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Analytical Method Development Validation and Degradation of Inosine Pranobex by Using RP-HPLC Method.

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ABSTRACT-
This study focuses on the development, validation, and degradation assessment ofan analytical method for inosine pranobex using Reverse Phase High-PerformanceLiquid Chromatography (RP-HPLC). The objective was to establish a reliable RP-HPLC method for the accurate quantification of inosine pranobex inpharmaceutical formulations and to evaluate its stability under various stressconditions.
Method Development: The RP-HPLC method was optimized using a C18 columnwithamobilephasecomposedofMethanol (with0.1%Water)andOPA(45%).
The flow rate was set at 0.8mL/min, and detection was performed at 259 nm.Samplepreparationinvolveddissolvinginosinepranobexinmethanol,followedbyfiltration.
MethodValidation: Thedevelopedmethodwasvalidatedforspecificity,linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation(LOQ). Specificity tests confirmed no significant interference from excipients ordegradation products. The calibration curve demonstrated linearity over theconcentration range of 10-50 µg/mL. Precision studies showed acceptablerepeatability and reproducibility. Accuracy was confirmed through recoverystudies, with results within acceptable ranges. The LOD and LOQ were determinedtobe 0.16µg/mL and0.49µg/mL,respectively.
Degradation Studies: Stability of inosine pranobex was assessed under acidic,alkaline, oxidative, photolytic, and thermal conditions. Degradation products wereidentified and quantified using the RP-HPLC method. Significant degradation wasobservedunderacidicandoxidativeconditions,withmultipledegradationproductsformed.
Conclusion: The RP-HPLC method developed is robust, accurate, and precise forthe analysis of inosine pranobex. It is suitable for routine quality control andstabilitytesting.Thedegradationstudiesprovidevaluableinsightsintothestabilityprofile of inosine pranobex, ensuring its effective application in pharmaceuticalformulations.
Keywords: Inosine pranobex, RP-HPLC, method development, validation,degradationstudies,stabilityanalysis.

I. INTRODUCTION-

Inosine pranobex, also known as inosine pranobex or inosine acedobendimepranol, is a pharmaceutical agent used primarily for its immunomodulatoryeffects. It is often utilized in the treatment of viral infections and certain chronicconditions due to its ability to enhance the immune response. Given its clinicalsignificance, it is crucial to have accurate and reliable methods for thequantification and quality control of inosine pranobex in pharmaceuticalformulations.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) is a widelyemployed analytical technique known for its high resolution, precision, andaccuracy. This method is particularly effective in separating and quantifyingcomplexmixturesofcompounds,makingitsuitableforanalyzingpharmaceutical products. The development and validation of an RP-HPLC method for inosinepranobex are essential for ensuring the consistency, safety, and efficacy of itspharmaceuticalpreparations.

Objective

The primary objective of this study was to develop and validate an RP-HPLCmethod for the determination of inosine pranobex. The method needed to becapable of accurately quantifying inosine pranobex in its dosage forms andassessing its stability under various stress conditions.

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Validation was essential toconfirmthatthemethodmetregulatoryrequirements andwassuitableforroutinequality control.

ImportanceofMethodDevelopmentandValidation

1. **Accuracy and Precision:** An accurate and precise RP-HPLC method iscritical for ensuring that the concentration of inosine pranobex inpharmaceutical formulations is within the specified limits, therebyguaranteeingthe therapeutic efficacyof the drug.
2. **Specificity:**Themethodmustbespecifictoinosinepranobex,effectivelydistinguishing it from other compounds and potential impurities ordegradationproducts.
3. **Robustness:**Themethodshouldberobustenough towithstandvariationsinoperating conditions, ensuring consistent performance under differentconditions.
4. **RegulatoryCompliance:**Validationensuresthat themethodcomplieswithregulatory standards, such as those set by the International Conference onHarmonisation (ICH)andtheUnited States Pharmacopeia(USP).

DegradationStudies

Understandingthedegradationprofileofinosinepranobexiscrucialforevaluatingits stability and shelf life. Degradation studies simulate various stress conditions,such as acidic, alkaline, oxidative, photolytic, and thermal environments, todetermine how the drug degrades over time and under different conditions. Thesestudies provide insights into the stability of the drug and the formation ofdegradationproducts,which areessentialforensuring drugsafetyand efficacy.

SignificanceoftheStudy

This study's significance lies in its contribution to the development of a reliableanalytical tool for inosine pranobex. The RP-HPLC method established will serveasacriticalqualitycontrolmeasureinpharmaceuticalmanufacturingandstabilitytesting, ensuring that inosine pranobex products maintain their quality throughouttheir shelf life. Furthermore, understanding the degradation behavior of inosinepranobex aids in optimizing formulation conditions and storage practices toenhanceproductstability andpatientsafety.

DrugProfile:InosinePranobex

1. **GeneralInformation:**

- **ChemicalName:**Inosinepranobex
- **Chemical Structure:** Inosine pranobex is a combination of inosine, a purinenucleoside, and the complex compound acedoben dimepranol. The chemicalformulaofinosinepranobexistypicallyrepresentedasC16H20N4O6.
- **MolecularWeight:**Approximately316.36g/mol.
- **Synonyms:** Inosine acedoben dimepranol, inosine pranobex, immuno-inosine.

2. **PharmacologicalClassification:**

- **Class:**Immunomodulator,antiviralagent.
- **MechanismofAction:**Inosinepranobexisbelievedtoenhancetheimmuneresponse by stimulating the activity of T-cells and other components of theimmune system. It also has potential antiviral effects, improving the host'sability tocombatviralinfections.

3. **TherapeuticUses:**

- **PrimaryIndications:**
- **ViralInfections:**Usedinthetreatmentofviralinfectionssuchasherpessimplex,influenza,and otherviral conditions.
- **ChronicConditions:**Sometimesusedtomanage chronicinfectionsorconditions thatinvolveimmunesystemdysfunction.

Off-LabelUses: Occasionallyusedinotherimmunological conditionsorchronicdiseasesbasedonclinicalexperienceandphysiciandiscretion.

4. **DosageFormsandAdministration:**

- **DosageForms:**Availableinvariousformulations includingtablets,oralpowders,andinjectables.
- **Administration Routes:** Typically administered orally in tablet form, butmayalsobeavailableasaninjectableformdependingontheindicationandformulation.
- **CommonDosageRegimens:**
- **Adults:** Often prescribed in doses ranging from 1 to 2 grams daily,divided intomultipledoses.
- **Children:**Dosageisusuallyadjustedbasedonbodyweightandspecificcondition.

5. **Pharmacokinetics:**

- **Absorption:**Inosinepranobexisabsorbedfromthegastrointestinaltract,thoughtheextentofabsorptioncanbeaffectedbyfoodandformulation.

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- **Distribution:**Onceabsorbed,itdistributesthroughoutthebody,includingtissues and fluidswhereitexerts itstherapeutic effects.
- **Metabolism:**Inosinepranobexismetabolizedprimarilyintheliver,withitsactivecomponentsbeing processed andbrokendown.
- **Excretion:**Excretedthroughurine,primarilyasmetabolites.

6. SideEffectsandAdverseReactions:

- **CommonSideEffects:**
- Gastrointestinalissuessuchasnausea,vomiting,ordiarrhea.
- Mildheadacheordizziness.

SeriousAdverseReactions:

- Allergicreactionsincludingrash,itching,orswelling.
- Rarely,severelivertoxicityorhematologicalabnormalities.

7. ContraindicationsandPrecautions:

- **Contraindications:**
- Hypersensitivitytoinosinepranobexoranyofitscomponents.
- Severeliverdysfunctionorpre-existingliverdisease.

Precautions:

- Use with caution in patients with pre-existing kidney or liverconditions.
- Monitoringmayberequiredforpatientswithahistoryofallergicreactions.

8. DrugInteractions:

- **PotentialInteractions:**
- Mayinteractwithotherimmunosuppressivedrugsorantiviralagents.
- Potentialinteractionswithdrugsaffectinglivermetabolism.

9. StabilityandStorage:

- **StorageConditions:**
- Typicallystoredatroomtemperature,awayfromlightandmoisture.
- Tabletsshouldbekeptintightlyclosedcontainerstopreventmoistureabsorption.

ShelfLife:

- Usually has a shelf life of 2-3 years from the date of manufacturewhenstoredunderrecommendedconditions.

10. RegulatoryStatus:

- **Approval:** Inosine pranobex is approved for use in several countries,includingvariousEuropeannationsandothers,forthetreatmentofspecificviralinfections andimmunological conditions.
- **Regulatory Classification:** It is classified as a prescription medication, anditsuseis regulatedbynationalandinternationalhealth authorities.

11. Summary:

Inosine pranobex is a notable immunomodulatory agent with applications intreating viral infections and managing immune system-related conditions. Itsefficacyissupportedbyitsabilitytoenhanceimmune responsesandcombatviralactivity. Proper formulation, administration, and monitoring are essential tomaximizingtherapeuticbenefitswhileminimizing potentialsideeffects.

MaterialsandMethods

1. Materials:

1. DrugSubstance:

- **InosinePranobex**:Purereferencestandardobtainedfrom aSunPharmaceuticalindustriesPvt.Ltd

ChemicalsandReagents:

- **MobilePhaseComponents:**
- **Water**:High-puritygrade,typicallydeionizedordistilled.
- **Acetonitrile**:HPLCgrade.
- **AmmoniumPhosphateBuffer**:ForpHadjustment,analyticalgrade.
- **SampleSolvents:**
- **Methanol**:HPLCgrade,forsampledissolution.
- **DimethylSulfoxide(DMSO)**:If needed,HPLCgrade.
- **StandardsandImpurities:**
- **InosineStandard**:Formethodvalidationandcalibration.

Equipment:

- **HPLC System**: Equipped with a UV detector, autosampler, and suitable RP-HPLCcolumn (C18, 250 mm x 4.6 mm, 5 µm).
- **PipettesandMicropipettes**:Foraccuratemeasurementofreagentsandsamples.
- **FiltrationApparatus**:0.45 µmfiltersfor sample and solutionfiltration.
- **pHMeter**:ForadjustingandmonitoringpHofmobilephasesandsamplesolutions.

Software:

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- **HPLCDataAnalysisSoftware**:Forpeakintegrationandquantification.

2. Methods:

1. MethodDevelopment

1. ColumnSelection and Optimization:

- Use a C18 column (250 mm x 4.6 mm, 5 µm) for the RP-HPLC analysis.Optimizethecolumntemperature(commonlyat30°C)forbestperformance.

MobilePhasePreparation:

- **Mobile Phase A**: Prepare an aqueous phase by adding 0.1% phosphoric acid todeionizedwater.
- **MobilePhaseB**: Prepareby usingacetonitrile.
- **Gradient Profile**: Develop a gradient elution method starting with 90% A and10%B,transitioningto50%Aand50%Bover 20minutes.Adjustasnecessaryforoptimal separation.

DetectionWavelength:

- SettheUVdetectorat259nmformonitoringinosinepranobex.

SamplePreparation:

- **Stock Solution:** Dissolve an accurately weighed amount of inosine pranobex inmethanolorDMSOtoprepareastocksolution(e.g.,1mg/mL).Filterthrougha 0.45µm filter.
- **Standard Solution:** Prepare a series of standard solutions at differentconcentrations(e.g.,0.5 µg/mLto50 µg/mL)fromthestocksolutionforcalibration.

2. MethodValidation

1. Specificity:

- Inject standard solutions, placebo (excipients without drug), and spiked samplestoconfirm no interferencewithinosinepranobexpeak.

Linearity:

- Plotthecalibrationcurveusingthestandardsolutions.Assesslinearitybycalculatingthecorrelation coefficient(r^2)of thecalibration curve.

Precision:

- **Repeatability:**Analyze multiple injections(n=6)ofthe samesampletodetermineintra-day precision.
- **IntermediatePrecision:**Performtheanalysison differentdaysand/orbydifferentanalysts to evaluate inter-day precision.

Accuracy:

- Perform recovery studies by spiking known amounts of inosine pranobex into theplacebo matrix. Calculate the percentage recovery and ensure it falls within theacceptablerange(typically 98-102%).

LimitofDetection(LOD)and LimitofQuantitation(LOQ):

- DetermineLODand LOQ basedonthe standard deviationoftheresponse andtheslope of the calibration curve. LOD is calculated as $3.3 \times \sigma/S$ and LOQ as $10 \times \sigma/S$,whereσisthe standard deviation andSisthe slope.

Robustness:

- Test the method by varying parameters such as flow rate (±0.1 mL/min), columntemperature(±5°C),andpH ofthemobilephase(±0.2units)to assesstheimpactonmethod performance.

3. DegradationStudies

1. AcidicDegradation:

- Expose a solution of inosine pranobex to hydrochloric acid (0.1 N) at 60°C for 2hours.Neutralize, dilute, and analyzeby RP-HPLC.

AlkalineDegradation:

- Exposethedrug solutiontosodium hydroxide(0.1 N)at60°C for2hours.Neutralize,dilute,and analyze.

OxidativeDegradation:

- Treat the drug with hydrogen peroxide (3%) at room temperature for 2 hours.Analyzeafter dilution.

PhotolyticDegradation:

- Exposethe drug toUV light (365nm) for 24hours.Analyzethe samplebeforeandafter exposure.

ThermalDegradation:

- Store the drug at elevated temperatures (e.g., 60°C) for 1 month. Analyze samplesperiodically.

AnalysisofDegradationProducts:

- IdentifyandquantifydegradationproductsbycomparingretentiontimesandUVspectrawith standards and using the developed RP-HPLC method.

4. DataAnalysis:

- UseHPLC softwarefordataacquisition,peakintegration,and quantification.Validateresults by comparing them to known standards and evaluating method performanceparameterssuch asresolution, peaksymmetry, and retention time.

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5. Documentation:

- Documentallprocedures,results,andobservation smeticulously.Includecalibrationcurves, validation data, degradation study results, and any deviations from standardprocedures.

This comprehensive approach ensures that the RP-HPLC method for inosine pranobex is robust,accurate,and suitablefor routine analysis and stabilitytesting.

6. MATERIALANDMETHODS

1. SelectionandProcurementofDrugDrugsample supplier

NameofDrug	DrugSupplier
InosinePranobex	Sunpharmaceuticalpvt.Ltd

Table3:DrugandDrugSupplier

Listofreagents&chemicalsused

Table4:ListofReagentsandChemicalsused

Sr.No.	Nameofchemicals	Manufacturer.
1.	Acetonitrile(HPLCgrade)	MerckLtd.,India
2.	Methanol(HPLCgrade)	MerckLtd.,India
3.	0.1%OPA(HPLCgrade)	LobaChemie
4.	Ammoniumphosphate Buffer	Avantorperformance materialindiaLtd
5.	water(HPLCgrade)	Merkspecialitiesprivatelimited,Mumbai

2. Selectionofformulation:

MarketedPreparation:

TableNo.5:ListofbrandnamesofcombinedformulationsofInosinePranobex

Sr.No	Brandname	Formulation	Availablestrength	Address ofmanufacturer
1.	Viralex	Tablet	Inosine Pranobex500 mg	Themismedicareltd

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The marketed preparation was obtained from local market and is referred here after in thisthesisby thename as such.

3. SelectionofAnalyticalTechnique

HPLCwasselectedasanalyticaltechniqueforestimationofInosinePranobex.

- **Instruments:**

TheanalysisofthedrugwascarriedoutonAgilentTechnologyGradientSystemwithAuto injector, (DAD) &Gradient Detector. Equipped with Reverse Phase (Agilent) C18column (4.6mm x 250mm; 5µm), a 20µl injection loop and DAD Absorbance detector andrunningchemstation 10.1 software.

- **Stockpreparations**:
- StockI:StandardSamplePreparation

Std.InosinePranobex 10mgin10mlMethanol=100µg/ml

- StockII:formulationsolutionPreparation:-

Take13.54mlin10mlMethanoli.e=100µg/ml

- **ForAccuracySolutionPreparations:-**
- Take10µg/mlFORMULATIONFORACCURACY,

80%=0.1MLFORMULATIONandADD0.8mlSTDInosinePranobexANDMAKEUP VOL 10 ML WITHMobile Phase

100%=0.1MLFORMULATIONandADD0.1mlSTDInosinePranobexANDMAKEUP VOL 10 ML WITHMobile Phase

120%=0.1MLFORMULATIONandADD0.12mlSTDInosinePranobexANDMAKEUP VOL 10 ML WITHMobile Phase

4. InstrumentsandEquipments

	NameofInstrument	CompanyName
1	HPLCInstrument	AgilentTechnologyGradientSystemwithAutoinjector(chemstationsoftware10.1)
2	UV-Spectrophotometer	AnalyticalTechnologiesLimited
3	Column(C18)	AgilentC18(250mmX4.6mm,5µm)
4	pHmeter	VSIpHmeter(VSI1-B)
5	Balance	WENSAR™HighResolutionBalance.
6	Sonicator	Ultrasonicselectronicinstrument

TableNo.6:Instrument(HPLC)DetailsusedduringMethodDevelopment

7. EXPERIMENTALWORK

7.1.Preliminarycharacterization

Preliminarycharacterizationinvolveddeterminingcolour,odour,andappearance,whichareneededfor Inosinepranobex analysis.

Thefollowingcriteriawereusedtoanalyzecommondrugs.

A. Meltingpoint

Thepurityandstandard ofadrugcan bedeterminedbythemeltingpoint.It'sdeterminedwith the aid of digital melting point equipment. The optimized Melting point of inosinepranobexis 143 compares to thestandard is 140-145 in range.

B. SolubilityStudies

This study was carried out to find an ideal solvent in which drugs are completely soluble.Various solvents were tried for checking solubility of Inosine pranobex. From solubilitystudies it was concluded that of Inosine pranobex is freely soluble in Methanol and poorlysolublein water.

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C. UV-Spectrophotometer

UV Spectrophotometer was used to determine the ʎmax and it was compared to thestandardvalueacquired from books and researcharticles.

1. Preparationofstandardstocksolution

For the preparation of stock solution, weighed 10mg of drug Inosine pranobex transferredinto 10ml volumetric flask, added 10 ml of methanol and sonicate to dissolve the standardcompletely and make up the volume with methanol (1000µg/ml). Further diluted from0.1mlto 10 ml methanol(10µg/ml).

2.Selectionofanalyticalwavelength

Methanol as a blank and drug standard solution (10µg/ml) was scanned from 400nm to200nm&absorptionmaximaweredeterminedfordrugInosinepranobexshowedmaximumabsorbanceat259 nm.**(Fig No:7).**

3. HPLC:

1. SelectionofAnalyticalTechnique

HPLCwasselectedasanalyticaltechniqueforestimationofInosinepranobex.

a. Instruments:

The analysisofthe drug wascarriedouton AgilentTech Gradient SystemwithAuto injector DAD Detector Equiped with Reverse Phase C18(Agilent) with250mm x4.6 (5µm), a SP930D pump,a 20µlinjection loopandDADAbsorbancedetector andrunning chemstation software.

b. Selectionofstationaryphase:

The column used in this method C18 Agilent The configuration of the column is 4.6x250mm,particlesize5µmC18columngiveshighnonpolarretentively,symmetricpeak shape,highly reproducibleandstable ideal forHPLC method.

c. **Mobilephaseselection**:

In order to produce a sharp peak with a spectrum the mobile phases of the Inosinepranobexweremixedatvaryingratiosandflowrate.Water(AmmoniumAmmoniumPhosphateBuffer PH-7.4with0.1%OPA),methanol,andothersolvents consisting of one or more of the different mobile phases. Different ratiosand Mobile phase were examined, but a suitable peak was produced by combiningmethanolandwater(10mMAmmoniumAmmoniumPhosphateBufferpH-7.4with0.1% OPA)as asolvent.

d. **Preparation of Stock Solution:**10 mg of Inosine pranobex were dissolved in 10mlofmethanolwhichgivesconcentrationofstocksolutionwas1000µg/mlrespectively.

e. PreparationofStandardSolution:

Pipette out 0.2ml from stock solution and dilute with 9ml methanol & 1ml water to give10mlsolution.

