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NOVEL RP- HPLC METHOD FOR ESTIMATION OF IDOXURIDINE IN PHARAMCUTICAL DOSAGE FORM

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INTRODUCTION

- Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals.
- Today HPLC (High performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. Theappropriate selection and chromatographic conditions ensure that the HPLC method will have the desired specificity. UV spectroscopy is also a simple analytical tool widely used for routine assay of drugs. Hence for the assay of the selected drugs HPLC and UV spectroscopy has been chosen for these proposed methods.

HPLC

- Adsorption chromatography employs high-surface area particles that adsorb the solute molecules. Usually a polar solid such as silica gel, alumina or porous glass beads and a non-polar mobile phasesuch as heptanes, octane or chloroform are used in adsorption chromatography.
- In partition chromatography, the solid support is coated with a liquid stationary phase. The relative distribution of solutes between the two liquid phases determines the separation. The stationary phase can either polar or non-polar. If the stationary phase is non-polar, it is called normal phase partition chromatography. If the opposite case holds, it is called reversed-phase partition chromatography. In normal phase mode, the polar molecule partition preferentially in to the stationary phase and are retained longer than non-polar

compounds. In reverse phase partition chromatography, the opposite behavior is observed.

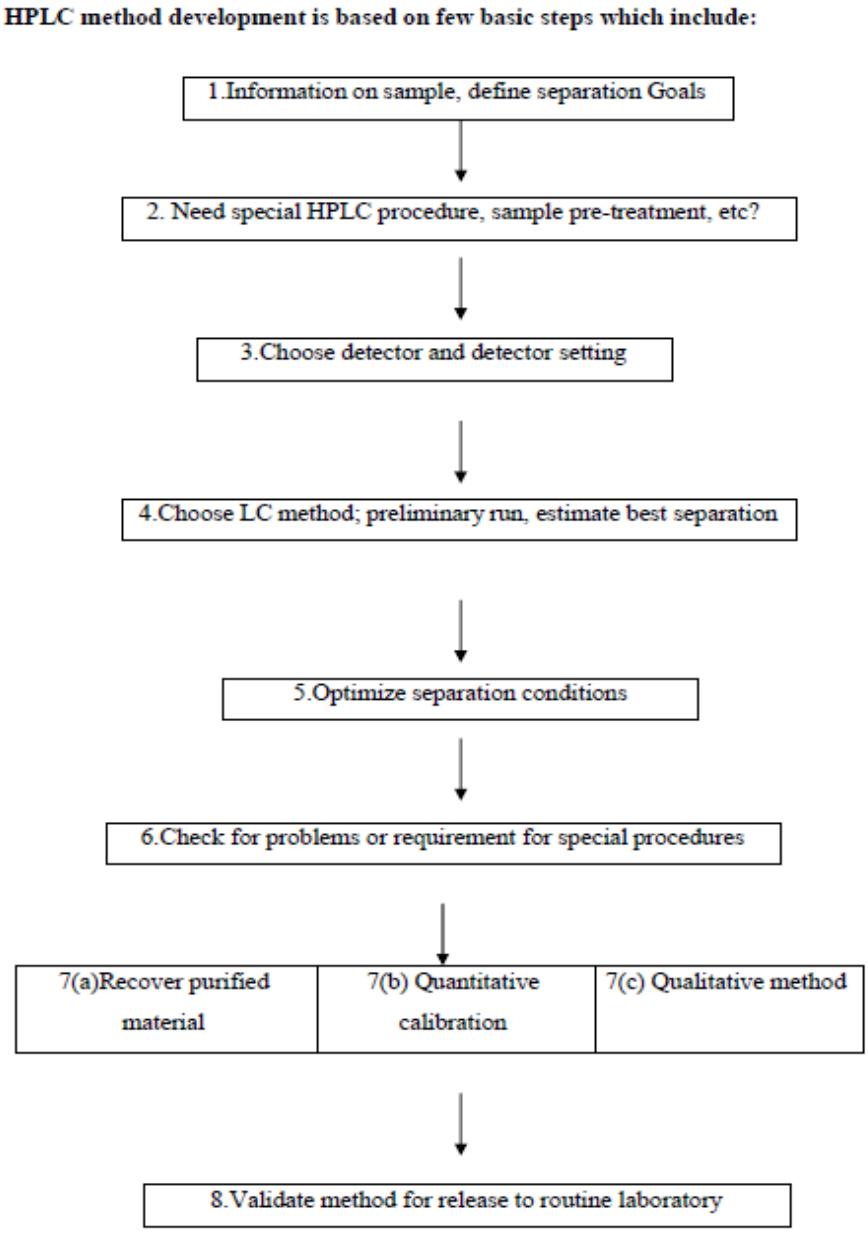


Figure 2. Steps in HPLC method development

ANALYTICAL METHOD VALIDATION

- Method validation can be defined as (ICH) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting is predetermined specifications and quality characteristics".
- Method validation study include system suitability, linearity, precision, accuracy,

specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

VALIDATION DEFINITION

- FDA defines validation as "Establishing documented evidence, which Provides a high degree of degreeof assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes.

OBJECTIVE OF METHOD VALIDATION

- The objective of validation is to form a basis for written procedure for production and control, which aredesigned to assure that the drug products have the identity, Quality, and purity.

TYPES OF ANALYTICAL PROCEDURES

