 0.500 mililiter of mobile phase.

Method Validation
The method performance was evaluated for selectivity, accuracy, precision, linearity, stability at various conditions including bench top stability, freeze thaw stability and recovery.

Results and Discussion
Chromatographic Optimization
The chromatogram was developed initially using separation condition such as mobile phase (methanol: water in the ratio of 10:90 increasing order). The system was used Anaytical technologies Lts. Model no. 3000Series. The optimized chromatographic conditions were optimized using a mobile phase methanol: water in the ratio of 70:30 at flow rate 0.9ml/min respectively with the stationary phase was used as

Grace C18 column (250mm x 4.6ID, partical size: 5 micron). The chromatograms of Prednisolone with IS have been shown in fig.1.

Selectivity

The desired method used RP-HPLC method for separation of Prednisolone from Metformin (IS) and was shown to be selective for the analyte and its is (retention times for prednisolone and metformin were 5.24 and 6.11 minutes respectively).No interfering peaks were observed with the same retention time of the analyte when different plasma samples were analysed.fig.2 and fig.3 represent the chromatograms of blank plasma and plasma sample spiked with drugs respectively.

Linearity

Linearity was demonstrated from 100-1600 ng/ml.fig.4 showes calibration curve of Prednisolone. The calibration curve includes 6 calibration standards which are distributed 0.999 with goodness of fit.

Accuracy and Precision

Accuracy and Precision was evaluated by analyzing 5 bathches, each batch consist of five replicates of LQC, MQC and HQC. The precision and accuracy of the method for each concentration levels are represented in Table 1.

Table 1: Intraday and Interday Precision and Accuracy of Prdnisolone

			Standard Deviation		Accuracy	Precision
Conc.	**Conc.**	**Area**	**Mean**	**SD**	**%SD**	**%RSD**
LQC	0.187	3.164	3.374	0.162158873	4.80613138	4.80613137
	0.187	3.525				
	0.187	3.41				
	0.187	3.25				
	0.187	3.521				
MQC	1.5	0.5295	0.555	0.039515351	7.11296242	7.112962423
	1.5	0.5982				
	1.5	0.5025				
	1.5	0.5621				
	1.5	0.5854				
HQC	2.5	0.3244	0.33466	0.011809234	3.52872577	3.528725774
	2.5	0.3521				
	2.5	0.3254				
	2.5	0.3302				
	2.5	0.3412				