7.4METHODDEVELOPMENTOFHPLC:

FollowingMobilephaseweretried

TableNo.8:SelectionofmobilePhase

Sr.No	MobilePhase
1.	Methanol+Buffer(90+10)259nm,20Mg,Flowrate0.7min /ml.
2.	Methanol+Buffer(80+20),259nm,Flowrate0.7ml/min.
3.	Methanol+Buffer(70+30),259nm,Flowrate0.7ml/min

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4.	Methanol+Buffer(60+40%v/v),259nm,Flowrate0.7ml/min
5	Methanol+Buffer(55+45%v/v),259nm,Flowrate0.7ml/min.
6	Methanol+10mmAmmoniumPhosphateBufferpH-7.4with0.1%OPA(45+55%v/v),259nm,andFlowrate0.8 ml/min.

7.4Studyonthechromatographicconditionsof Inosinepranobex:

Accuratelyweighandtransfer10mgInosineP ranobexworkingstandardinto10mlvolumetric flask as about dilute Methanol prepared in completely and make volume up tothe mark with the same solvent to get 1000µg/ml standard (stock solution) and 15 minsonicate to dissolve it and from the resulting solution 0.1-05ml was transferred to 10 mlvolumetricflaskandthevolumewasmadeuptothem arkwithmobilephaseMethanol:(10mM Ammonium Ammonium Phosphate Buffer pH adjusted 7.4 with OPA)Water solvent. The resulting 10-50µg/ml of solution was subjected to chromatographicanalysesusingmobilephasesofdiffe rentstrengthswithchromatographicconditionsmentio nedbelow: **(Table No: 11).**

Analyticalcolumn :AgilentC18Column(250mmx4.6mm),5µ mparticlesize.Injectionvolume : 20µl
Flowrate:0.8ml/min
Detection:259nm
RunTime:10min

1. Preparationofstandardstocksolution:-

- **Inosinepranobexstandardstocksolution:(Sto ck I)**

An accurately weighed quantity, 10 mg of Inosine Pranobex (ISP) was dissolved inMethanol in a 10 ml volumetric flask and volume made up to 10.0 ml to produce asolutionof 1000 µg/ml.

a)Take 0.1-05 ml from stock solution and volume make up with mobile phase (4.5mlMethanol+5.5mlBuffer) Solutioncontains 10-50 µg/mlof Inosinepranobex.

8. StudiesofCalibrationplot:-

1. OptimizationofChromatographiccondition:

Thefollowingchromatographicconditionswereestabl ishedbytrialanderrorandwerekeptconstantthroughou t theanalysis.

Analyticalcolumn :AgilentC18Column(250mmx4.6mm)parti calsize:5µm
Injectionvolume :20µl
Flowrate:0.8ml/min
Detection:259nm
RunTime:15min
Mobilephase:Methanol:(10mMAmmoniumPhosph ateBufferpHadjusted7.4withOPA)(45%:55%)

9. ProcedureforcalibrationcurveofInosinepran obex:

Themobilephasewasallowedtoequilibratew ithstationaryphaseuntilsteadybaselinewas obtained. From the freshly prepared standard stock solution, pipette out 10 mg Inosinepranobexin10mlofvolumetricflaskanddilute dwithmobilephase.Fromit0.1,0.2,0.3,
0.4 and 0.5ml of solution were pipette out in 10 ml volumetric flask and volume was madeup to 10 ml with mobile phase to get final concentration 10, 20,30,40,50 µg/ml of Inosinepranobex. Sample were injected and peaks were recorded at 259 nm as the graph plotted asconcentrationofdrugversespeak areais depictedin**(fig. no.15)**respectively.

10. Studyofsystemsuitabilityparameters:

The system suitability is used to verify, whether the resolution and reproducibility of thechromatographic system are adequate for analysis to be done. The test was performed

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bycollectingdata from tworeplicateinjections of standard solution.

1. CalibrationExperiment:

- **RP-HPLCMethod:**

a. PreparationofCalibrationcurvestandard:

The above standard stocksolution(1000µg/ml) of Inosine pranobexwasdiluted withmobilephasetoyieldFivecalibrationcurve(cc)standardswithconcentrationsof10,20,30,40,50 µg/ml of Inosine pranobex. The calibration curve of Inosine pranobex isdepictedin (**FigNo.15)**.

b. SelectionofdetectionWavelength:

Standard solutions were scanned in the range of 200-400nm ,against 10 ml Methanol andvolumemakewithwatersolventsystemasreferenceInosinepranobexwereshowedabsorbancemaxima (lamdamax)at 259 nm**(FigureNo:7**).

c. Calibrationstandarddrugandregression equationdata:

FromthestandardstocksolutionofInosinepranobex,differentconcentrationwereprepared respectively in the range of 10-50 µg/ml for Inosine pranobex **(Figure No:18)** andmeasuredat259nm.The calibrationcurveswere plottedun Regressionequationdatapresentedin **(Table No:22).**

d. Calibrationrunsandregression analysis:

Thesecalibrationstandardsolutionswereanalyzedinthreereplicatesusingtheundermentionedchromatographic conditions.

Analyticalcolumn :C18Column(250mmx4.6mm,5µmpartical size).Injectionvolume :20µl.
Flowrate:0.8ml/min.

Mobilephase :Methanol:Water(10mMAmmoniumPhosphateBufferpHadjusted7.4 withOPA)(45:55%V/V).
Detection :259nm.

11. ValidationofmethodforanalysisofInosine pranobex:

- Thedevelopedmethodwasvalidatedasper ICHguidelines.

1. Linearity:

Linearity of an analytical method is its ability to elicit test results that are directly or by awell defined mathematical transformation, proportional to the concentration of analyte insampleswithin agiven range, TheResult areshown in;**(Table No:9).**

Determination:

The linearity of the analytical method is determined by mathematical treatment of testresults obtained by analysis of samples with analyte concentrations across the claimedrange. Area is plotted graphically as a function of analyte concentration. **(Fig No. 19-29)**Percentage curve fittings are calculated**.** The Result are shown in; **(Table No.34 and TableNo.35).**

AcceptanceCriteria:

Theplotshouldbelinearpassingthroughtheorigin.
CorrelationCoefficient shouldnotbelessthan0.999.TheResult areshownin;

Preparationofstandardstocksolutionforlinearity:

10mgofInosinepranobexwereweighedandtransfered to10mLvolumetricflask&diluentwasaddedtomakeupthevolume.Sonicatedfor10minwithoccasionalswirling.
0.1mlofthissolutiondilutedupto10mlvolumetric flaskwithdiluentswasaddedtomakeup the volume.

Preparationoflinearitysolution:

Aseriesofstandardpreparationsofworkingstandardofwereprepared.

TableNo.9:TableoflinearityforRp-HPLCMethod

LinearityofInosinepranobex HPLC	
Sr.No.	**Concentratin(µg/mL)**
1	10
2	20
3	30
4	40
5	50

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2. Accuracy(recovery):

Theaccuracyofananalyticalmethodistheclosenessoftestresultsobtainedbythatmethod to the true value. Accuracy may often the expressed as percent recovery by theassayofknownaddedamountsofanalyte.Theaccuracyofananalyticalmethodisdetermined by applying the method to analyzed samples, to which known amounts ofanalyte have been added. The accuracy is calculated from the test results as the percentageofanalyterecoveredby theassay, TheRP-HPLCResult areshown in;**(Table No:39,40).**

AcceptanceCriteria:

Meanrecoveryshouldbeintherangeof98-102%.

TheRelativeStandardDeviationshouldnotbemorethan2.0%.

Preparationofstandardstocksolution:

10 mg of Inosine pranobex working standards were weighed and transfered to 10 mLvolumetric flask & diluent was added to make up the volume 0.2 ml of this solution dilutedupto 10ml with diluent.

Application of proposed method for analysis of tablet formulation:Accuracy

The accuracy was determined by Inosine pranobex (equivalent to 10 mg (80 %, 100 % and120 % of the label claimed, respectively) to quantity equivalent to average weight ofmarketed tablets. This powder containing 10 mg of Inosine pranobex were triturated andthen subjected to chromatographic analysis using the described method. The resulting wasanalyzed in triplicates over three days. The % recovery of added drug was taken as ameasureofaccuracy**.**
TheResultareshownin;**(FigNo:31,32,33).**

TableNo.10:TableofAccuracyforRp-HPLCMethod

Sample	**AmountAdded(mg)**
	Inosinepranobex
Accuracy80%	8
Accuracy 100%	10
Accuracy 120%	12

3. Repeatability:

Precision of the system was determined with the sample of RP-HPLC for. Three replicatesofsamplesolutioncontaining10mgofInosinepranobexwereinjectedandpeakareaswere measured and %RSD was calculated. Was repeated for five times result are shown in;**(TableNo : 43) &(FigNo :34,35).**

- **Applicationofproposedmethodforanalysisof Repeatability:**

10 mg was weighed and transfered to 10 mL volumetric flask & diluent was added to makeup the volume. Sonicated for 10 min with occasional swirling. The above solution wasfiltered through 0.45µm membrane filter 0.5 ml of this solution diluted upto 10 ml withdiluent.

4. Precision:

Precision of an analytical method is the degree of agreement among Individual test resultswhen the procedure is applied repeatedly to multiple Samplings of a homogenous sample.Precision of an analytical method is usually expressed as standard deviation or relativestandard deviation. Also, the results obtained were subjected to one way ANOVA andwithin-daymeansquareandbetween-daymeansquarewasdeterminedandcomparedusingF-test. **(Fig No:36).**

- **ResultofIntradayandInterdayPrecisionstudiesonRP-HPLCmethodforInosinepranobex**

1. Intra-dayprecision:

Samplesolutionscontaining10mgof Inosinepranobexthreedifferentconcentration(10µg/ml, 30µg/ml, 50µg/ml Inosine pranobex were analyzed three times on the same dayand%R.S.D

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wascalculated. TheResult areshownin;**(Table No.50)&(Fig No:36-38).**

2. Inter-dayprecision:

Samplesolutionscontaining10mgofInosine pranobexthreedifferentconcentration(10µg/ml,30µg /ml,50µg/ml)inHPLCandthreedifferentconcentratio ninInosinepranobexdifferentdaysand%R.S.Dwascal culated.Itisusuallyexpressedasstandard deviationorrelativestandarddeviation.TheResultares hownin;**(TableNo.50)&(FigNo:39-41).**

- **Acceptancecriteria:**

TheRelativeStandardDeviationshouldnotbemoretha n2%fortest

- **Preparationofstandardstocksolution:**

10 mg of Inosine pranobex working standards were weighed and transfered to 10 mLvolumetricflask&diluentwasaddedtomakeupthe volume.0.1mlofthissolutiondilutedupto 10 ml with diluent.

5.Robustness:

The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion and the flow ratewas**(FigNo:42,43)**ofMethanol:Water(10mMA mmoniumPhosphateBufferpHadjusted 7.4 with OPA) in the mobile phase composition (±1ml/ min^{-1}**)** and the change indetection wavelength (±1 ml/ min-1**)** and the effect of the results were examined**.(Fig No:44,45)**and **(Fig No:46,47)** it was performed using 30µg/ml solution of Inosine pranobex inreplicate.TheResult are shown in;**(Table No:57).**

5. DetectionLimit

Based on the S.D. of the response and the slope of calibration curve, the detection limit(DL)was calculated as,

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

Where,

σ=theS.D.ofthey-interceptsofregressionlines.S=the slopeof thecalibration curve.
TheslopeSmaybeestimatedfromthecalibrationcurve andS.D.wasusedshouldbecalculated from they-interceptsofregression linein calibration curve.
Theresultareshownin:**(chapter:8).**

6. QuantitationLimit

Based on the S.D. of the response and the slope of calibration curve, the quantitation limit(QL)was calculated as,

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

Where,
σ=theS.D.ofthey-interceptsofregressionlines.S=the slopeof thecalibration curve.
TheslopeSmaybeestimatedfromthecalibrationcurve andS.D.wasusedshouldbecalculated from they-interceptsofregression linein calibration curve.
Theresultareshownin**(chapter:8)**

7.12Analysisofmarketedformulation

Weigh Equivalent weight of tablet 13.54 mg. add about 10 ml of diluent and sonicate todissolve it completely and make volume up to the mark with diluent. Mix well and filterthrough 0.45 µm filter. Further pipette 0.3 ml of the above stock solution into a 10 mlvolumetricflaskanddiluteuptothemarkwithdiluent s(30µg/ml).Thesimplechromatogram of test Inosine pranobex Shown in **(Fig No: 48)** the amounts of Inosinepranobex per tablet were calculated by extrapolating the value of area from the calibrationcurve.Analysisprocedurewasrepeatedfive timeswithtabletformulation.TabletAssayfor%Lable claim for%RSDCalculated, Result was shownin**(Table No. 59).**

BrandName:Viralex(500mg)[ThemisMedicareL td]

Totalweightof 20tabPowderwt.=13.54 gms

AvgPowder Weight =0.677 gms./TabEq.Weight10 mg= 10xAvg.Wt. 10x677
_____=_____=13.54mgLableclaim 500
Take13.54mgtabletpowderwt.in10mlMEOH=1000 µgm/ml

Ruggedness

The degree of reproducibility of test result obtains by the analysis of same sample undervariety of Condition. Such as different analyst, laboratory Different instrument. Result wasshownin **(Table No. 59), (Fig No:48).**

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Forceddegradationstudies:

Stress degradation of the method was performed to measure the analyst response in thepresence of its potential impurities. Stress testing of the individual drug substance and thecombination was performed to measure the resolution factors of the drug peak from itsnearest resolving peak and also from all other peaks. The drugs were subjected to acidic,alkaline,oxidizingandphotolyticconditions.Foracidicdegradation,thedrugsweresubjected to 0.1 N hydrochloric acid for 2 hr. for the alkaline degradation the drugs treatedwith0.1Nsodiumhydroxidefor1hr.Oxidativestudieswerecarriedoutusing3%hydrogenperoxidefor 2hr.Thermaldegradationthedrugtreatedwith1hr.Photodegradation was performed by exposing the solution to light for 24h. After the completionof the treatment, the solutions were left to return to room temperature, neutralized (foracidicandbasicdegradation)anddilutedwiththemobilephasetoobtainthefinalconcentrationof30 µg/mL.

Degradationbehavior

Forced degradation studies of both the drugs namely Inosine pranobex were carried outindividuallyandinunderdifferentstressconditionslikeacidhydrolysis,alkalinehydrolysis, hydrogen peroxide oxidation and photolysis. The results are shown in (**Tableno: 60).**

1. Acidhydrolysis:

The acid hydrolysis performed using 0.1N HCl for 2 hr for Inosine pranobex indicateddegradation.7.20 %anddegradationwas observed 2 hr.**(Figno: 53).**

2. Alkalinehydrolysis:

The alkaline hydrolysis condition was performed using 0.1N NaOH for 1 hr and 2 hrInosinepranobex.DegradationofInosinepranobex2 hrwasfoundtobe5.44%.**(Figno :50).**

3. Oxidation:

In the oxidation condition with 3% H2O2 for 1 hr and 2 hr Inosine pranobex show anyoxidative stress degradation peak in the chromatogram. Degradation of Inosine pranobexwereobserved 3.16%for 2 hrrespectively (**Fig no: 56).**

4. Neutral:

There was no major degradation observed for Inosine pranobex and hence they were notsensitiveto light for2hr. **(Fig no: 59).**

II. RESULTANDDISCUSSION:

1. PreliminarystudiesonInosinePranobex

TableNo.7PreliminarystudiesonInosinePranobex

1	**Appearances**	**Solid**
2	**Color**	**WhitetoOffwhitePowder**
3	**Taste**	**BitterTaste**

1. Meltingpoint

TheprocuredreferencestandardofInosinePranobexwasfoundtomeltintherangeof140-145 ^{0}C.

2. Solubility

Thedrugwasfoundtobe

- FreelysolubleinMethanol,Ethanol,DMSO.
- PracticallyinsolubleinEther.
- **8.1.3.UVSpectroscopy**

Standard solutions were scanned in the range of 200-400nm ,against 10 ml methanol andvolume make with water solvent system as reference Inosine Pranobex in Methanol wasfoundto be selected wavelength is 259 nm**(FigureNo:07).**

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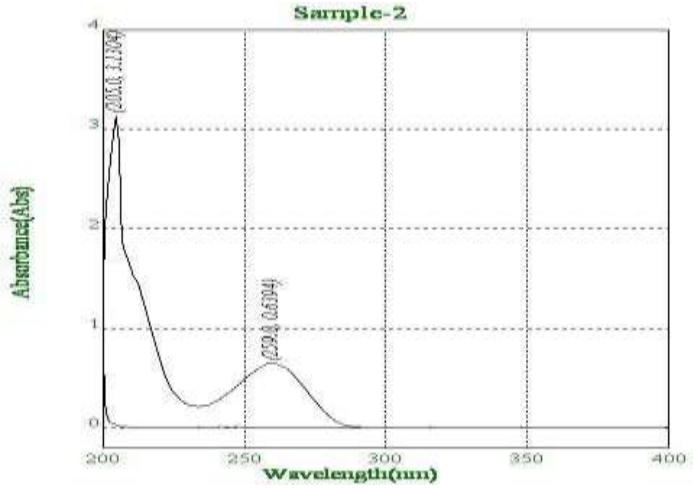

Fig.7:UVSpectrum ofInosinePranobex

TableNo.8:ChromatographicbehaviorofInosinePranobexmobilephaseofvarious.

FigNo	Columnused	Mobile phase, FlowRateandWavelength	Inj.Vol.	Observation	Conclusion
1.	C18(Agilent)(4.6mmx 250mm), 5.µm)	90% Methanol +10%AmmoniumPhosphatebuffer pH-7.4 with0.1%OPA259nm 10Mcg,Flowrate 0.7ml/min	20µl	SharpPeakswerenotobtained	Hencerejected
2.	C18 (Agilent)(4.6 mm x 250mm), 5µm)	80%Methanol+ 20%AmmoniumPhosphate buffer(80+20)pH-7.4with 0.1%OPAWater,Ph-3 259nm 20Mcg,Flowrate 0.7ml/min	20µl	Sharp Peakswere notobtained	Hencerejected
		70%Methanol +			

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3.	C18(Agilent)(4.6mmx 250mm), 5µm)	30%AmmoniumPhosphatebuffer(70+30), pH-7.4 with0.1%OPA,259nm, Flowrate 0.7ml/min	20µl	SharpPeakswerenot obtained	Hencerejec ted
4.	C18(Agilent)(4.6mmx 250mm),5.0µ)	60% Methanol +40%Ammonium Phosphatebuffer(60+40), pH-7.4	20µl	SharpPeakswerenotobt ained	Hencerejec ted

		with0.1%OPA259nm,Flow rate 0.7ml/min.			
5.	C18(Agilent)(4.6mmx250 mm), 5µm)	Methanol + AmmoniumPhosphatebuffe rpH-7.4with0.1% OPA (60+40%v/v),259nm, Flowrate0.7ml/min	20µ l	SharpPeakswerenot obtained	Hencereject ed

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6.	C18(Agilent)(4.6mmx250mm),5.0µ)	Methanol + AmmoniumPhosphatebufferpH-7.4with0.1%OPA (45+55%v/v),259nm, Flowrate0.8ml/min	20µl	Sharp andwellresolvedPeakswereobtained	Henceselected

Thus,fromtheabove,ithasbeenobservedthat ,usingmobilephaseofMethanol+Ammonium Phosphate buffer pH-7.4 ,with 0.1% OPA water (45:55 % v/v) 259nm, Flowrate 0.8 ml/min gave adequate retention time is 5.555 min with good shape peak will beobtained.