i. Identification tests

ii. Quantitative tests for impurities content

iii. Limit test for control of impurities

iv. Quantitative tests of the active moiety in samples of drug substances or drug product or

v. Other selected components(s) in the drug product.

vi. Dissolution testing for drug products

vii. Particle size determination for drug substances.

LITERATURE REVIEW

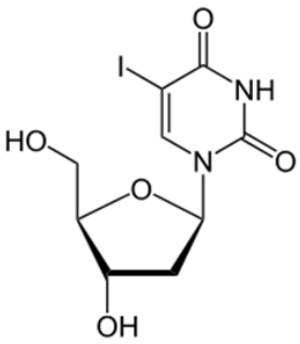
- **Asha Eluru et al., (2020)** developed a reliable, accurate and simple RP-HPLC method was developed for the simultaneous estimation of idoxuridine validated according to ICH guidelines and degradation studies were done by LC-MS/MS/QTOF in tablet dosage form. A column of Symmetry C18 (150x4.6mm, 3.5µm) with a flow rate of 1ml/min was used. The combination of 0.1% Tri ethyl amine and Acetonitrile in 70:30 ratio was used as a mobile phase. Idoxuidine peaks were eluted at a retention time of 2.770min, 5.118min respectively.
- **Phani et al., (2017)** developed a new High- performance liquid chromatographic bio-analytical method has been developed and validated [The combination of TFD and TPC are used in the treatment of unresectable advanced or recurrent colorectal cancer. The method was developed with UV detector (at 260 nm wave length), Intersil ODSC 18 column (250 mm × 4.6 mm×5µ), at flow rate of1.0mL/min. The mobile phase consisted

of 15% NaClO4 buffer (PH 4.5 v/v), 85% Methanol. The retention times of TFD and TPC are 3.4 min and 7.4 min respectively.

- **Hazra et al., (2018)** developed a simple, accurate and precise HPLC method for simultaneous determination of Trifluridine in pure and tablet dosage form has been developed. To develop and validate analytical method for simultaneous estimation of Idoxuridine in pharmaceutical formulation by RP-HPLC. HPLC of Waters (Model: Alliance 2695) with Phenomenex Luna C18 (4.6 mm I.D. × 250 mm, 5 µm) column was used for chromatographic separation. It contains waters injector and PDA Detector (Deuterium).

DRUG PROFILE

- **IDOXURIDINE**[19-21]
- **Molecular formula:** $C_9H_{11}IN_2O_5$
- **Molecular Weight:** 354.100 $g \cdot mol^{-1}$
- **Solubility:** The substance is a white crystalline powder. It is freely soluble in methanol and acetone; soluble in water, ethanol, 0.01 M hydrochloric acid, and 0.01 M sodium hydroxide; sparingly soluble in isopropyl alcohol and acetonitrile; slightly soluble in diethyl ether; and very slightly soluble in isopropyl ether.