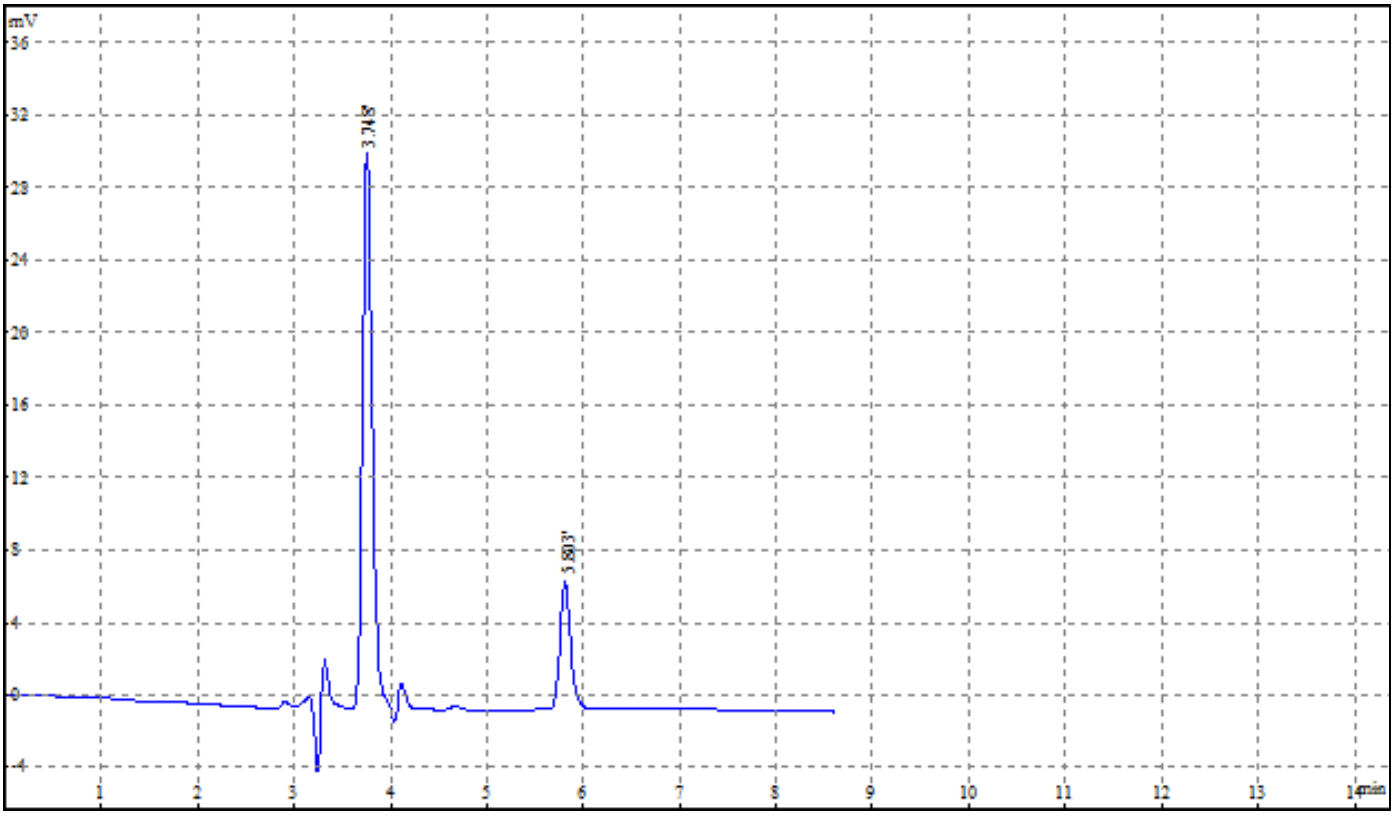

Fig 1: Typical chromatogram of Prednisolone with Metformin

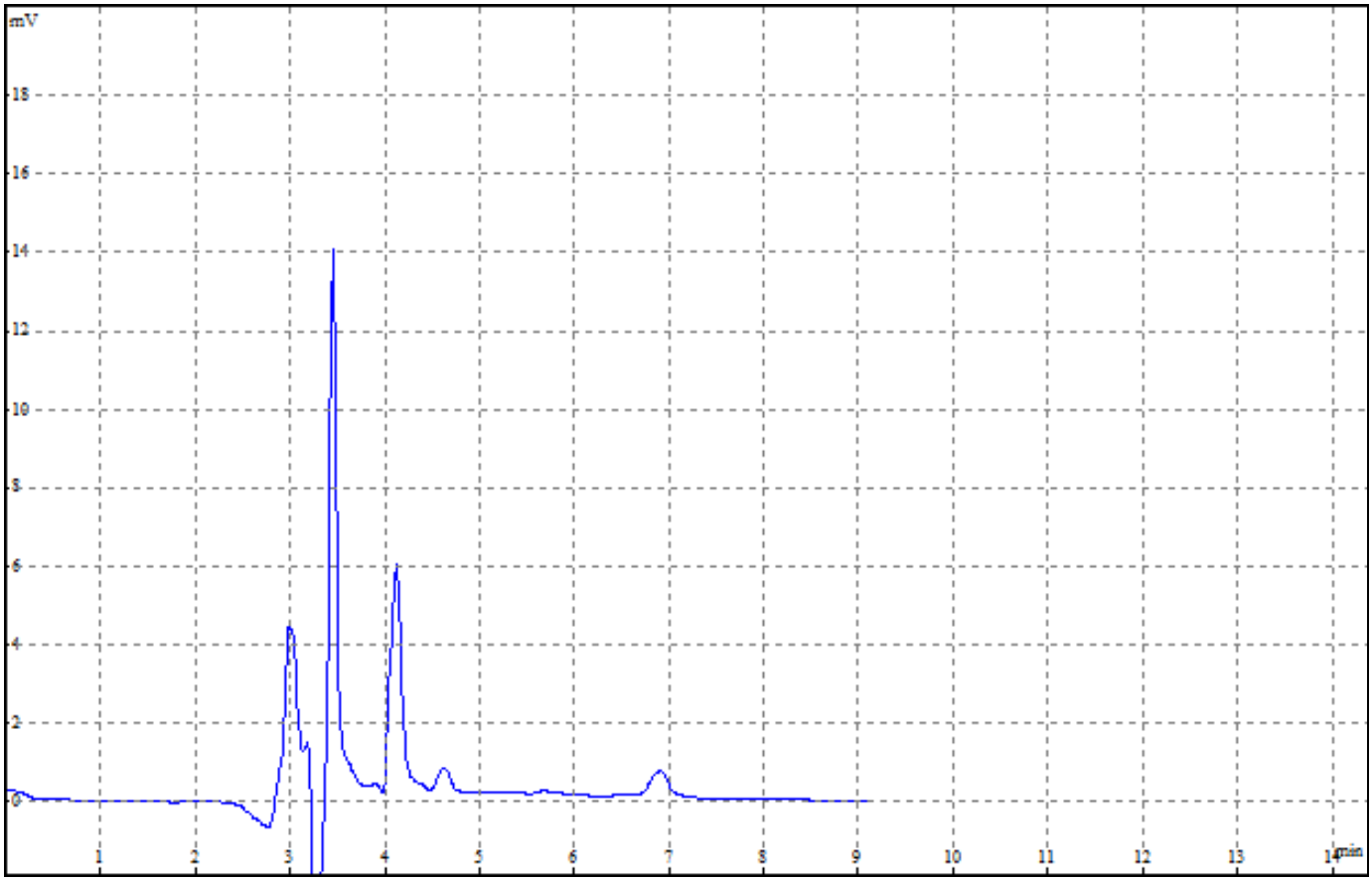

Fig 2: Typical chromatogram of blank plasma sample

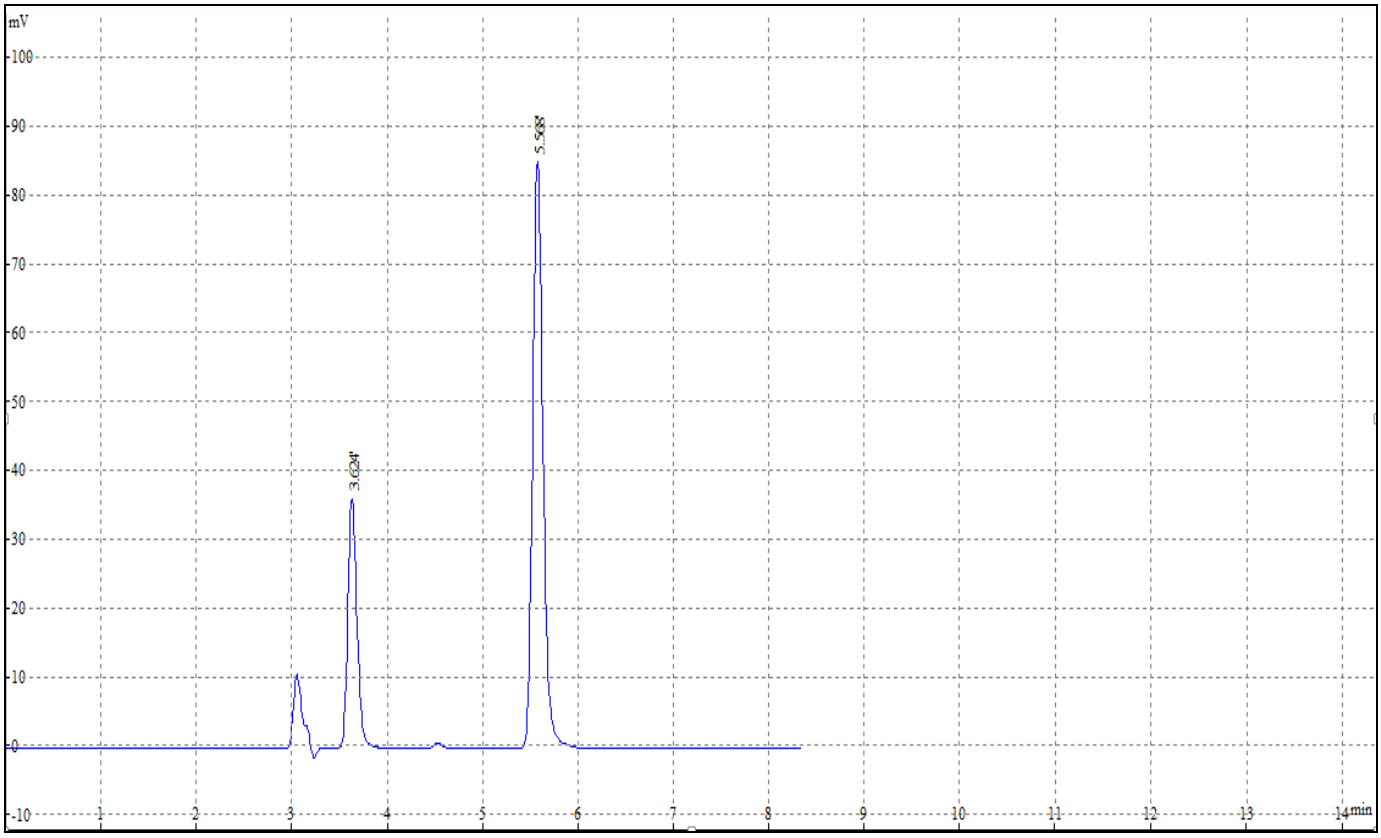

Fig 3: Typical chromatogram of plasma sample spiked with Prednisolone and Metformin

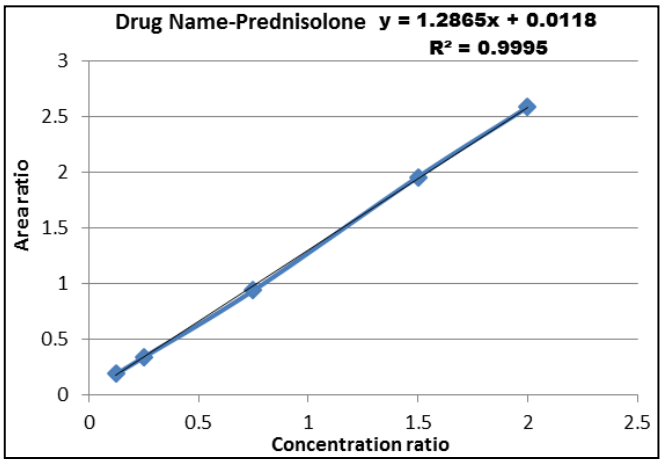

Fig 4: Calibration curve of Prednisolone

Recovery

The recovery was evaluated by comparing response of extracted and un-extracted samples. The average recovery for Prednisolone in plasma was ranged from 72.2 to 77.6% for the low, medium and high quality control samples with an average of 74.2%.

Stability Studies

Stability studies were performed to evaluate the stability of Prednisolone both in aqueous solution and in plasma after exposing to various stress conditions. The stability studies performed include bench top stability, freeze thaw stability, long term and short term stock stability. Prednisolone was found to be stable for three freeze and thaw cycles.

Table 2: Validation Parameters of Prednisolone by RP- HPLC method

S. No	Parameters	Results
1.	Selectivity	Pass
2.	System suitability	Pass
3.	Accuracy & precision	Pass
4.	Linearity	Prdnisolone-R^2 = 0.999
5.	Recovery	Pass
6.	Bench top stability	Short term stock stability-(2hrs,12hrs,24 hrs) Long term stock stability-(10days,20days,30 days)
7.	Freeze thaw stability	Pass (3 cycles)

Conclusion

The current validated bio analytical RP-HPLC method for Prednisolone offers good accuracy and significant advantages in terms of linearity, stability & selectivity. The separation method developed produce acceptable values of recovery. The chromatograms developed has well resolved peaks of Prednisolone without any interference. From the results we conclude that the developed method can be used in bio analytical, bioequivalence & pharmacokinetic studies with desired precision and accuracy.

Acknowledgement

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