ChromatogramofTrial1:

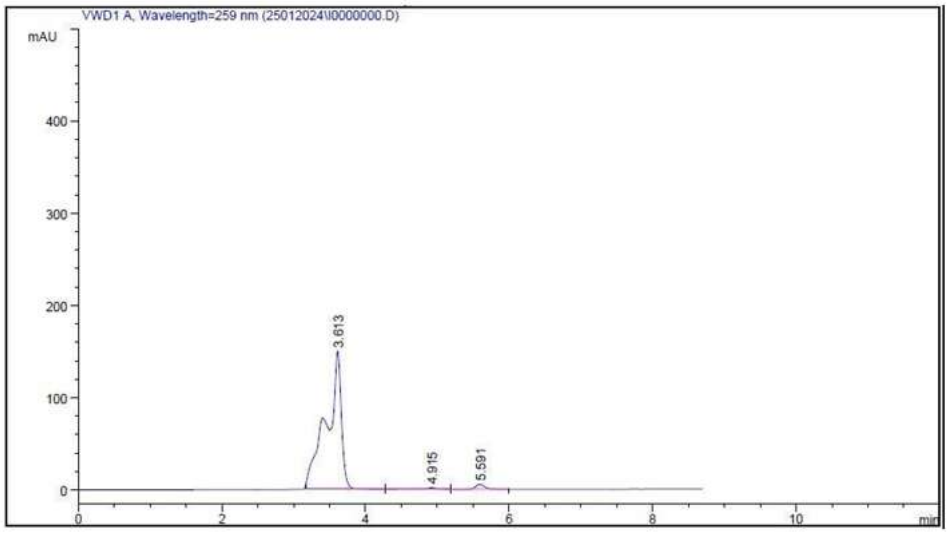

Fig.8:ChromatogramofTrial1

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.613	2170.05469	928	2.94	--
2	4.915	20.59516	9092	1.96	3.82
3	5.591	48.1525	10083	0.90	3.14

TableNo9 Resultofchromatogram oftrial 1

Observation:sharppeakwerenotobtained,sothismethodwasrejected.Unsatisfactoryresultis obtained.

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ChromatogramofTrial2:

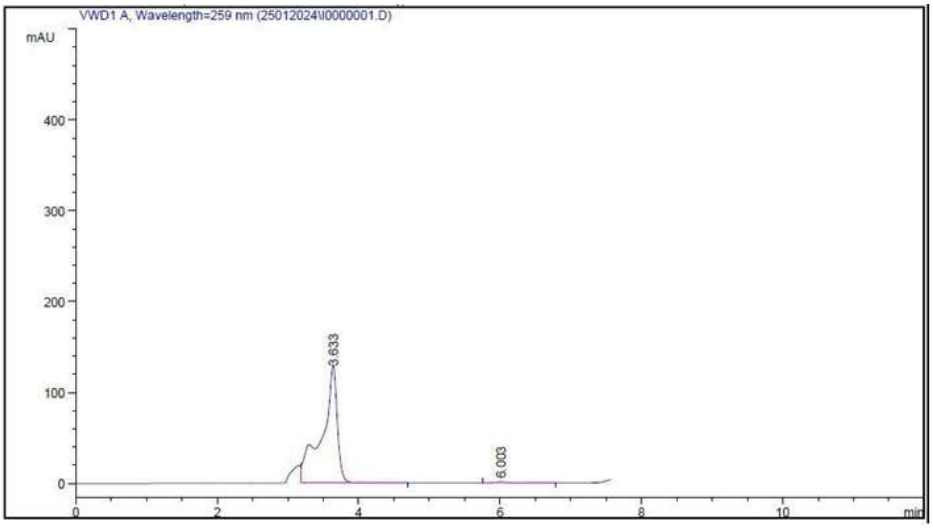

Fig.9:Chromatogramof Trial2

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.633	1969.28125	3031	2.37	--
2	6.003	14.44654	7783	0.40	8.83

TableNo.10Resultofchromatogram oftrial 2

Observation:sharppeakwerenotobtained,peaksplittingareobserved,sothismethodwasrejected. Unsatisfactory result is obtained.

ChromatogramofTrial3:

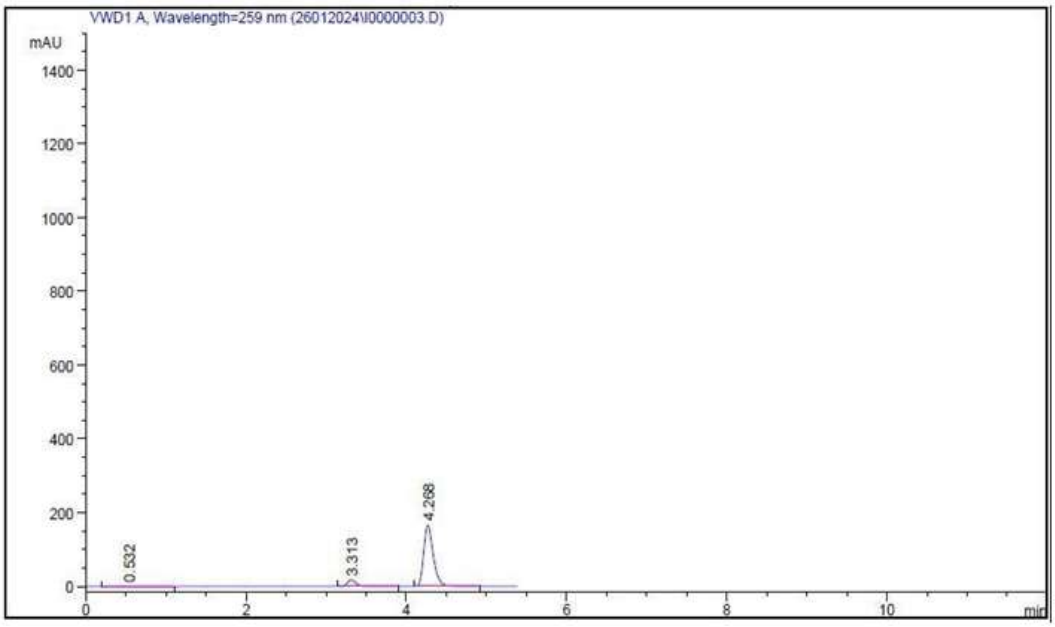

Fig.10:ChromatogramofTrial 3

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	0.532	29.02412	5308	0.71	--
2	3.313	152.35164	9261	2.31	2.29
3	4.268	120.81380	-	4.30	-

TableNo.11Resultofchromatogram oftrial 3

Observation:sharppeakwerenotobtained,peaktelling areobserved,sothismethodwasrejected. Unsatisfactory result is obtained.

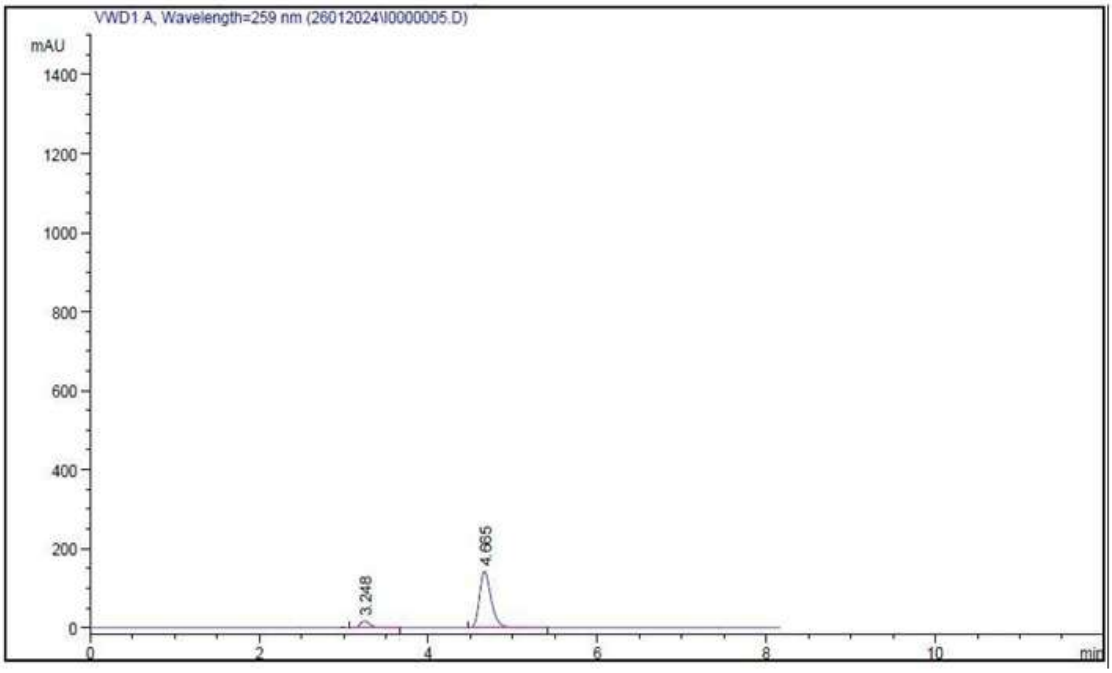

Fig.11:ChromatogramofTrial 4

.No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.248	137.13821	3969	0.79	-
2	4.665	1373.50842	5883	0.71	6.30

TableNo.12Resultofchromatogram oftrial 4

Observation:sharppeakwerenotobtained,peaksplittingareobserved,sothismethodwasrejected. Unsatisfactory result is obtained.

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ChromatogramofTrial5:

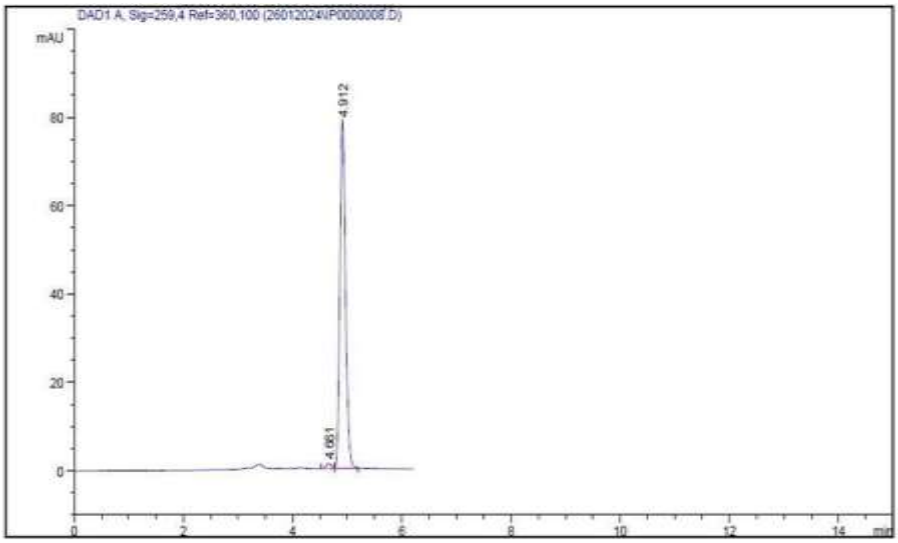

Fig.12:ChromatogramofTrial 5

.No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	4.661	8.39332	9370	0.91	-
2	4.912	558.01556	11746	0.79	1.34

TableNo.13Resultofchromatogram oftrial 5

Observation:sharppeakwerenotobtained,peaksplittingareobserved,sothismethodwasrejected. Unsatisfactory result is obtained.

ChromatogramoffinalTrial6:

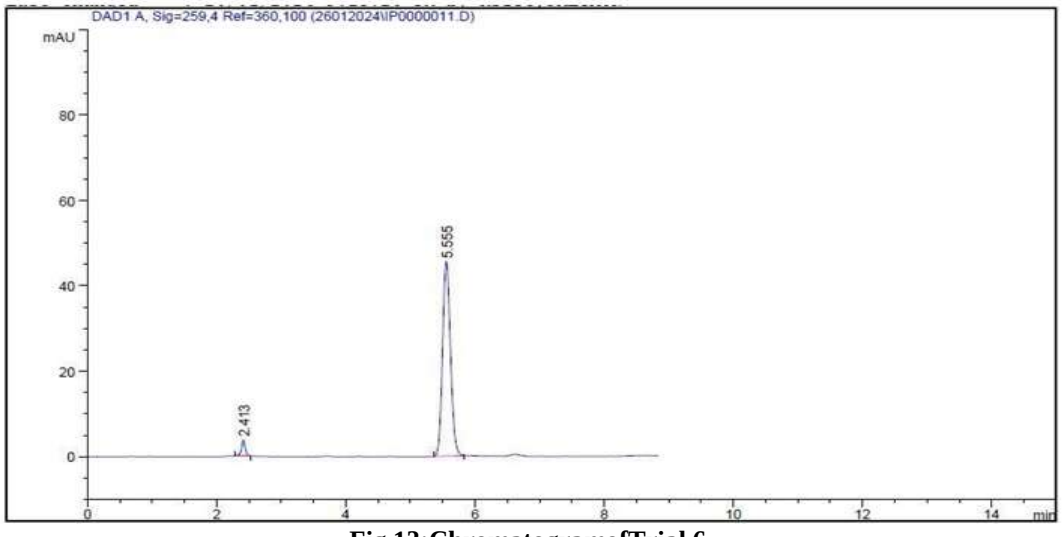

Fig.13:ChromatogramofTrial 6

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.No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	2.413	16.44549	9216	1.03	--
2	5.555	391.08365	10115	0.85	19.51

TableNo.14Resultofchromatogram oftrial 6

Observation: Sharppeakwasobtainedsothismethod wasselected.

- **Thefinalchromatographicconditionsselected wereasfollow:**
- **Analytical Column**: Agilent C18 Column (250mm x 4.6mm, 5µmparticalsize).
- **Injectionvolume** :20µl.
- **Flowrate** :0.8ml/min.
- **Mobilephase** :Methanol:(PhosphatebufferpH-7.4with1%OPA)Water (45:55%V/V)
- **Detectionwavelength**:259nm.
- **RunTime** :15min.
- **PreparationofStandardchromatogramofInosinePranobex**

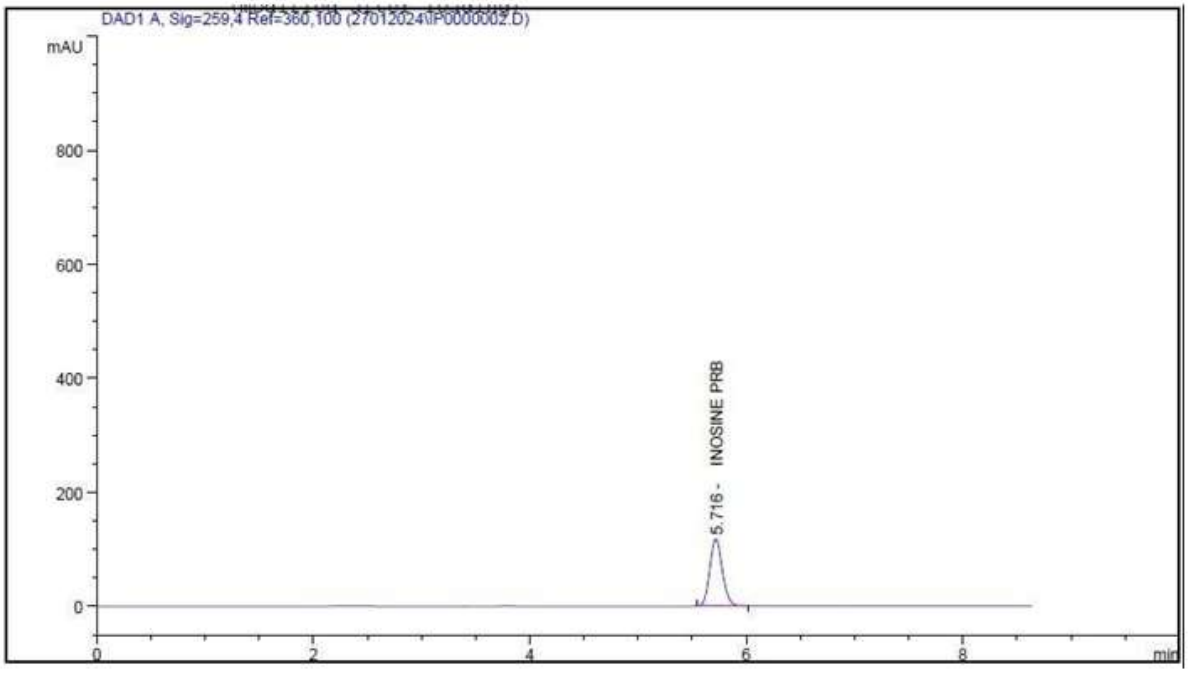

Fig.14:ChromatogramofstandardInosinePranobex

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.716	956.42035	12031	0.84	--

Table.No15DetailsofchromatogramofstandardInosinePranobex

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In the standard of Inosine Pranobex theoretical plates were found above 2000 i.e. forInosinePranobex is 12031 at minimum RT 5.716.

5. Calibrationexperiment

- **RP-HPLCMethod:**

Thedataobtainedinthecalibrationexperimentswhensubjectedtolinearregressionanalysis showed a linear relationship between peak areas and concentrations in the range10-50µg/mlforInosinePranobex(**TableNo:16)**depictthecalibrationdataofInosine Pranobex .The respective linear equation for Inosine Pranobex was Y= 93.82 x – 9.564wherexistheconcentrationandyis areaofpeak.Thecorrelationcoefficientwas0.999.The calibration curveofInosinePranobex is depicted in (**FigNo.15).**

Method	Concµg/ml	Peakarea(µV.sec)		Averagepeak area(µV.sec)	S.D. ofPeakArea	%RSD of PeakArea
		1	2			
RP-HPLC Method	10	956.0263	956.450	305.685	0.77	0.25
	20	1894.0279	1896.92	718.485	1.82	0.25
	30	2791.7861	2799.79	1140.14	2.88	0.25
	40	3746.9580	3767.88	1606.42	1.44	0.09
	50	4716.0210	4716.87	2036.725	6.26	0.31
	Equation		Y=93.82x–9.564			
	$_R2$		0.999			

TableNo16LinearitydataforInosinePranobex

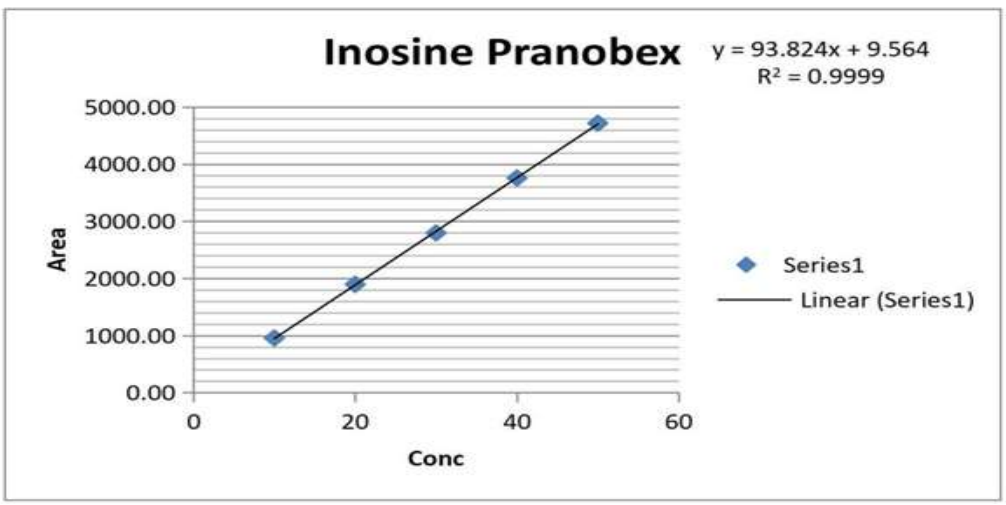

Fig.15:CalibrationcurveofInosinePranobex(HPLC)

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TheRP-HPLCMethodforrespectivelinearequationforInosine Pranobexwasy=93.8

x – 9.564 where x is the concentration and y is area of peak. The correlation coefficientwas0.999. Thecalibration curveofInosinePranobex is depicted in**Fig15.**\

2. AnalyticalofMethodValidation:

1. Linearity:

From Inosine Pranobex standard stock solution, different working standard solution (10-50µg/ml) were prepared in mobile phase20 µl of sample solution was injected into thechromatographic system usingmixedvolumeloopinjectorChromatograms wererecorded.The areas foreachconcentration wererecorded(**TableNo.17)**TheCalibration curvesareshownin**[Fig. No.16].**