- **Mechanism of action:** The mechanism of action of Idoxuridinee is an antiviral agent has not been fully elucidated, but appears to involve the inhibition of viral replication. Idoxuridinee gets incorporated into viral DNA during replication, which leads to the formation of defective proteins and an increased mutation rate. Idoxuridinee also mediates antineoplastic activities via this mechanism; followinguptake into cancer cells, Idoxuridinee is rapidly phosphorylated by thymidine kinase to its active monophosphate form. Subsequent phosphorylation produces trifluridine triphosphate, which is readily incorporated into the DNA of tumour cells in place of thymidine bases to perturb DNA

function, DNA synthesis, and tumour cell proliferation. As Idoxuridinee is subject to rapid degradation by TPase and readily metabolised by a first-pass effect following oral administration. Idoxuridinee monophosphate also reversibly inhibits thymidylate synthetase (TS), an enzyme that is necessary for DNA synthesis and which levels are shown to be elevated different cancer cell lines. Up-regulation of the expression of the TS enzyme may also lead to the resistance to antineoplastic therapies, such as 5-fluorouracil (5-FU). However, this inhibitory effect is not considered to be sufficient enough to fully contribute to the cytotoxicity in cancer cells.

AIMAND PLAN OF WORK

AIM

- HPLC method was there for simultaneous estimation of Idoxuridine in bulk and combination form.
- Therefore, in proposed project a successful attempt has been made to develop, simple, Accurate, andeconomic methods for analysis of Idoxuridine tablets validated.

OBJECTIVE

- In the method development of Idoxuridine we have decided to carry out our project work by incorporatingthe Reverse phase High performance Liquid chromatography (HPLC).
- Then the developed method will be validated according to ICH guidelines for its various parameters.

METHODOLOGY

- **Instruments**
- WATERS HPLC, Model: E2695, Photo diode array detector (PDA) 2998, with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. Supelco C18 (250mm X 4.6, 5mm,) column was used for separations.

List of Equipments

S.NO	Equipment's	Model	Company
1	Electronic Balance	ER200A	ASCOSET
2	Ultra-Sonicator	SE60US	ENERTECH
3	Heating Mantle	BTI	BIO TECHNICS INDIA
4	Thermal oven	---------	NARANG
5	pH Meter	AD102U	ADWA
6	Filter Paper 0.45microns	---------	MILLI PORE

Chemicals and Reagents

S. No.	Chemicals/standards and reagents	Grade	Make
1	Na2SO4	AR	Finar
2	ACETONITRILE	HPLC	Merck
3	WATER	HPLC	Loba Chemi
4	Potassium Dihydrogen phosphate	AR	Dr. Reddy's
5	IDOXURIDINE	NA	HETERO

METHOD DEVELOPMENT TRAILS[28-32]

Trail: 1

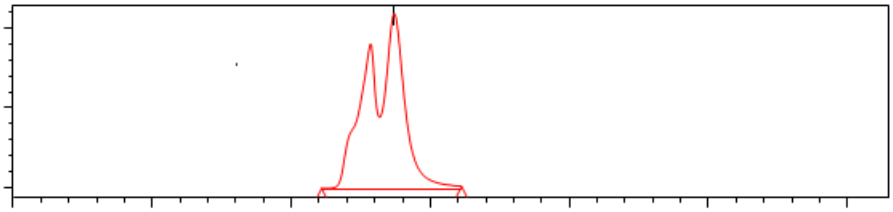
- Mobile Phase: OPA: Methanol (60:40)
- Column : ACE, C18, 250cmx4.6mm, 5µm
- Flow Rate : 1.0ml/Min
- Temperature : 25˚C
- Volume : 10µl
- Run time: 6min
- Detector: PDA

Observation: One peak was detected & peak shape is not good.

Reason: may be column efficiency is low.

Corrective Action: Change the column.

Typical chromatogram of trail 1



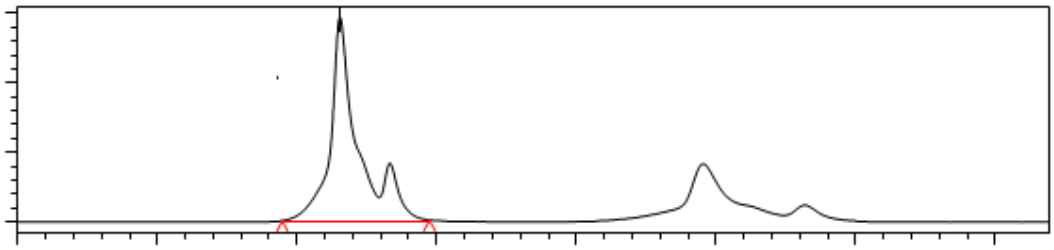
	Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
1	2.741	3801813	100.00	216746		0.81	539

Trail: 2

- Mobile Phase: OPA: Methanol (60:40)
- Column : Phenomenex, C18,250cm x 4.6mm, 5µm
- Flow Rate : 1.0ml/Min
- Temperature : 25˚C
- Volume : 10µl

- Run time: 7min
- Detector: PDA

Typical chromatogram of trail 2



	Retention Time	Area	% Area	Height	USP Resolution	USP Tailing	USP Plate Count
1	2.309	4023912	66.54	292203		1.44	1553

Observation: Peak was not detected & peak shape is not good.

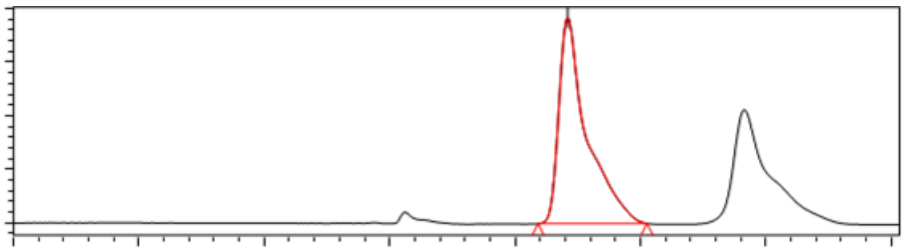
Reason: May be column efficiency is low.

Corrective Action: Change the colum and Buffer.

Trail: 3

- Mobile Phase: NaH2PO4: Methanol (60:40)
- Column : KROMASIL, C18, 250cmx4.6mm, 5µm
- Flow Rate : 1.0ml/Min
- Temperature : 25˚C
- Volume : 10µl
- Run time: 10min
- Detector: PDA

Typical chromatogram of trail 3



	Retention Time	Area	% Area	Height	Int Type	USP Resolution	USP Tailing	USP PlateCount
1	4.419	2988154	58.64	192703	BB		2.17	2701

Observation: peaks were detected & peak Shape is not good.

Reason: May be column efficiency is low and buffer.

Corrective Action: Change the column & Buffer.

OPTIMIZED METHOD

Mobile Phase: Na2SO4: Methanol (60:40)