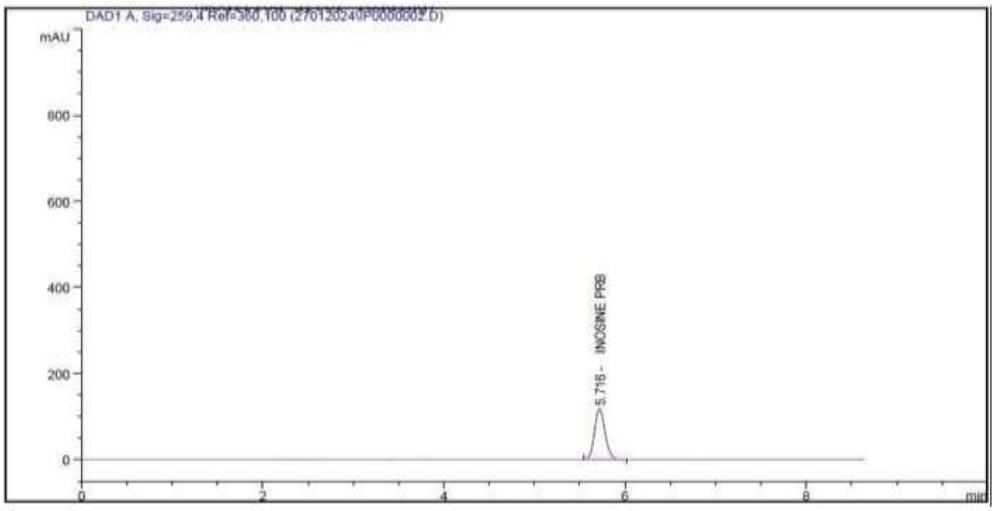

Fig.16:Chromatogramoflinearity

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.716	956.42035	12031	0.84	--

TableNo.17DetailsofchromatogramofLinearity

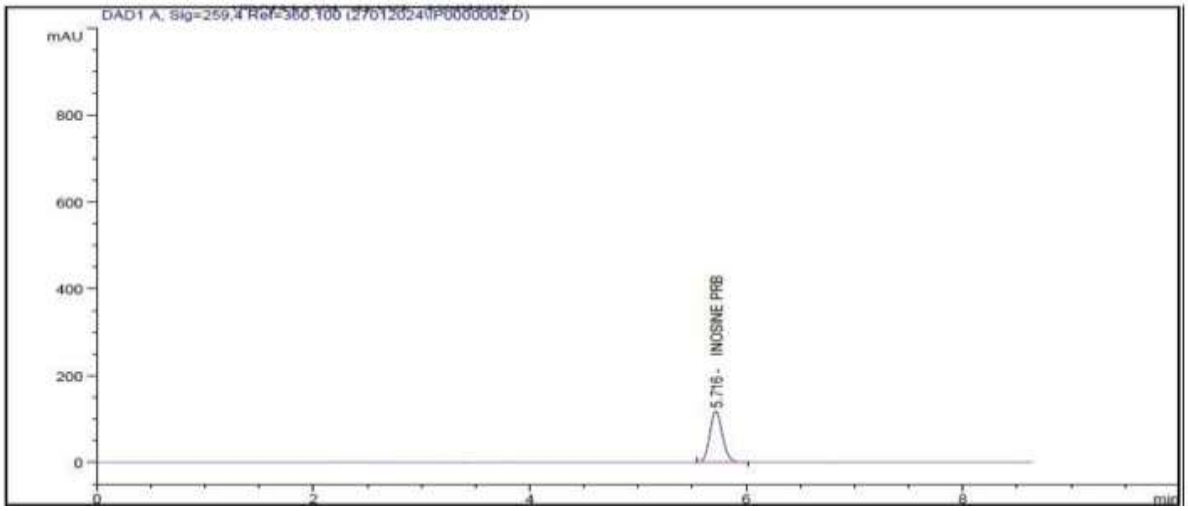

Fig.`17Chromatogramoflinearity(10mcg)-01

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.716	956.42035	12031	0.84	--

TableNo.18Detailsofchromatogram ofLinearity(10mcg)-01

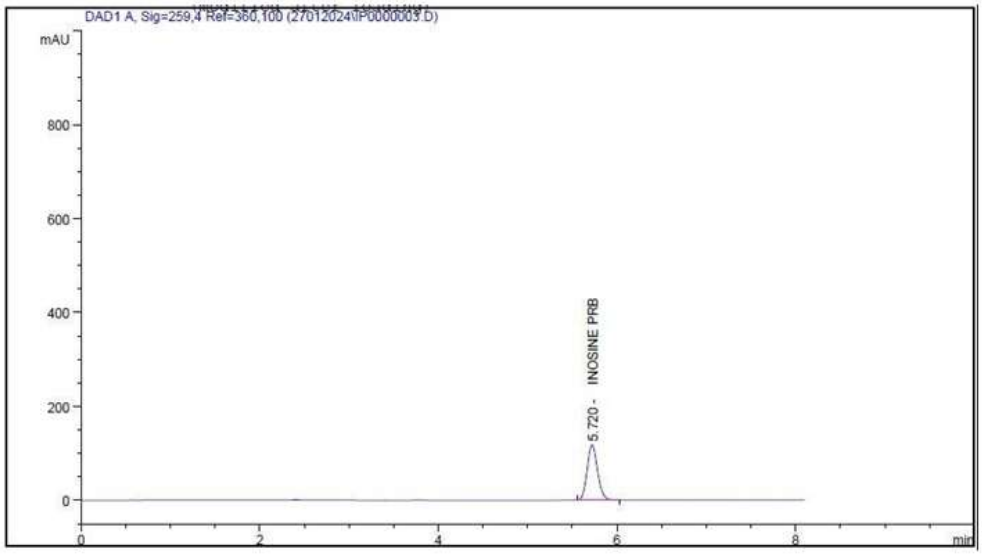

Fig.18Chromatogramoflinearity(10mcg)-02

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.720	957.35382	11788	0.84	--

TableNo.19DetailsofchromatogramofLinearity(10mcg)-02

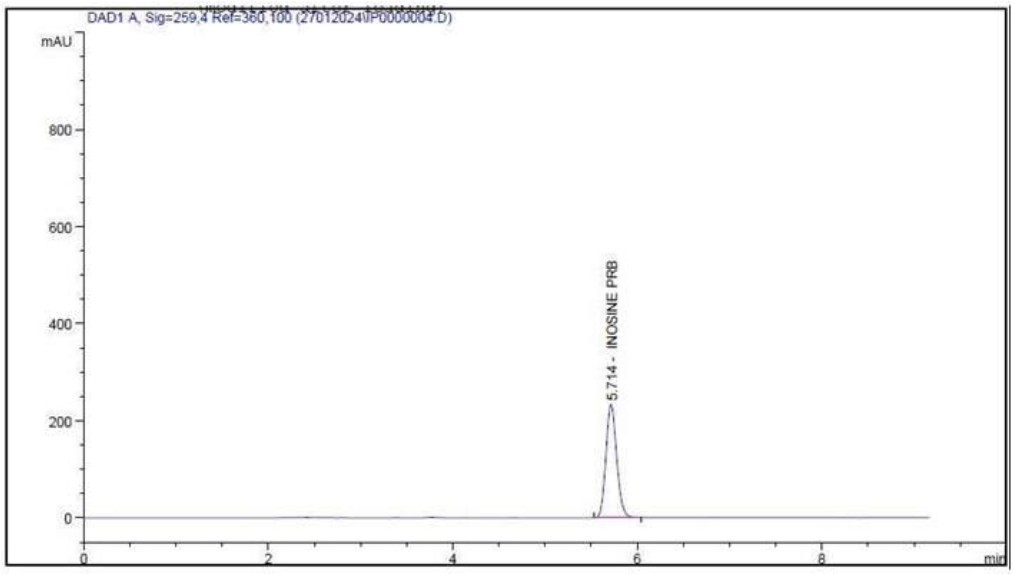

Fig.19Chromatogramoflinearity(20mcg)-01

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.714	1894.02795	11762	0.84	--

TableNo.20Detailsofchromatogram ofLinearity(20mcg)-01

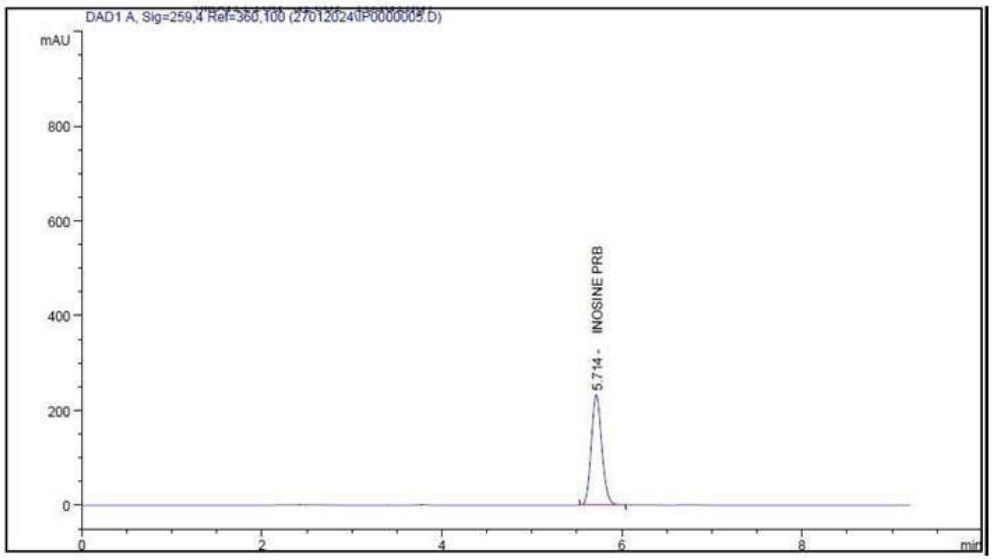

Fig.20Chromatogramoflinearity(20mcg)-02

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.714	1896.92249	11762	0.84	--

TableNo.21Detailsofchromatogram ofLinearity(20mcg)-02

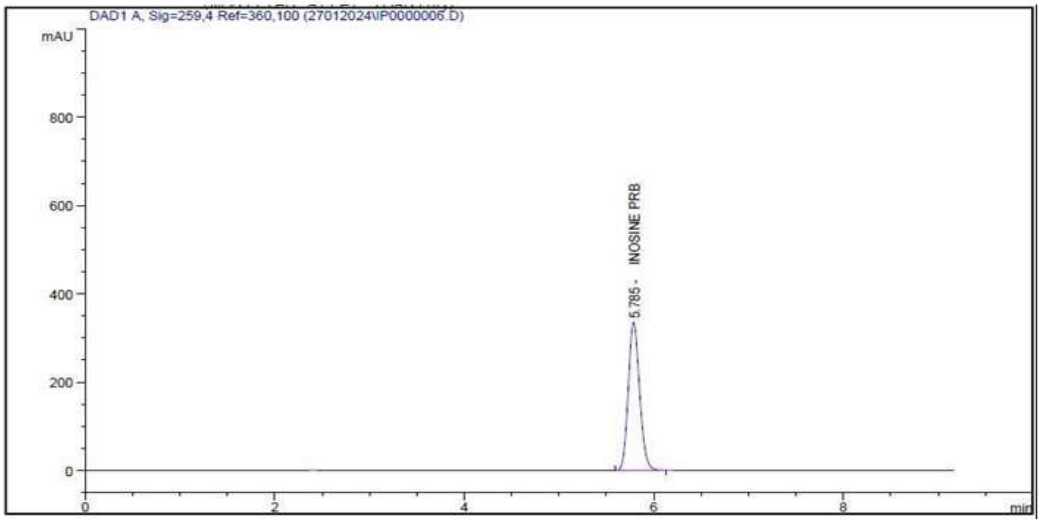

Fig.21Chromatogramoflinearity(30mcg)-01

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.785	2791.78613	11557	0.84	--

TableNo.22Detailsofchromatogram ofLinearity(30mcg)-01

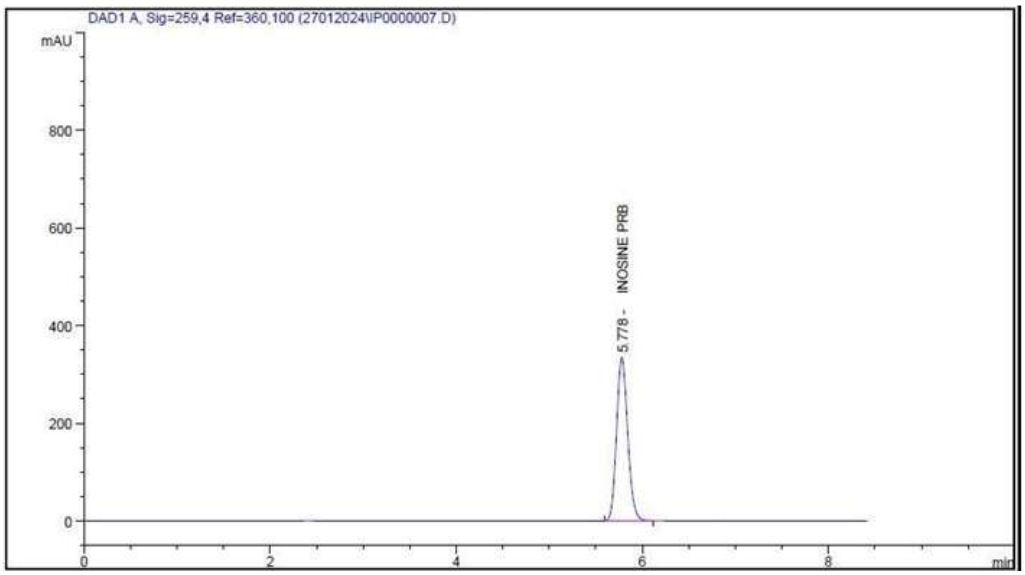

Fig.22Chromatogramoflinearity(30mcg)-02

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.778	2789.79639	11526	0.84	--

TableNo.23Detailsofchromatogram ofLinearity(30mcg)-02

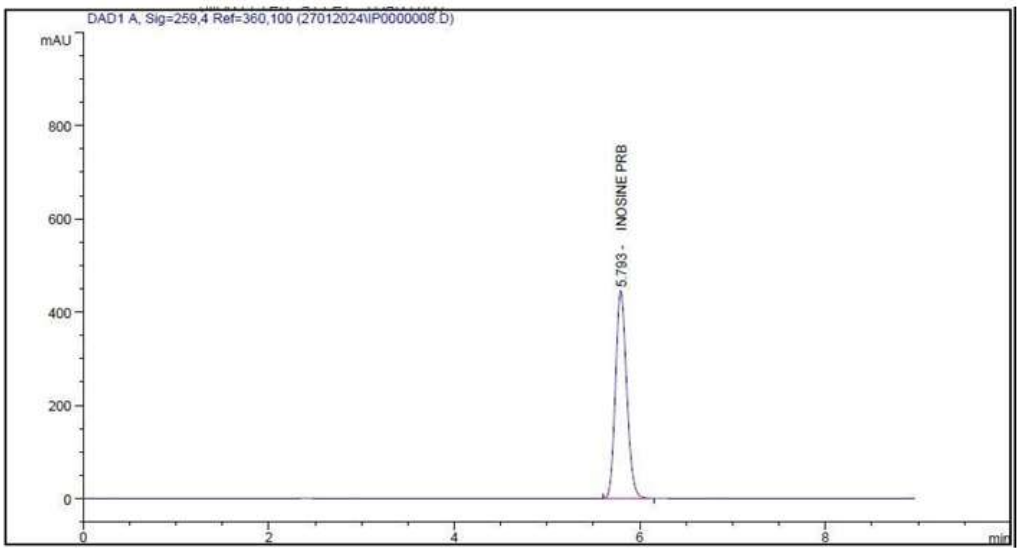

Fig.23Chromatogramoflinearity(40mcg)-01

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.793	3746.95801	11587	0.83	--

TableNo.24Detailsofchromatogram ofLinearity(40mcg)-01

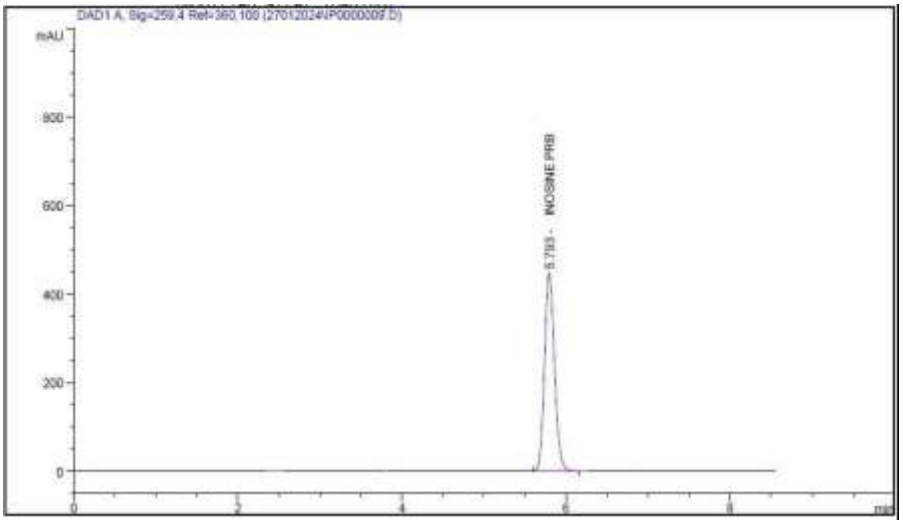

Fig.24Chromatogramoflinearity(40mcg)-02

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.793	3767.88013	11587	0.83	--

TableNo.25Detailsofchromatogram ofLinearity(40mcg)-02

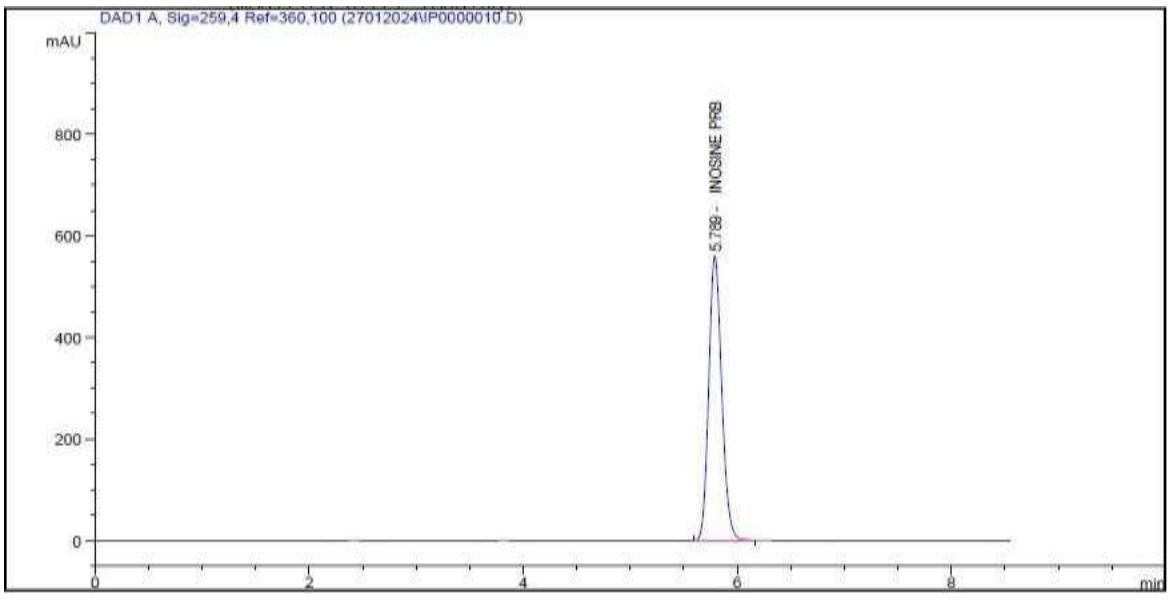

Fig.25Chromatogramoflinearity(50mcg)-01

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.789	4716.02100	11271	0.82	--