Column : SUPELCO, C18, 250X4.6mm, 5µm

Flow Rate : 1.0ml/Min

Temperature : 25˚C

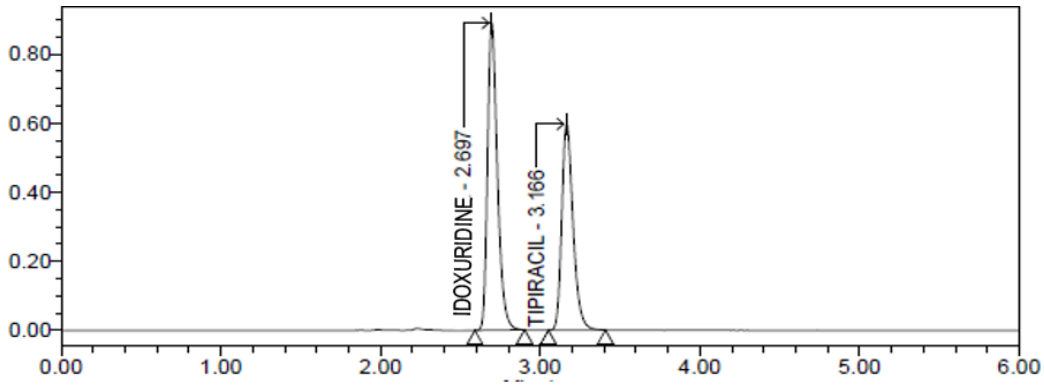
Volume : 10µl

Run time : 6min

Detector 265

pH : 4.1

Chromatogram for optimized method



Procedure

Inject 10µL of standard, sample into chromatographic system and measure the areas for the Idoxuridine peaks and calculate the % assay by using the formula.

Observation: RT was found to be good and the peak symmetry of drugs was good. And the resolution theoretical plate count and tailing were within the limits and it is used for validation of the method.

METHOD VALIDATION

- **SYSTEM SUITABILITY**

Tailing factor for the peaks due to Idoxuridine in standard solution should not be more than 2.0. Theoretical plates for the Idoxuridine peaks in standard solution should not be less than 2000.

SPECIFICITY

- Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into theHPLC system.

LINEARITY

- Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard Vs the actualconcentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

PRECISION

Preparation of sample

- Transfer the 284mg of sample into a 100 ml of volume at flask and add 10 ml of water and 10 ml of Acetonitrile and sonicate 20min and makeup with water. Transfer the above solution into 1ml into 10ml volume metric flaskdilute to the volume with water.
- The method precision parameters were evaluated from sample chromatograms obtained, by calculating the %

RSD of peek areas from 6 replicate injections.

RECOVERY/ACCURACY

- Recovery study can be performed in the concentration range of 80% to 120% of the target concentration of the test. Minimum 3 concentrations are recommended.

LIMIT OF DETECTION

- The sensitivity of measurement of Idoxuridine by use of proposed method was estimated in terms of the limit of detection (LOD). The LOD was calculated by the use of signal to noise ratio. In order to estimate the LOD value, the blank sample was injected six times and peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level.

$$LOD = 3.3\ \sigma / S$$

Where,

σ = standard deviation of intercepts of calibration curvesS = mean of slopes of the calibration curves

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

LIMIT OF QUANTITATION

- The sensitivity of measurement of Idoxuridine by the use of proposed method was estimated in termsof limit of quantitation (LOQ). The LOQ was calculated by the use of signal to noise ratio. In order to estimate the LOQ value, the blank sample was injected six times and the peak area of this blank was calculated at noise level. The LOQ was calculated as ten times the noise value gave the LOQ.

$$LOQ = 10\ \sigma / S$$

- Where,
- σ = standard deviation of intercepts of calibration curves
- S = mean of slopes of the calibration curves.
- The slope S may be estimated from the calibration curve of the analytic.

RESULTSAND DISCUSSION

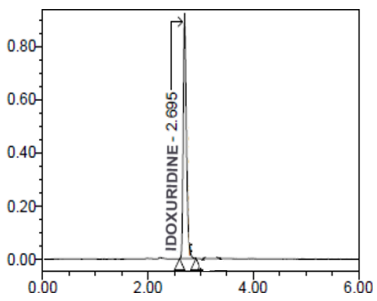
- **SYSTEM SUITABILITY**

Tailing factor for the peaks due to Idoxuridine in standard solution should not be more than 2.0.

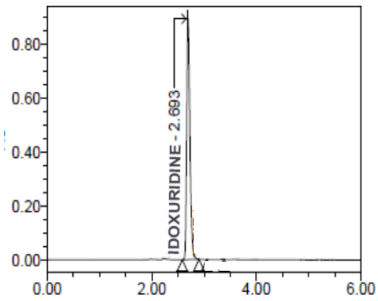
Theoretical plates for the Idoxuridine peaks in standard solution should not be less than 2000.