TableNo.26Detailsofchromatogram ofLinearity(50mcg)-01

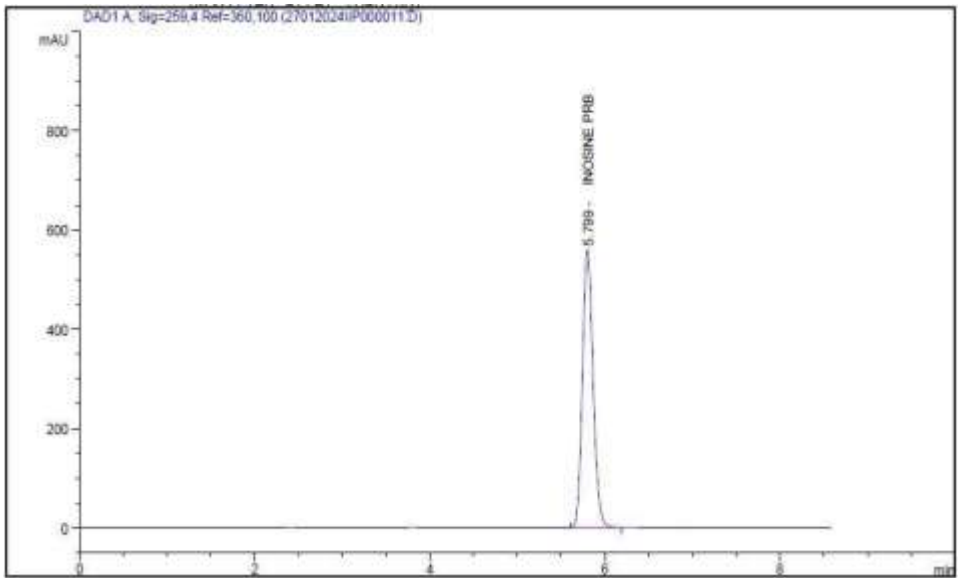

Fig.26Chromatogramoflinearity(50mcg)-02

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.799	4716.87402	11313	0.83	--

TableNo.27Detailsofchromatogram ofLinearity(50mcg)-02

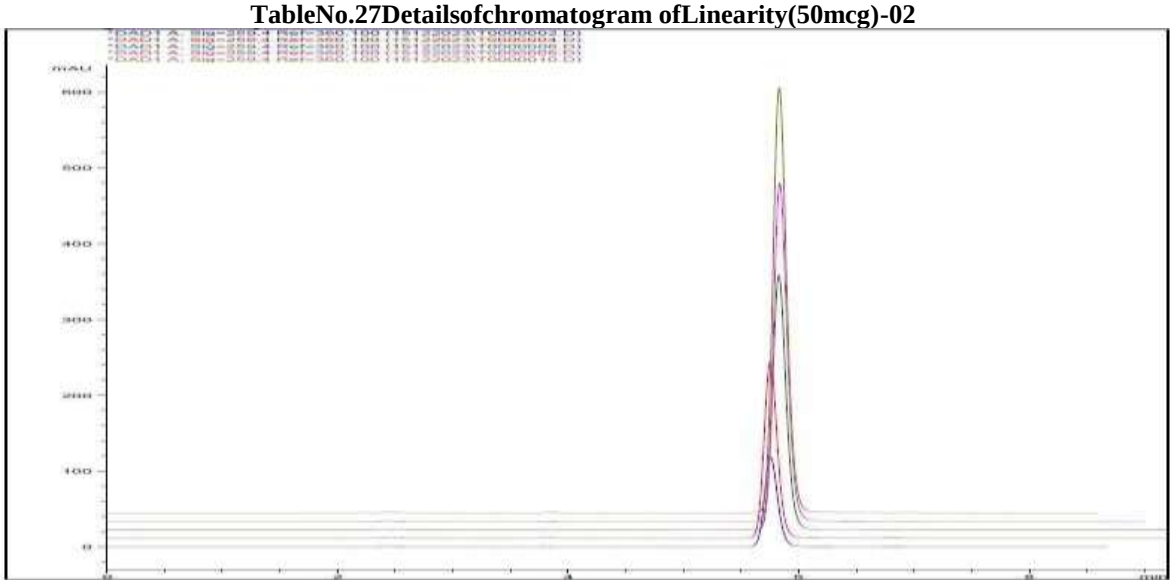

Fig.27Chromatogramofoverlaylinearity

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***meanofeach5readingforRP-HPLCmethodblue,red,green,pink,Peanutcoloursdenotedby concentration 10,20,30,40,50 mcg.**

Sr.No.	Concentration µg/mlInosinePranobex	Area InosinePranobex
10	956.0263	956.4506
20	1894.0279	1896.9224
30	2791.7861	2799.7963
40	3746.9580	3767.8801
50	4716.0210	4716.8740

TableNo.38LinearityofInosinePranobex

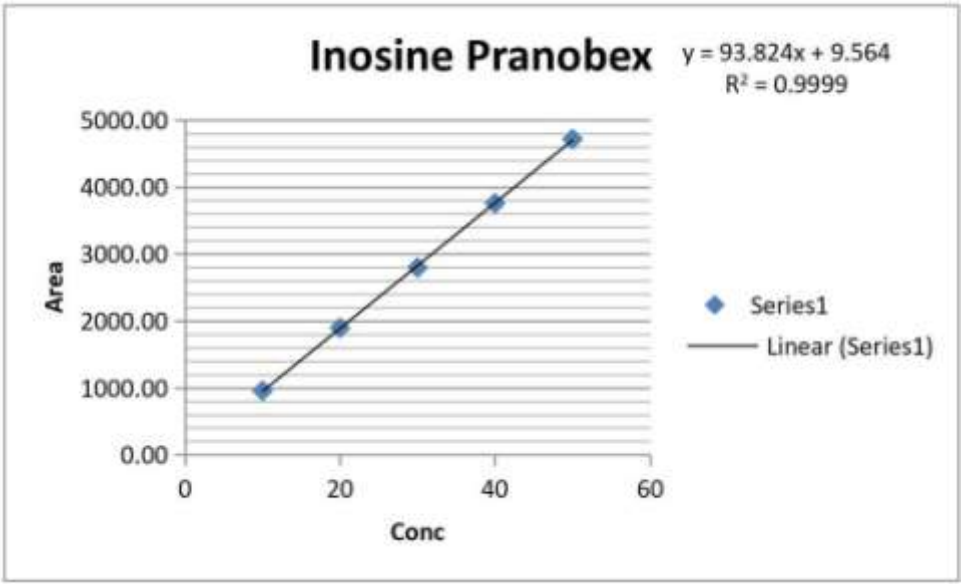

Fig.28CalibrationcurveofInosinePranobexforHPLCmethod

LinearityofofInosinePranobexwasobservedintheran geof10-50µg/ml.Detectionwavelengthusedwas 259nm.

Theplotshould belinearpassing throughtheorigin;CorrelationCoefficient shouldnotbe less than 0.999.that concluded. **(Table. No.29).**

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RegressionEquationDataY=mx+c	
Slope(m)	93.82
Intercept(c)	9.564
CorrelationCoefficient	0.999

TableNo.29RegressionequationdataforInosinePranobex

2. Accuracy:-

Recovery studies were performed to validate the accuracy of developed method. Topre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and120%) was added and then its recovery was analyzed **(Table No.32).** Statistical validationofrecovery studies shown in**(Table No. 34).**

Accuracy80%

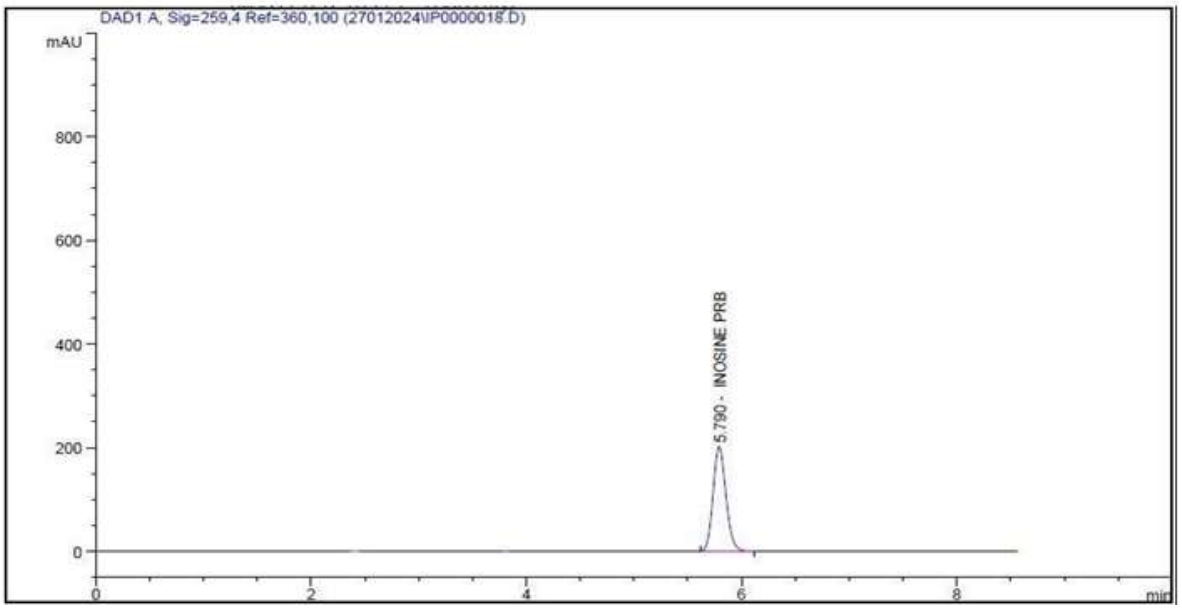

Fig.29ChromatogramofAccuracy80%

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.790	1695.55920	11822	0.84	--

TableNo.30DetailsofchromatogramofAccuracy80%

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Accuracy100%

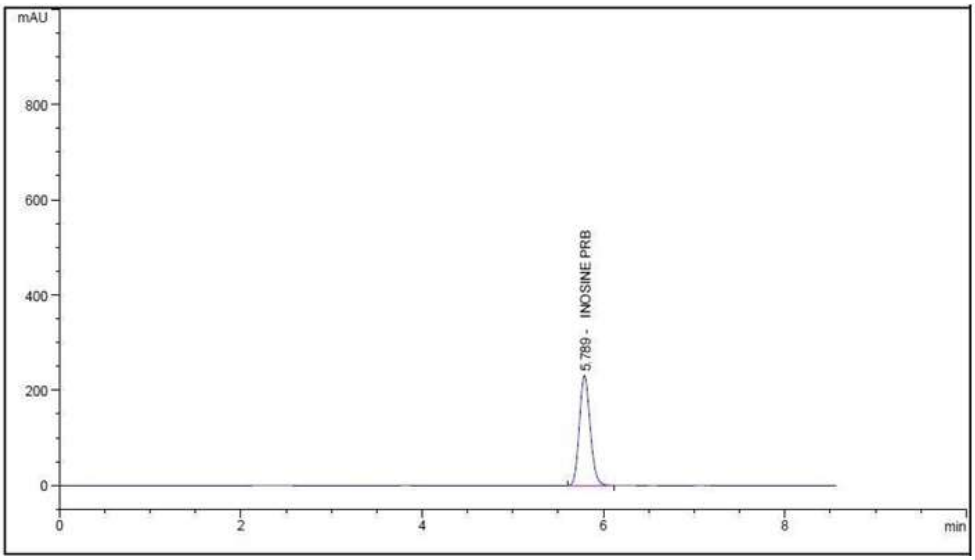

Fig.30ChromatogramofAccuracy100%

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.789	1895.37378	11571	0.84	--

TableNo.31DetailsofchromatogramofAccuracy100%

Accuracy120%

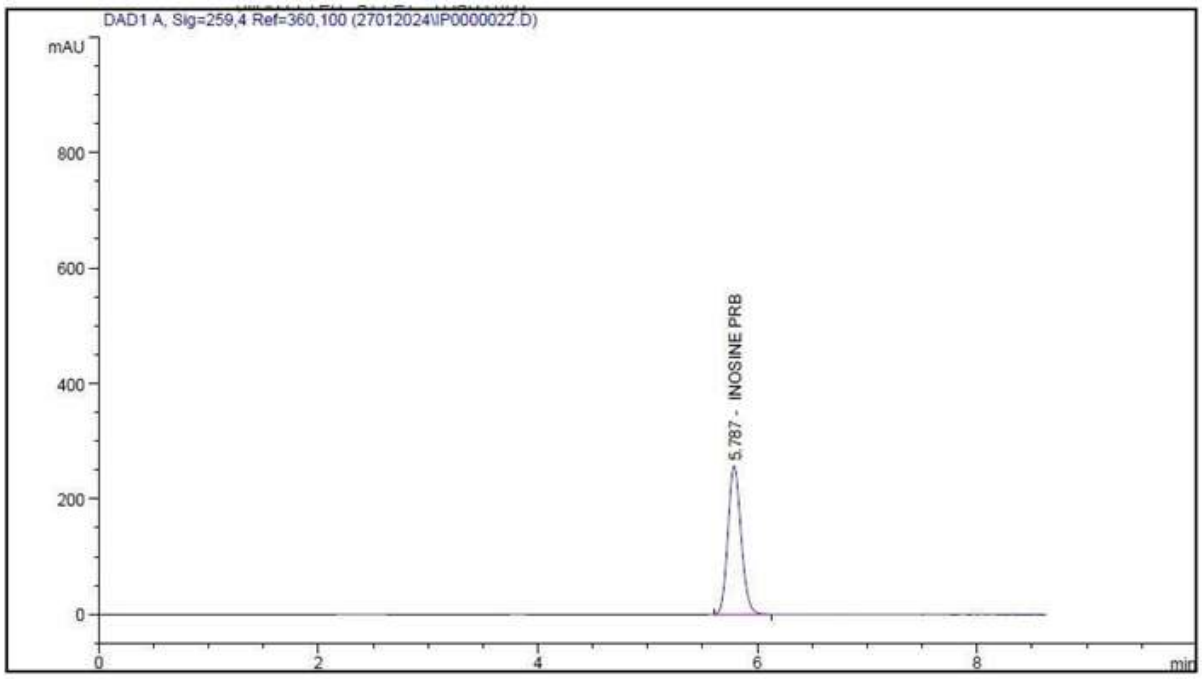

Fig.31ChromatogramofAccuracy120%

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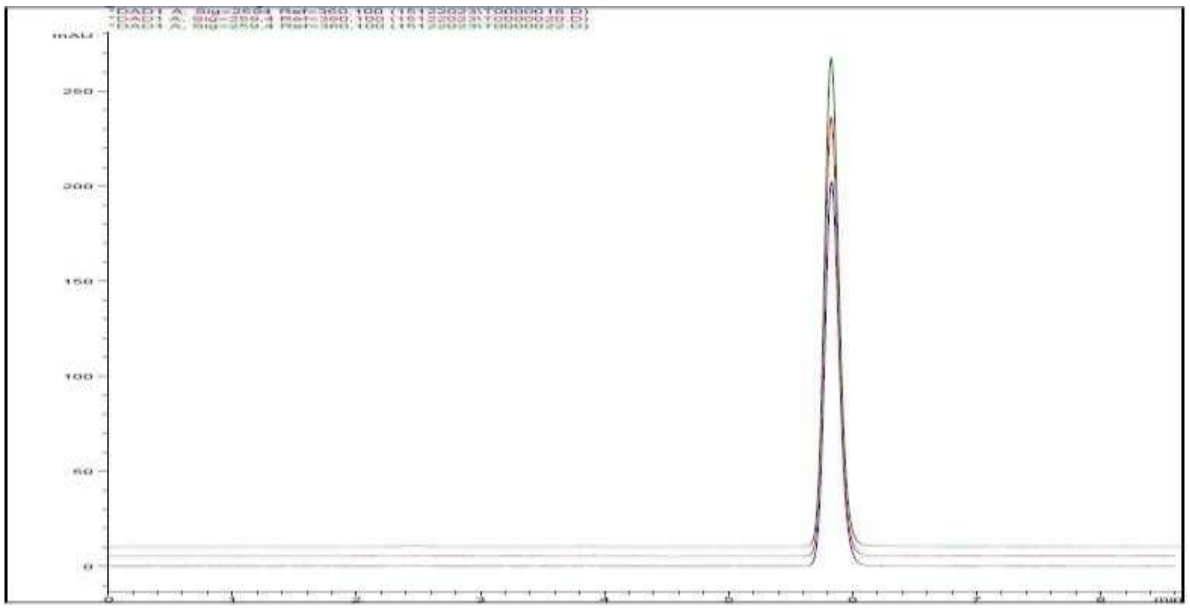
No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.787	2068.91992	11563	0.84	--

TableNo.32DetailsofchromatogramofAccuracy120%



Figno:32ChromatogramofOverlayAccuracy

***meanofeach3readingforRP-HPLCmethodblue,red,greencoloursdenotedbyconcentration 80%,100%120% forISP.**

METHOD	Drug	Level(%)	Amt.taken(µg/ml	Amt.Added(µg/ml	A.foundMean* ±S.D.	Amt.recoveredMean *±S.D.	%RecoveryMean*±S.D.
RP-HPLC Method	ISP	80%	10	8	17.94±0.03	7.94±0.03	99.29±0.48
		100%	10	10	20.08±0.03	10.08±0.03	100.75±0.36
		120%	10	12	21.93±0.02	11.93±0.023	99.45±0.19

***meanofeach 3readingforRP-HPLCmethod.**

TableNo.33ResultofRecoverydataforInosinePranobex

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METHOD	Drug	Level(%)	Mean %Recovery	StandardDeviation*	%RSD
RP-HPLC Method	ISP	80%	99.29	0.48	0.49
		100%	100.75	0.36	0.35
		120%	99.45	0.19	0.19

***Denotes average of three determinations for RP-HPLC methodTableNo.34StatisticalValidationofRecoveryStudiesInosinePranobex**

Accuracy of RP-HPLC method is ascertained by recovery studies performed at differentlevels of concentrations (80%, 100% and 120%). The % recovery was found to be within98-101%**(Table No. 33,34)**.

3. Systemsuitabilityparameters:(Repeatability)

To ascertain the resolution and reproducibility of the proposed chromatographicsystem for estimation of Inosine Pranobex system suitability parameters were studied. Theresultshown in below **(Table No.35)**

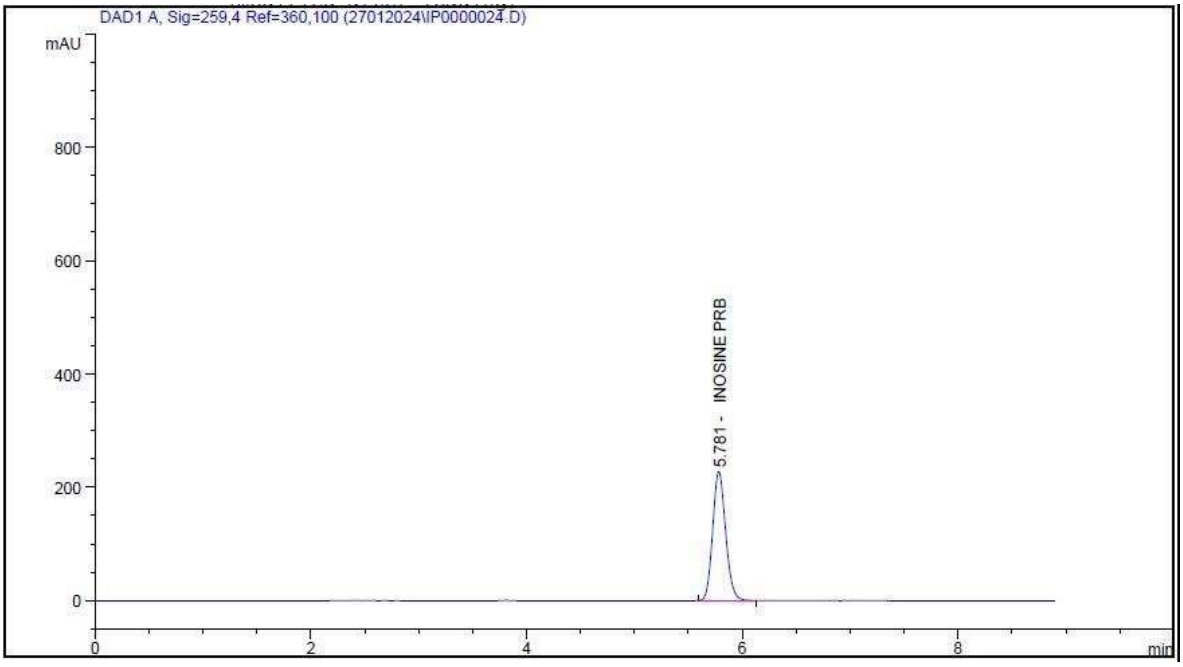

Fig.34ChromatogramofSystem suitability-1

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.781	1902.08423	11540	0.84	--

TableNo.35Detailsofchromatogramofsystemsuitability-1

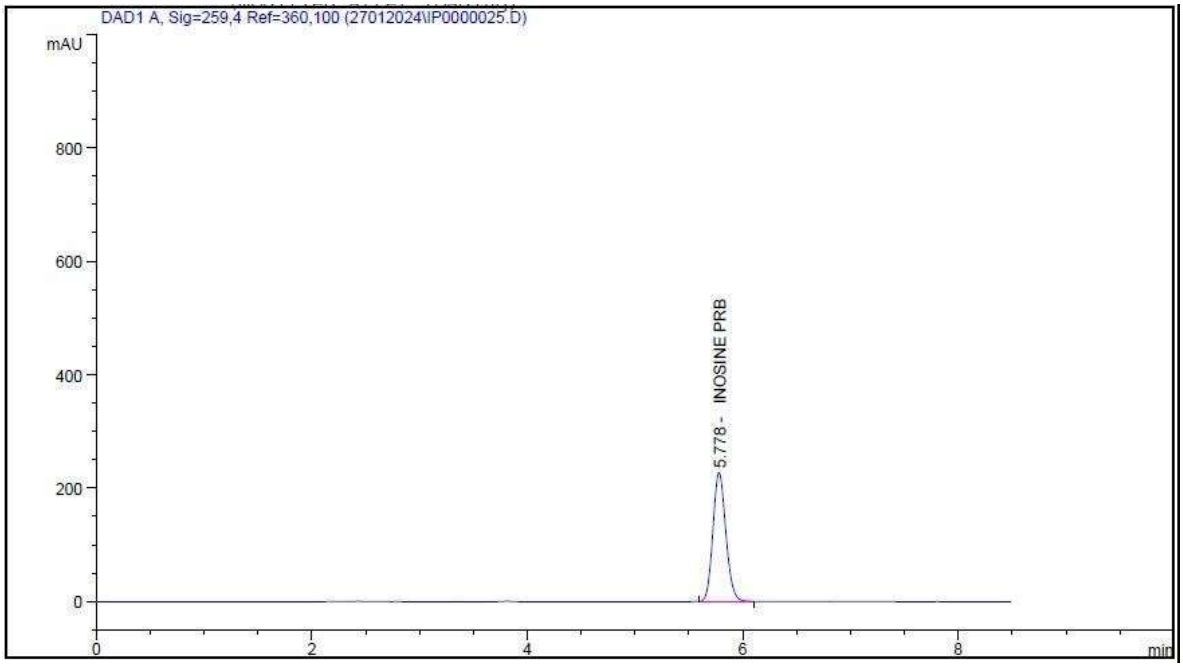

Fig.35ChromatogramofSystemsuitabilityNo-2

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.778	1902.49988	11529	0.84	--

TableNo.36Detailsofchromatogramofsystemsuitability-2

Sr.No.	ConcentrationofInosine Pranobex(mg/ml)	Peakarea	Amountfound(mg)	% Amountfound
1	20	1902.084	20.09	100.46
2	20	1902.500		
		Mean	20.09	100.46
		SD	0.29	0.29
		%RSD	0.02	0.02

TableNo.37:RepeatabilitystudiesonRP-HPLCforInosinePranobex

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RepeatabilitystudiesonRP-HPLCmethodforInosinePranobexwasfoundtobeThe %RSD was less than 2%, which shows high percentage amount foundin between98%to 102% indicates theanalyticalmethod thatconcluded**(TableNo.37).**

4. Precision:-

The method was established by analyzing various replicates standards of InosinePranobex. All the solution was analyzed thrice in order to record any intra-day & inter-dayvariationintheresultthatconcluded.Theresultobtainedforintradayisshownin**(TableNo. 44)** respectively.

ChromatogramofIntra-DayPrecision:

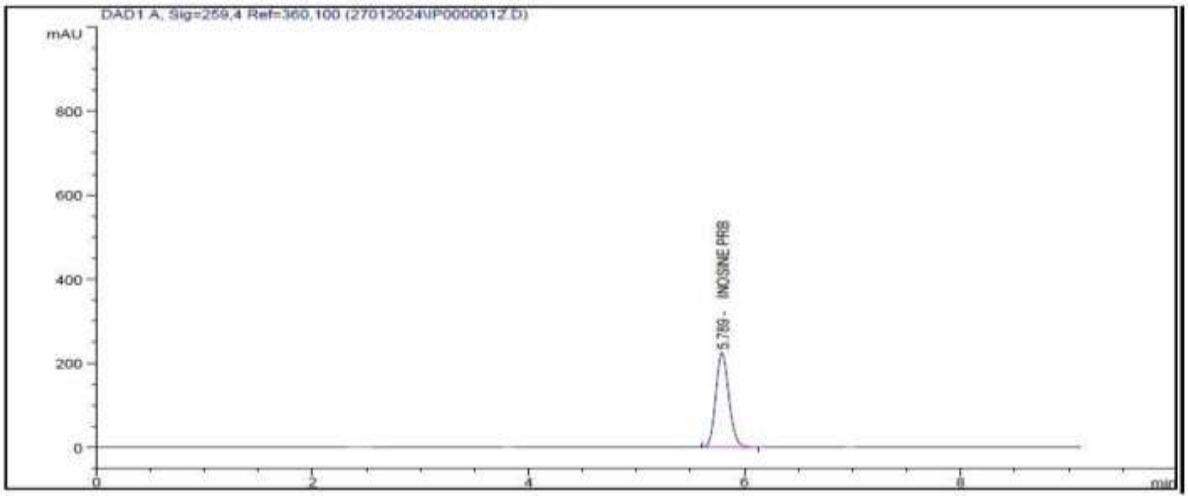

Fig.36ChromatogramofIntra-DayPrecision(20mcg)

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.789	1898.54761	11573	0.84	--

TableNo.38DetailsofchromatogramofIntra-Dayprecision (20mcg)

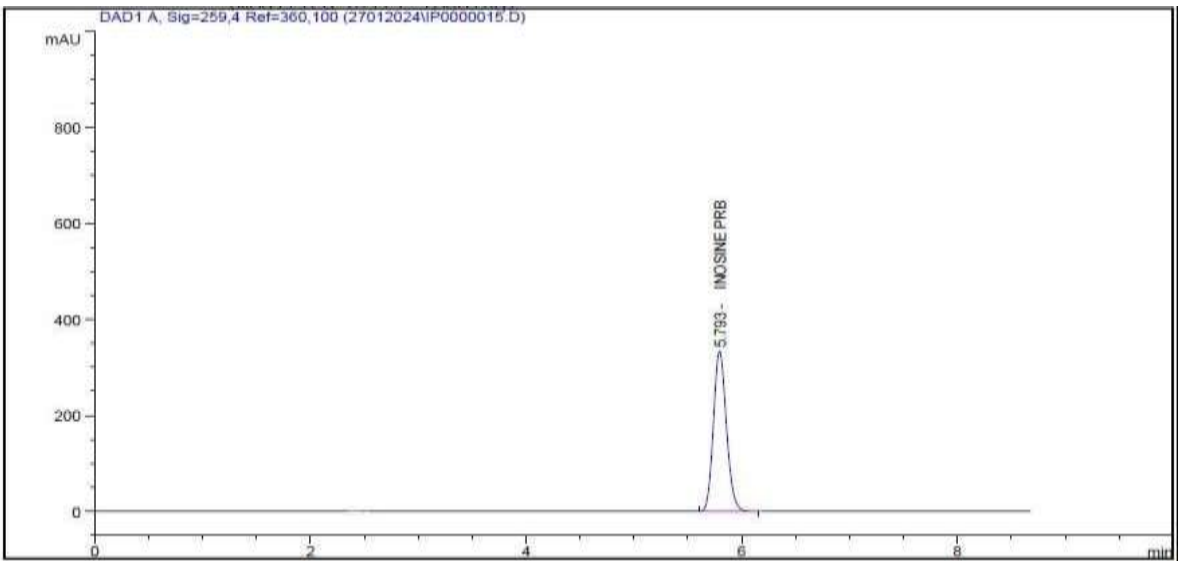

Fig.37ChromatogramIntra-dayprecision-(30mcg)

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.793	2802.84131	11588	0.84	--

TableNo.39DetailsofchromatogramofIntra-Dayprecision (30mcg)

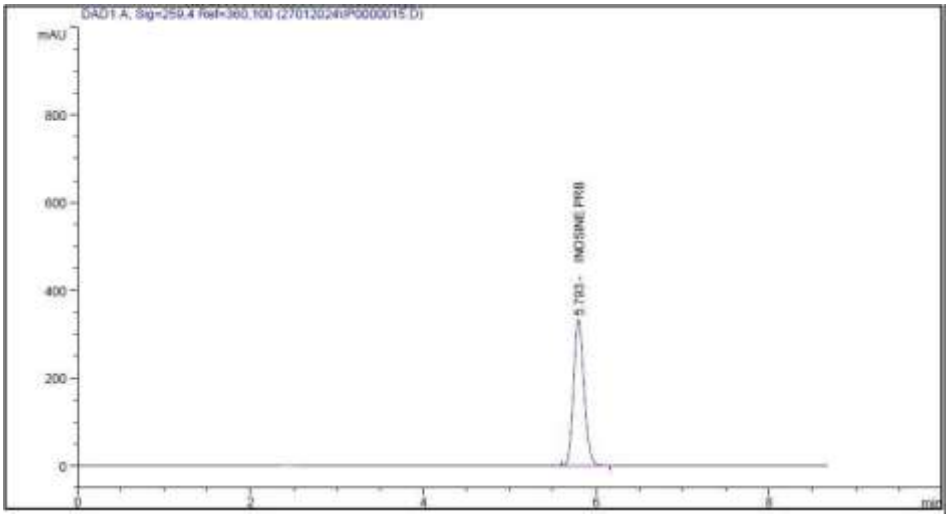

Fig.38ChromatogramIntra-dayprecision-(40mcg)

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.793	2802.84131	11588	0.84	--

TableNo.40DetailsofchromatogramofIntra-Dayprecision (40mcg)

ChromatogramofInter-DayPrecision:

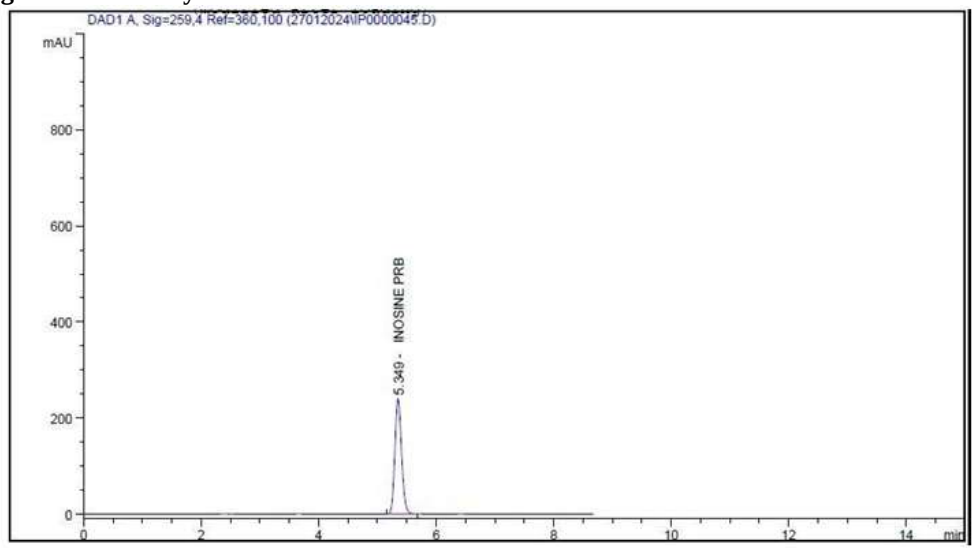

Fig.39ChromatogramInter-dayprecision(10mcg)

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.349	1897.52820	11009	0.83	--

TableNo.41DetailsofchromatogramofInter-Dayprecision(10mcg)

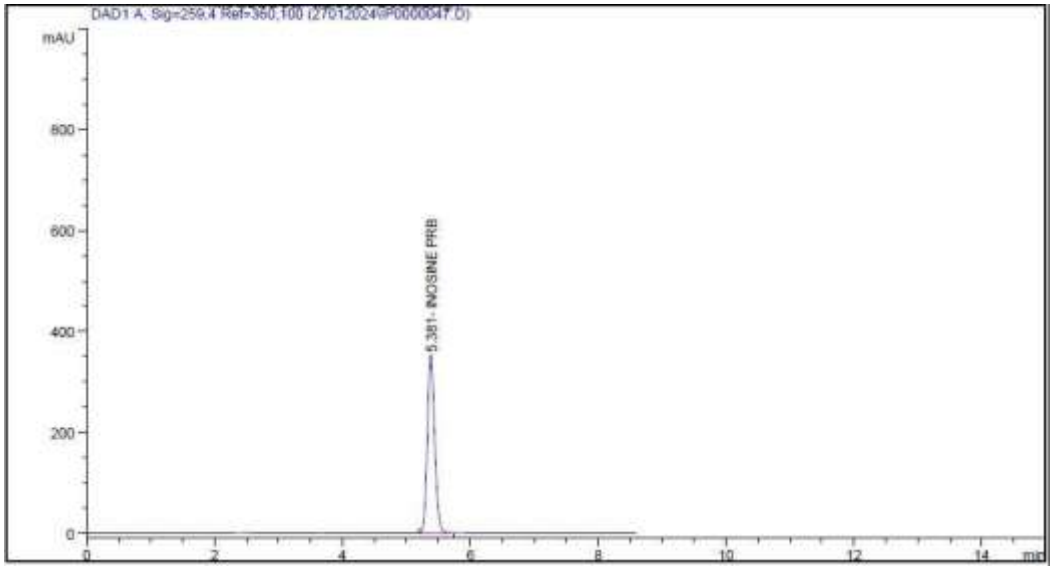

Fig.40ChromatogramInter-dayprecision(30mcg)

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.381	2802.16553	11142	0.83	--

TableNo.42DetailsofchromatogramofInter-Dayprecision(30mcg)

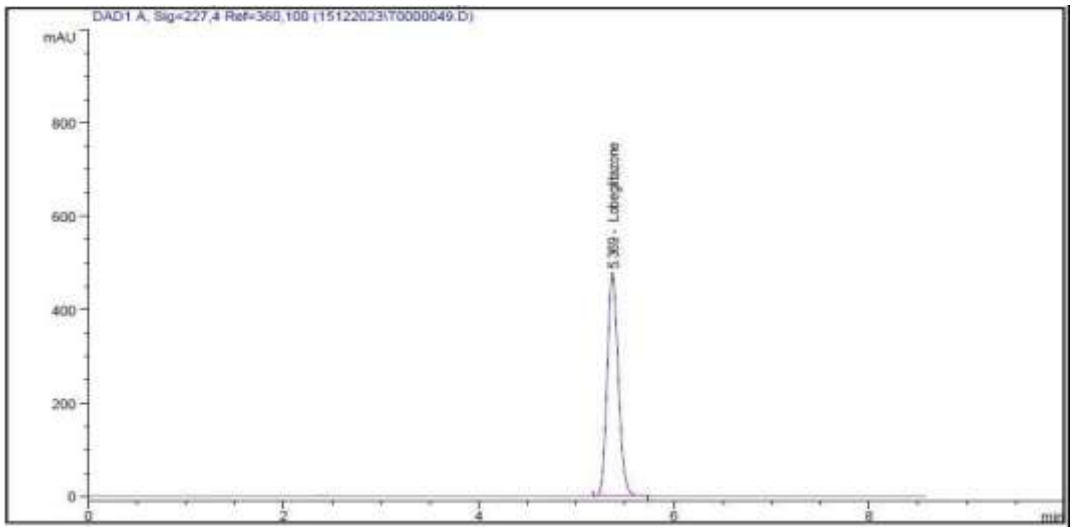

Fig.41ChromatogramInter-dayprecision(40mcg)

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.369	3783.32275	11088	0.83	--

TableNo.43DetailsofchromatogramofInter-Dayprecision(40mcg)

METHOD	Drug	Conc^{n}(µg/ml)	IntradayPrecision		InterdayPrecision	
			Mean±SD	%AmtFound	Mean±SD	%AmtFound
HPLCMETHOD Rp-	ISP	20	1899.4±1.24	100.72	1897.97±0.63	100.64
		30	2801.37±2.08	99.19	2802.49±0.46	99.23
		40	3779.43±1.18	100.45	3784.03±1.01	100.58

***Meanofeach3readingforRP-HPLCmethod**

Table No .44: Result of Intraday and Inter day Precision studies on RP-HPLCmethodforInosinePranobex

Intra day and Inter day Precision studies on RP-HPLC method for Inosine Pranobex whichshowsthehighprecision%amountinbetween98 %to102%indicatestoanalyticalmethodthat concluded.

5. Robustness:

The Robustness of a method is its ability to remain unaffected by small deliberatechanges in parameters. To evaluate the robustness of the proposed method, small butdeliberate variations in the optimized method parameters were done. The effect of changesin mobile phase composition and flow rate, wavelength on retention time and tailing factorofdrug peakwas studied.

Themobilephasecompositionwaschangedi n(±1ml/min^{-1})proportionandtheflowratewasvariedby(±1ml/min^{-1}),andwavelengthchange(±1ml/min^{-1})ofoptimizedchromatographic condition. The results of robustness studies are shown in **(Table No.45**).Robustness parameters were also found satisfactory ,hence the analytical method would beconcluded.