System suitability data of Idoxuridine

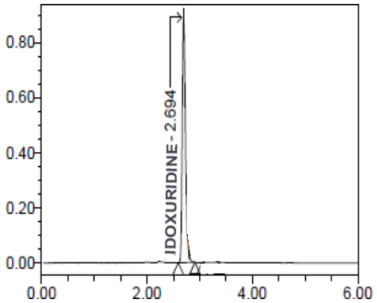
Parameter	Idoxuridine	Acceptance Criteria
Retention time	2.698	±10
Theoretical plates	9303	>2500
Tailing factor	1.37	<2.00
% RSD	0.2	<2.00



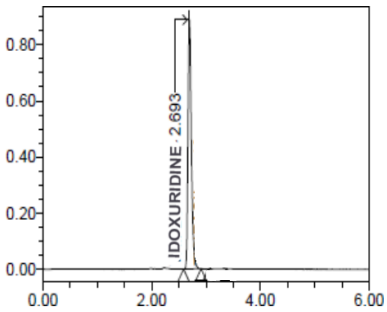
Typical Chromatogram of Standard-2; Injection-1



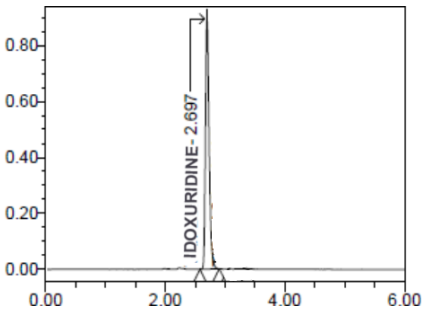
Typical Chromatogram of Standard-2; Injection-2



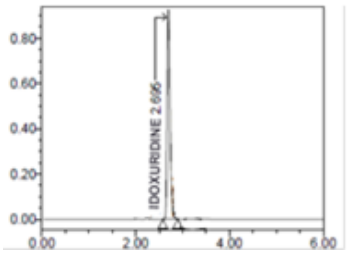
Typical Chromatogram of Standard-2; Injection-3



Typical Chromatogram of Standard-2; Injection-4



Typical Chromatogram of Standard-2; Injection 5



Typical Chromatogram of Sample-1

RESULT

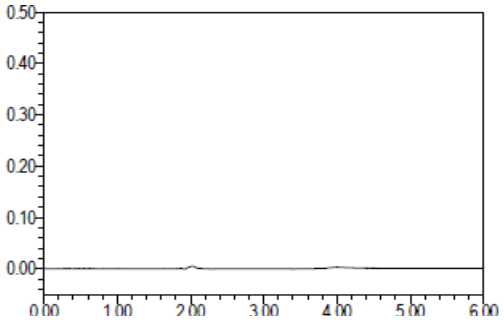
Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicatea good system for analysis.

SPECIFICITY

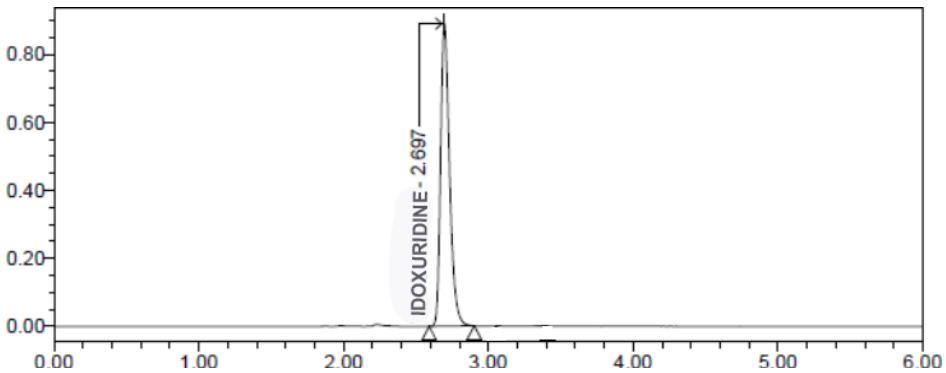
Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into the HPLC system.

Specificity data for Idoxuridine

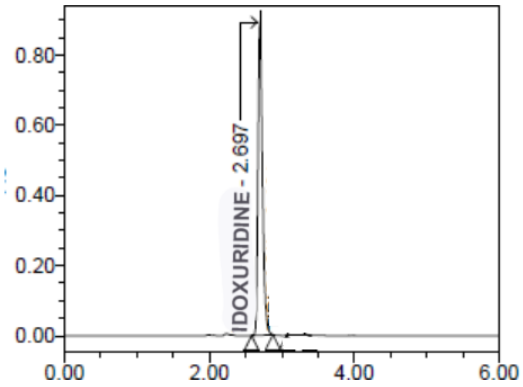
S. no	Sample name	Idoxuridine Area	Rt
1	Standard	3830577	2.698
2	Sample	3815897	2.696
3	Blank	-	-
4	Placebo	-	-



Graph 1: Typical chromatogram of the blank.



Graph 2: Chromatogram Representing Specificity of Standard.

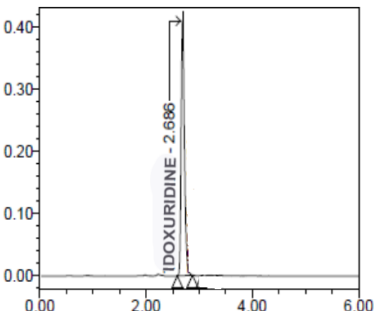


Graph: 3 chromatogram representing specificity of sample.

ACCURACY

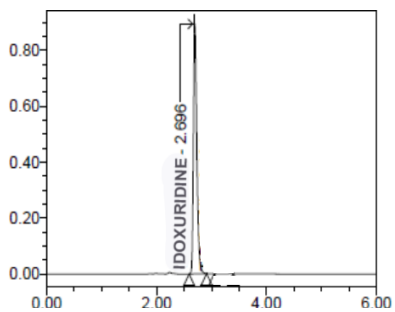
Accuracy level	injecton	Sample area	RT
50%	1	1895241	2.687
	2	1902958	2.683
	3	1899373	2.685
100%	1	3812611	2.697
	2	3815695	2.694
	3	3826479	2.697
150%	1	5730992	2.703
	2	5747674	2.702
	3	5737249	2.704

Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
50%	1	142.00	9.9	9.85	99	100
	2	142.00	9.9	9.89	100	
	3	142.00	9.9	9.87	100	
100%	1	284.00	19.8	19.81	100	100
	2	284.00	19.8	19.83	100	
	3	284.00	19.8	19.88	100	
150%	1	426.00	29.7	29.78	100	100
	2	426.00	29.7	29.87	101	
	3	426.00	29.7	29.81	100	



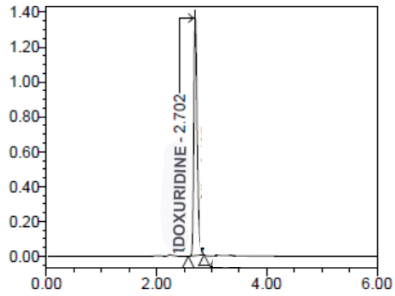
Typical chromatogram for Accuracy 50 %

Graph : 1



Typical chromatogram for Accuracy 100 %

Graph : 2



Typical chromatogram for Accuracy 150 %

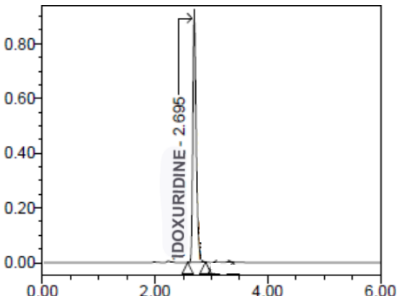
Graph :3

RESULT

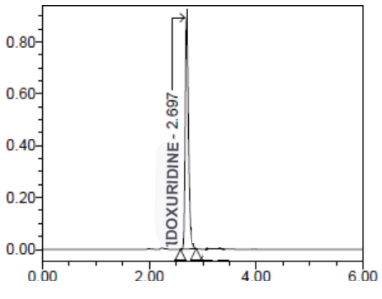
Results of accuracy study are presented in the above table. The measured value was obtained byrecovery test. Spiked amount of both the drug were compared against the recovery amount. % Recovery was 100% for Idoxuridine. All the results indicate that the method is highly accurate**.**