1. FlowRateChange0.9 ml

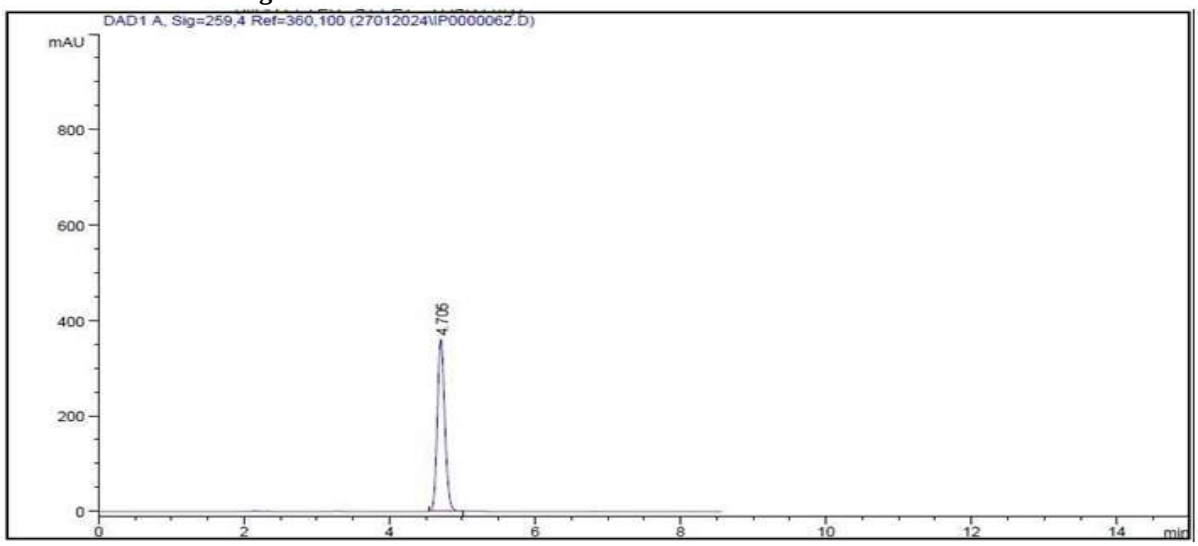

Fig.42ChromatogramofFlowratechange0.9ml

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	4.705	2581.92017	10258	0.84	--

TableNo.45DetailsofchromatogramofFlowratechange0.9ml

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2. FlowRateChange0.7 ml

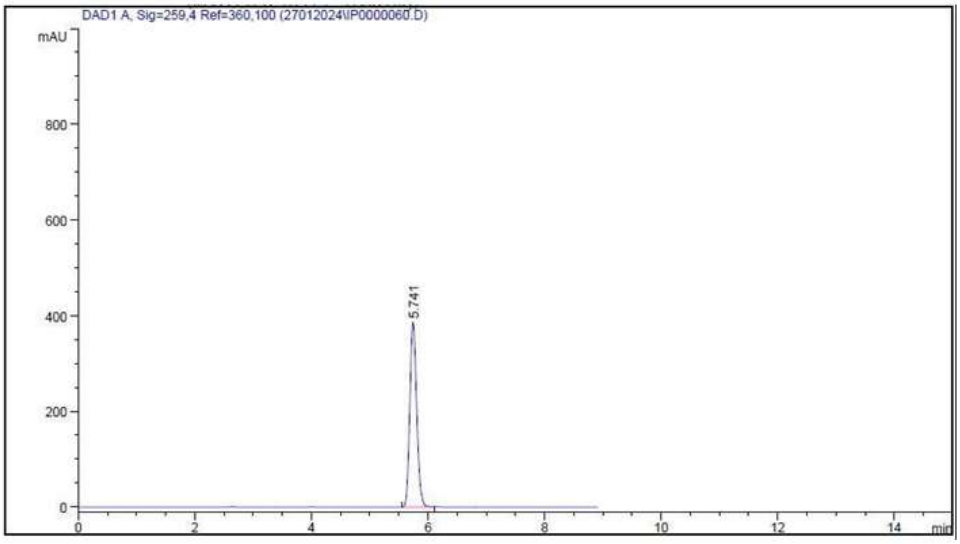

Fig.43.ChromatogramofFlowratechange0.7ml

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	4.705	2581.92017	10258	0.84	--

TableNo.46DetailsofchromatogramofFlowratechange0.7ml

3. MobilephasecompositionChange:44mlMeoH+56mlWater

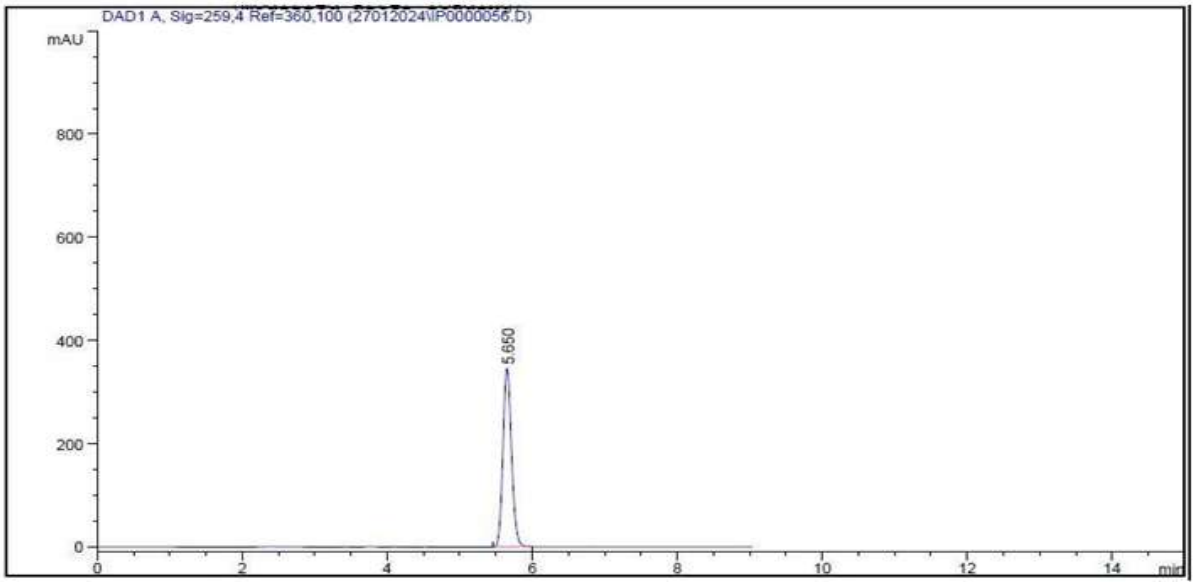

Fig.44ChromatogramofMobilephasecompositionchange:44mlMeoH+56mlWater

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.650	2848.25928	11258	0.84	--

TableNo.47DetailsofchromatogramofMobilephasecompositionchange

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4. MobilephasecompositionChange:46mlMEOH+54mlWater

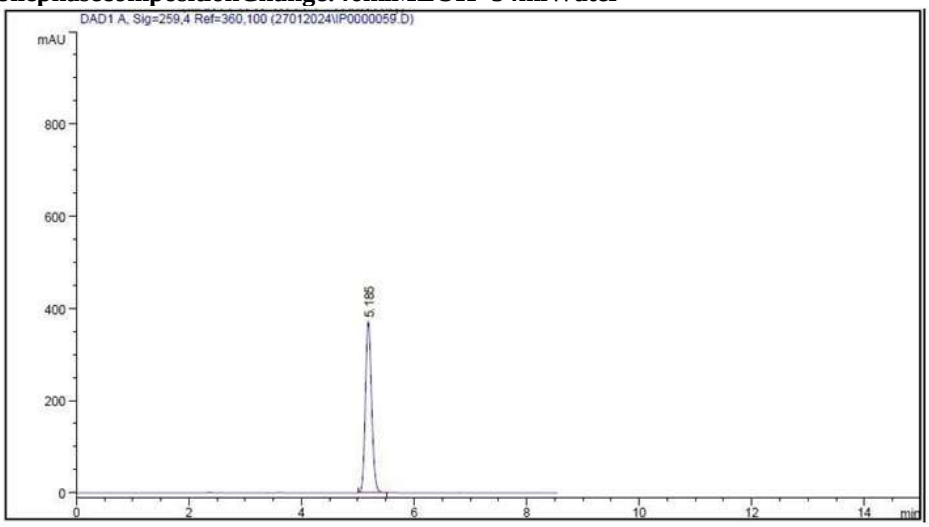

Fig.45ChromatogramofMobilephasecompositionchange46mlMEOH+ 54 ml water

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.185	2844.2246	11070	0.84	--

TableNo.48DetailsofchromatogramofMobilephasecompositionchange

5. WavelengthChange258nm

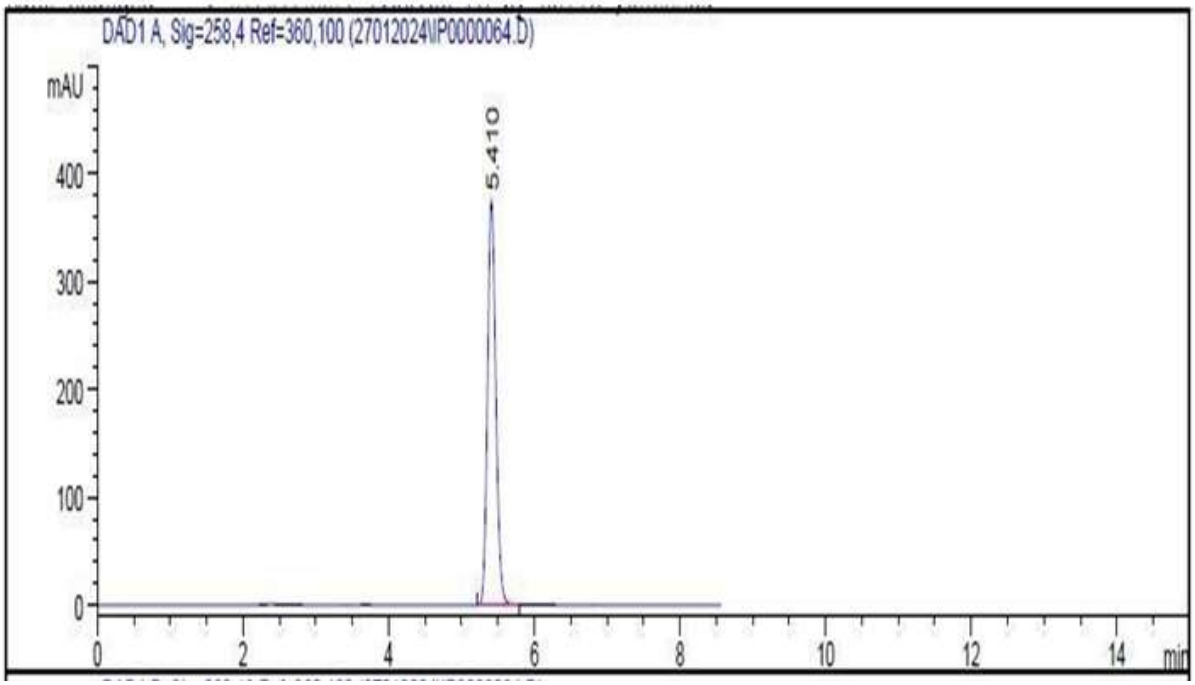

Fig.46Chromatogramofcompchangewavelengthchange258nm

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.410	2975.02930	11014	0.84	--

TableNo.49Detailsofchromatogramofwavelengthchange258nm

6. WavelengthChange228nm

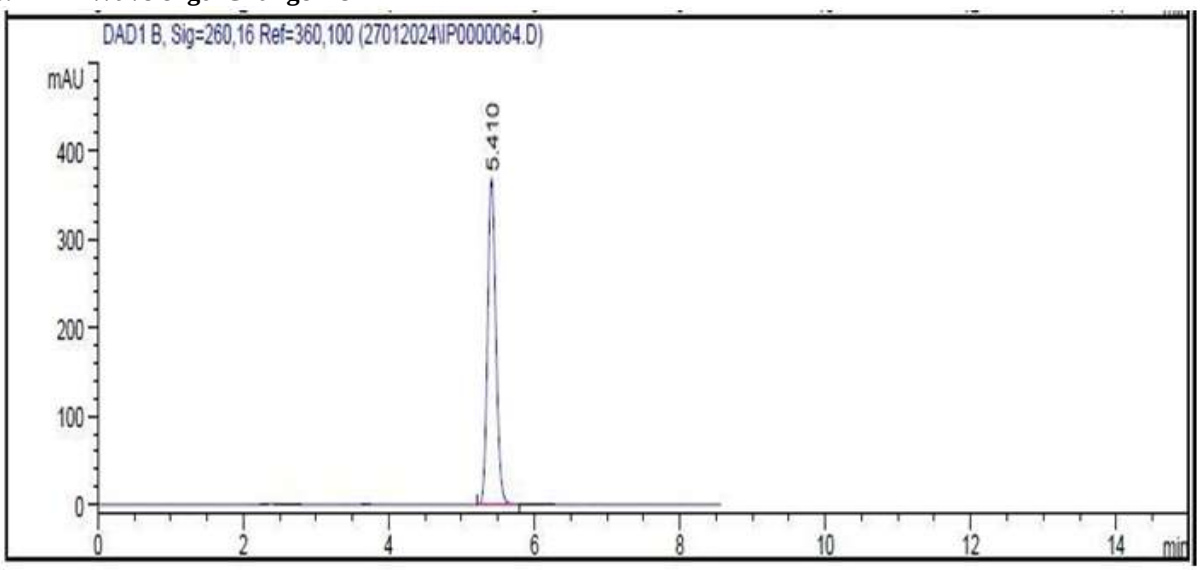

Fig.47Chromatogramofcompchangewavelengthchange260nm

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.410	2921.20142	11014	0.84	--

TableNo.50Detailsofchromatogramofwavelengthchange260nm

Parameters	Conc.	Amount ofdetected(mean±SD)	%RSD
Mobilephasecomposition-(44+56)	30	2849.4230±1.64	0.06
Mobilephasecomposition-(46+54)	30	2845.03±1.14	0.04
Wavelengthchange258nm	30	2974.2±1.12	0.04
WavelengthChange260nm	30	2920.70±0.71	0.02
Flowratechange(0.7ml)	30	3165.55±1.48	0.05
Flowratechange(0.9ml)	30	2583.00±1.52	0.06

TableNo.51Resultof Robustness StudyofInosine Pranobex

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RobustnessStudyofInosinePranobex:

The changes were did flow rate (±1 ml/ min^{-1}), PH of mobile phase composition (±1 ml/min-1), and Wavelength(±1 ml/ min-1). %RSD for peak area was calculated which shouldbeless than 2%.theresult shown in analyticalmethod that concluded.(**Table No.57).**

6. LimitDetection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of theresponseand theslope thelimit of detection (LOD)may beexpressedas:

LOD= 3.3XAvd.SD/Slope
=3.3x4.68/93.82
=0.1646

Where,SD=StandarddeviationofYinterceptS =Slope

- TheLODof InosinePranobexwasfoundtobe0.1646(µg/mL)analyticalmethodsthatconcluded.

8. LimitQuantification

TheLOQisthelowestconcentrationthatcanbequantitativelymeasured.BasedontheS.D.deviationof theresponseand the slope,
Thequantitationlimit(LOQ)maybeexpressed as:
LOQ= 10(SD)/ S
=10X4.68/93.82
=0.4990

Where,SD=StandarddeviationYintercept
S=Slope

- The LOQ of Inosine Pranobex was found to be 0.4990 (µg/mL) analytical methodthatconcluded.

8.3 Analysis of tablet formulation:-Procedure:

Weigh equivalent weight of Inosine Pranobex 13.54 mg in 10 ml volumetric flask.Add about 10 ml of diluent and sonicate to dissolve it completely and make volume up tothe mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.3 ml ofthe above stock solution into a 10 ml volumetric flask and dilute up to the mark withdiluents (30 µg/ml). The simple chromatogram of test Inosine Pranobex Shown in **(Fig No:48)**the amounts of Inosine Pranobex per tablet were calculated by extrapolating the valueof area from the calibration curve. Analysis procedure was repeated two times with tabletformulation. Tablet Assay for %Lable claim for %RSD Calculated, Result was shown in**(TableNo. 52).**
BrandName:Viralex(500MG)[ThemismedicareLtd]
13.54mgSAMPLEIN10MLMETHANOL=1000**µg m/mlINOSINEPRANOBEXSTOCK–II**
Take 0.3mlin10mlMobilePhasei.e.=30 µg/mltabsolution

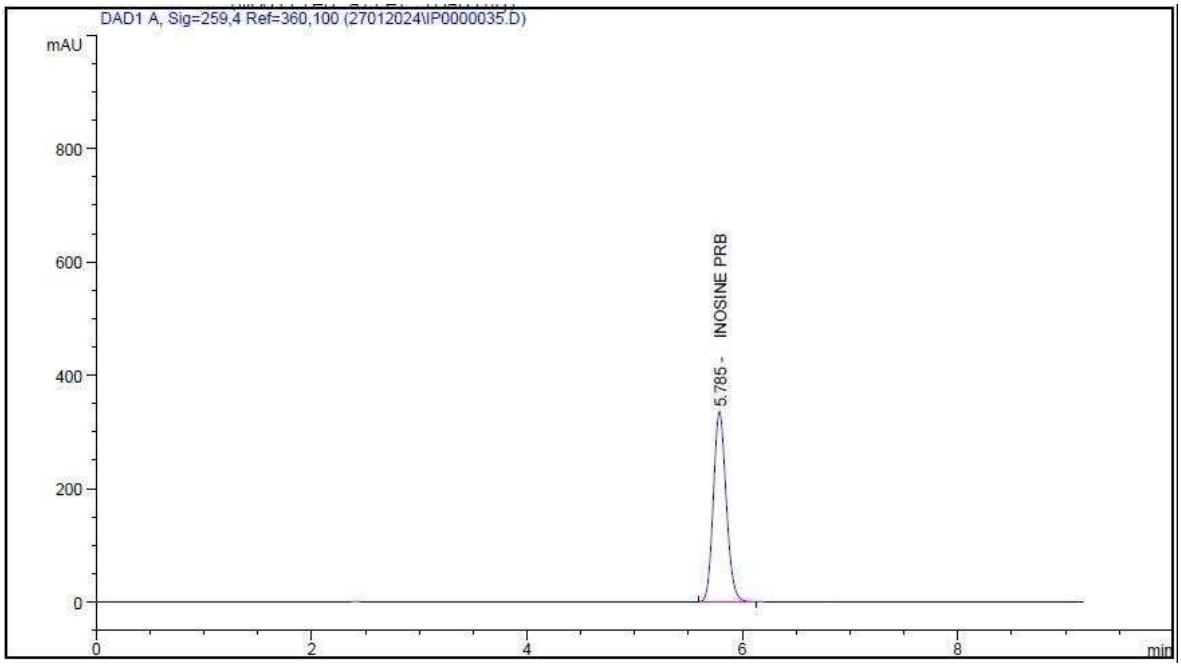

Fig.48ChromatogramforMarketedFormulation

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No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	5.785	2801.11613	11597	0.89	--

TableNo.52DetailsofchromatogramofMarketedFormulation

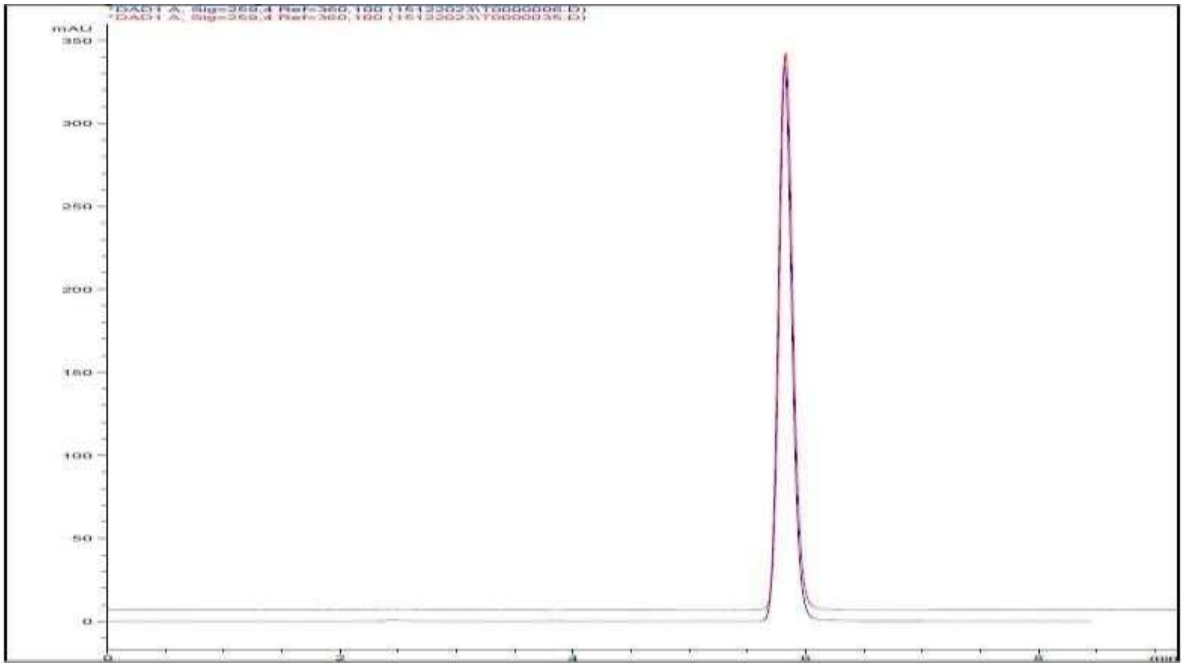

Fig.49ChromatogramforoverlayofMarketedFormulationandSTD.

Assy	Drug	LableClaimed	Amt.Found	%LableClaim	SD	%RSD
Rp-HPLC Method	ISP	30	29.6723	98.91	0.11	0.35
		30	29.8215	99.41	0.35	0.35

TableNO.53Analysisofmarketedformulation

Analysisofmarketedformulationwerealso%LableClaimwasfoundtobe98-102%Satisfactoryareconcluded.**(Table No.53).**

9. Ruggedness

Thedegreeof reproducibilityoftestresultobtainsbytheanalysisofsamesampleundervarietyof Condition.Such asdifferent analyst,laboratory Differentinstrument.

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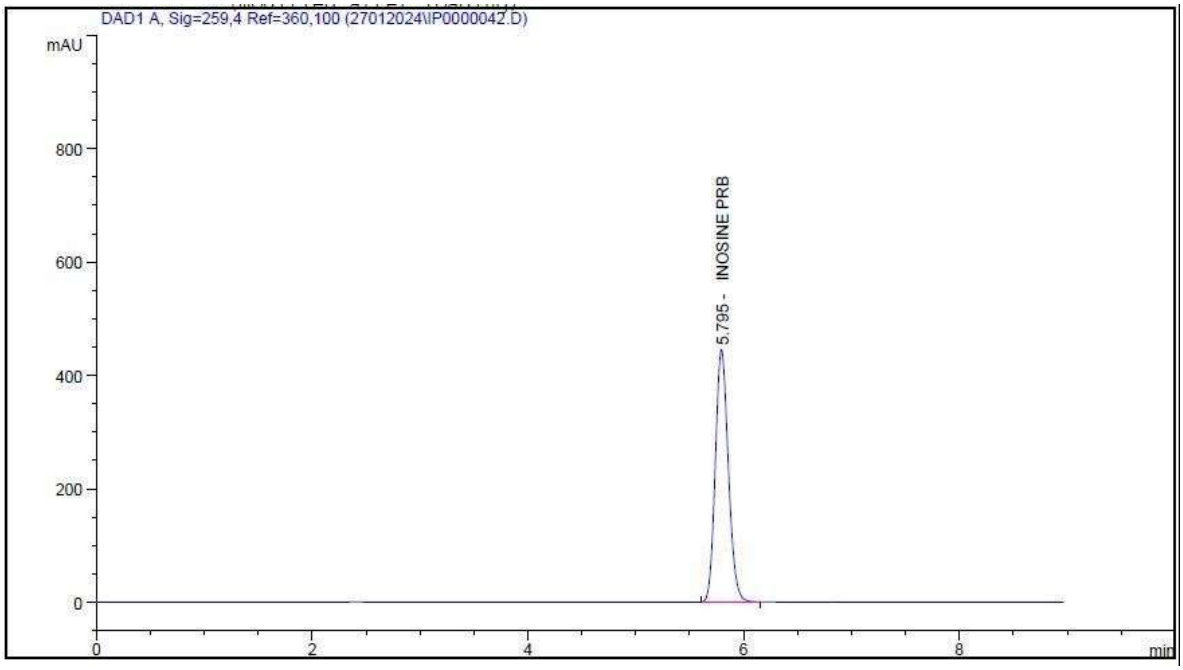
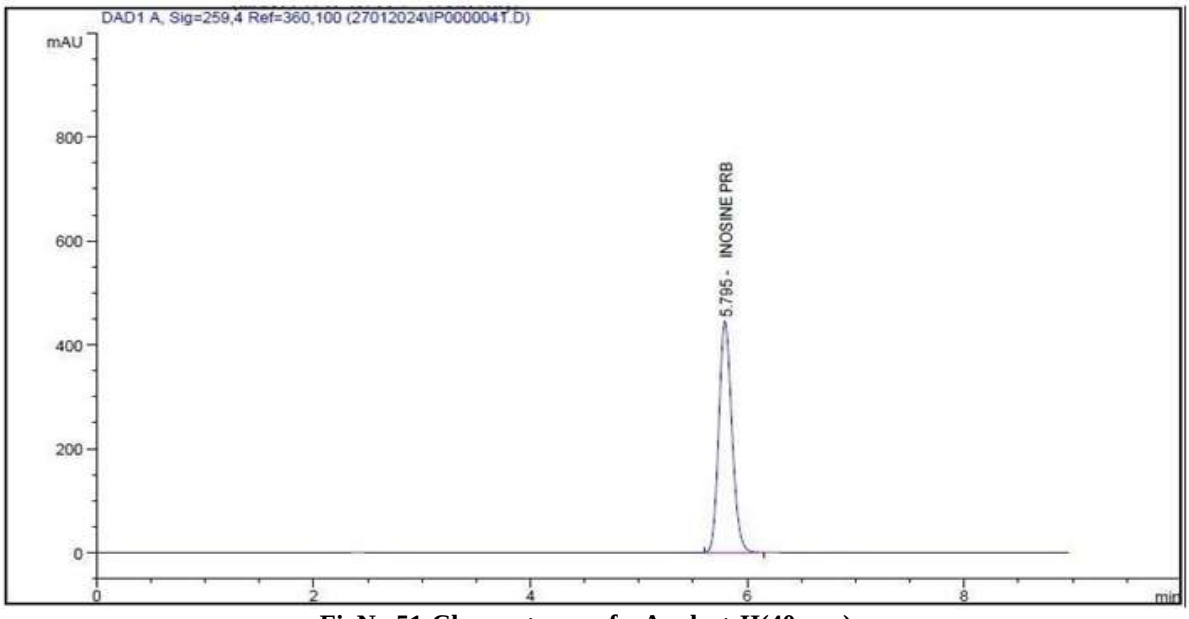


FigNo.50:ChromatogramforAnalyst-1(40mcg)



FigNo.51:ChromatogramforAnalyst-II(40mcg)

Drugname	R.T	AREA	TH.PLATES	SYMM
Analyst-I	5.795	3740.1210	12956	0.80
Analyst-II	5.795	3745.98001	12456	0.84

Table.no.55.AnalysisofAnalyst-1(40mcg)

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ForceofDegradation:

Degradationbehavior:

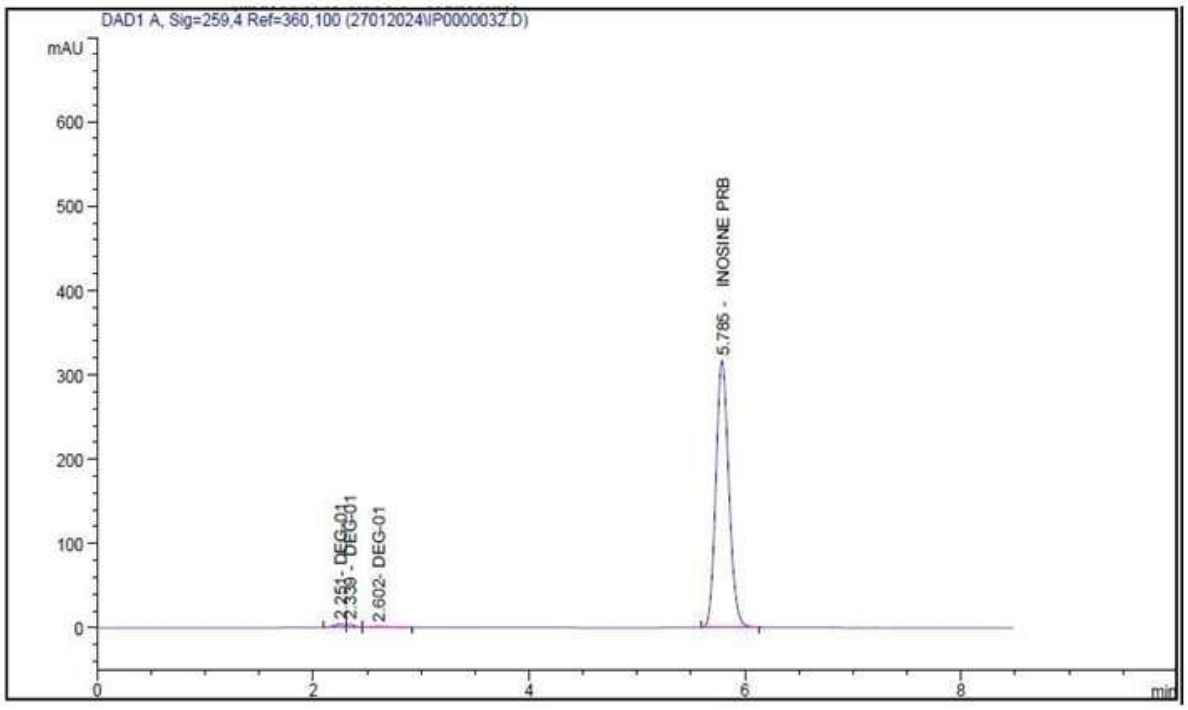
Forceddegradationstudiesofthedrugsnamely Inosine Pranobexwerecarriedoutindividuallyandincombinat ionunderdifferentstressconditionslike acidhydrolysis,alkaline hydrolysis, hydrogen peroxide oxidation and photolysis. The results are shown in(**FigureNo:52,53,54,55and Table no: 56)**

Sr no	Degradationparameter	%Degradation 2hr
1	AlkaliDEG.0.1 N NAOH-AFTER1hr30mcg	5.44
2	AcidDEG.0.1NHCL-AFTER 1hr(30mcg)	7.20
3	3%H202DEG AFTER1hr-30mcg	3.16
4	NeutralAfter1hr-30mcg	6.23
5	Photolytic24Hr.	7.35

Tableno:56Degradationofdifferentstresscondition

1.Alkalinehydrolysis:

Thealkalinehydrolysisconditionwasperformedusing0.1NNaOHfor2hrInosinePranobex. Degradation of Inosine Pranobex. The major degradation products for InosinePranobexwereobservedat 2 hr%degradation 5.44%respectively.



FigNo:52Degradationfor0.1NNAOHat2hr

Acidhydrolysis:

The acid hydrolysis performed using 0.1N HCl 1hr min for all three Inosine Pranobexindicated degradation. The major degradation products for Inosine Pranobex were observedat 2 hr, % degradation 7.20% the

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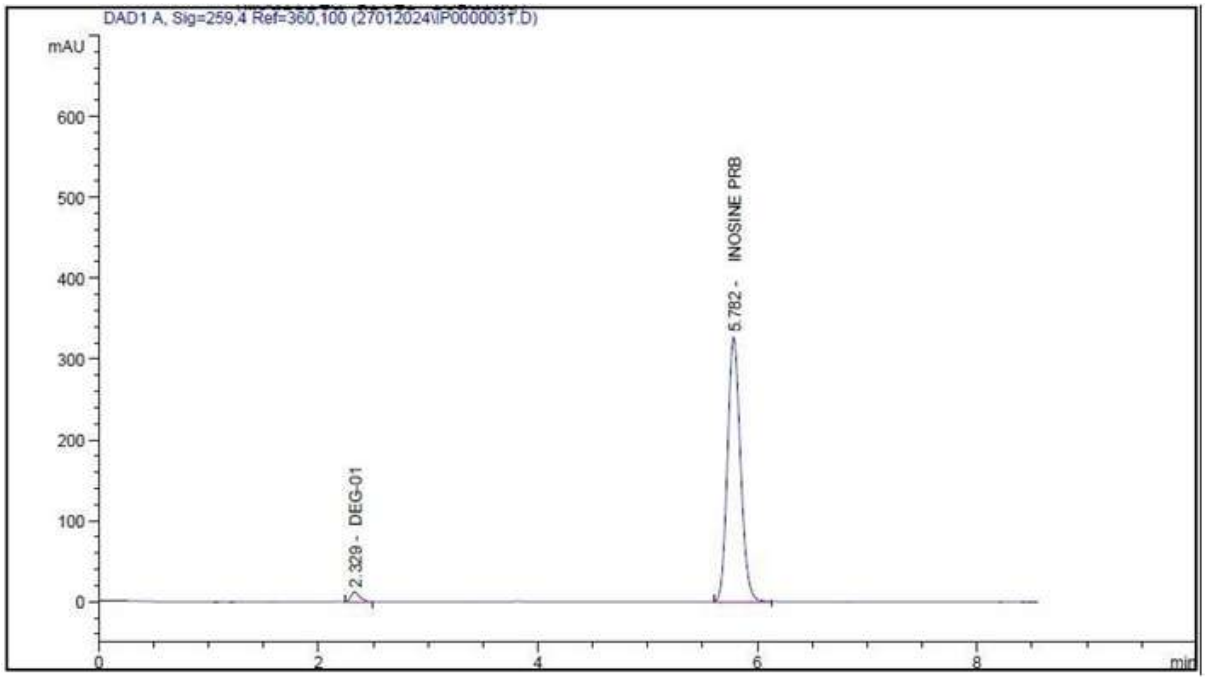
degradation product was observed at RRT of 2 hr. Theseimpuritieswerealso detected inInosinePranobex.



FigNo:53Degradationfor0.1NHCLat2Hr

3. Oxidation

Intheoxidation conditionwith3%H2O2for2hrInosinePranobexshow oxidative stressdegradationpeakinthechromatogramobservedat2hr %degradation(2hrfor3.16%).

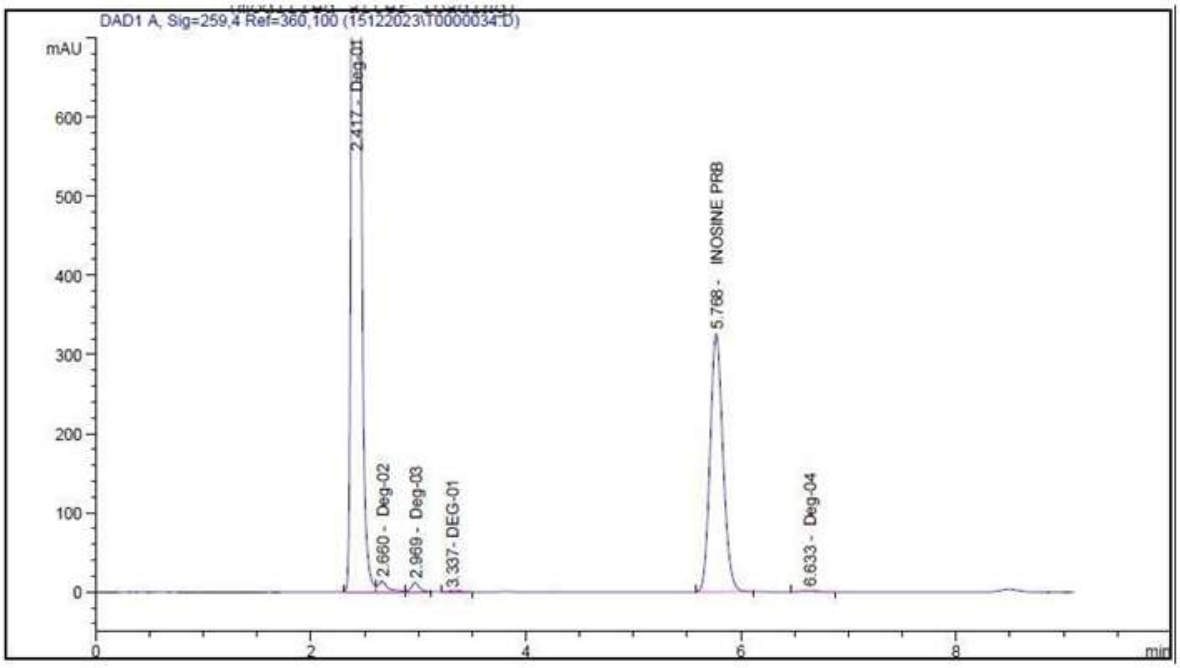


Fig.No.54Degradationfor3%H2O2at2hr.

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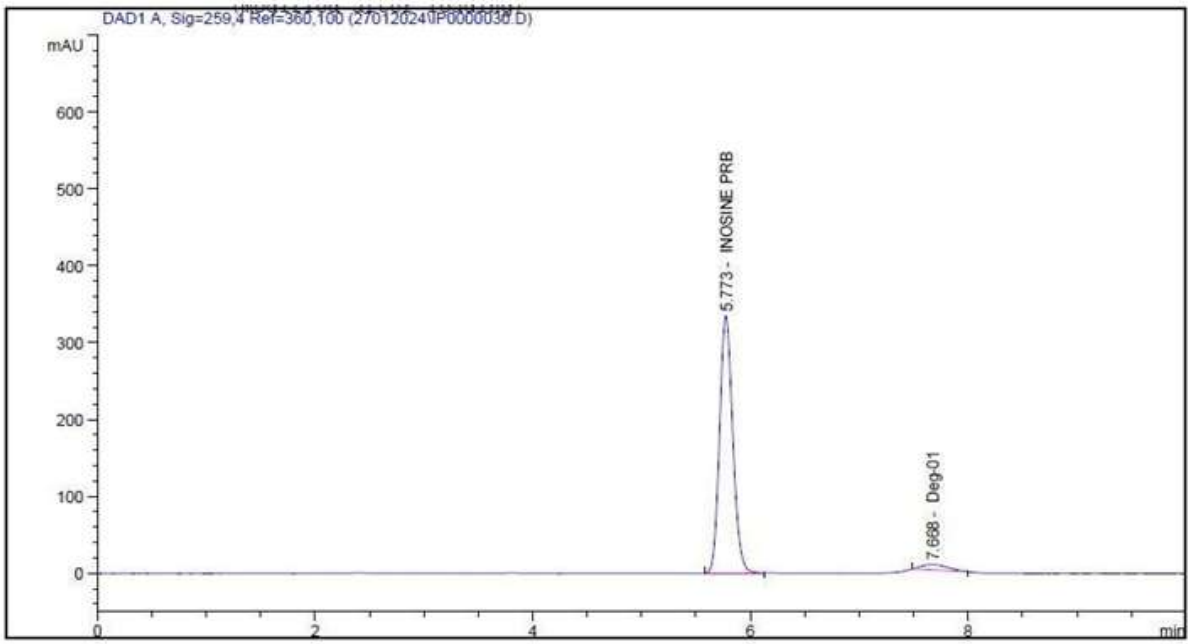
4. NeutralStudies:

TherewasnomajordegradationobservedforInosinePranobexandhencetheywerenotsensitive tolight.



FigNo.55:Degradationforneutralat2hr.

III. SUMMARY AND CONCLUSIONS

The present work deals with the Development and validation of RP-HPLC using fordeterminationofInosinePranobexbypureand Tablet dosageform.

SummaryofRP-HPLCmethod

Attemptswere made todevelopRP-HPLCfor estimationof Inosine Pranobexfromtablet. For the RP - HPLC Agilent (S.K) method Gradient System UV Detector andC18 column with 250mm x4.6 mm I'd and 5µmparticlesize methanol: 0.1% water withOPA(45%:55%v/v)wasusedasthemobilephasef orthemethod.Thedetectionwavelength was 259 nm and flow rate was 0.8 ml/min. In the developed method, theretentiontimeofInosine Pranobex werefound tobe3.613 and5.555 min

The developed method was validated according to the ICH guidelines. The linearity,precision, range, robustness was within the limits as specified by the ICH guidelines.Hencethemethodwasfoundtobesimple,ac curate,precise,economic andreproducible.

So,itisworthwhilethat,theproposedmethodscanbesu ccessfullyutilizedfortheroutinequalitycontrolanalysi sInosinePranobexinbulkdrug aswellasinformulations.

Conclusion

Simple, rapid, accurate and precise RP-HPLC have been developed and validated for theroutine analysis of Inosine Pranobex in API and tablet dosage forms. Both methods aresuitable for the simultaneous determination of Inosine Pranobex in Single-componentformulationswithoutinterferenceofeacho ther.Thedevelopedmethodsarerecommended for routine and quality control analysis of the investigated drugs in twocomponent pharmaceutical preparations. The amount found from the proposed methodswasingoodagreementwiththelabelclaimofth eformulation.Also the value ofstandarddeviationandcoefficientofvariationcalcul atedweresatisfactorilylow,indicating the suitability of the proposed methods for the routine estimation of tabletdosageforms.

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