PRECISION

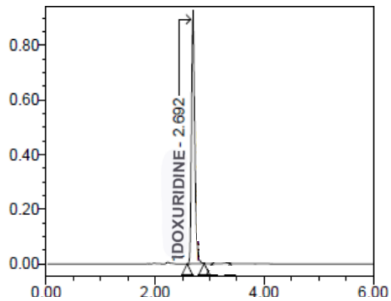
1. Transfer the 284mg of sample into a 100 ml of volume at flask and add 10 ml of water and 10 ml of Acetonitrile and sonicate 20min and makeup with water. Transferthe above solution into 1ml into 10ml volume metric flask dilute to the volume with water.
2. The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injections.



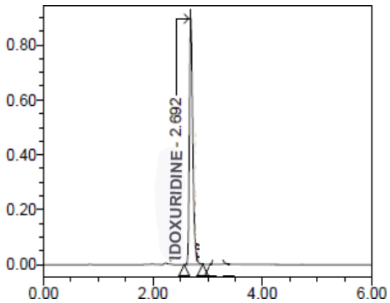
Chromatogram for precision injection 1



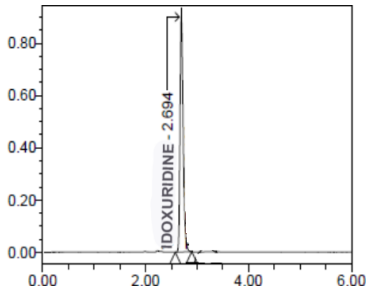
Chromatogram for precision injection 2



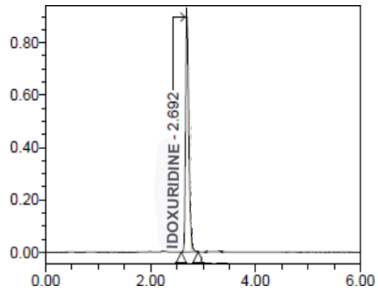
Chromatogram for precision injection 3



Chromatogram for precision injection 4



Chromatogram for precision injection 5



Chromatogram for precision injection 6

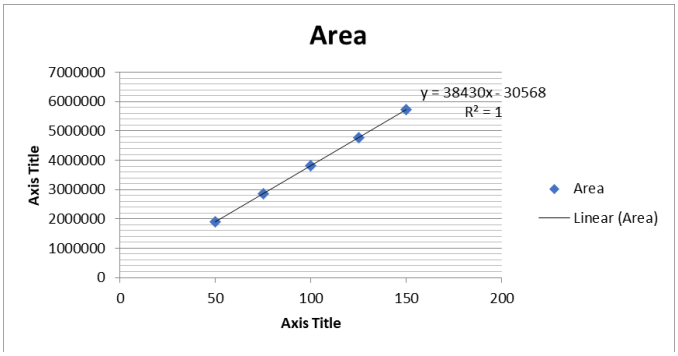
RESULT

Results of variability were summarized in the above table. % RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which provesthat method is precise**.**

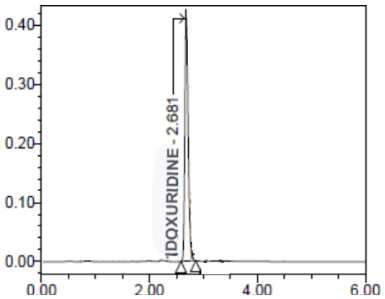
LINEARITY

Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard Vs the actual concentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

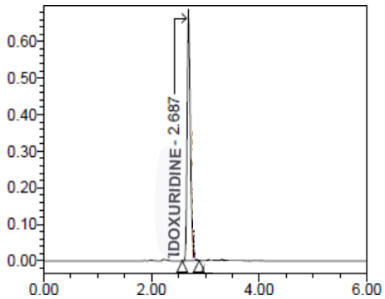
S.No	Conc (µg/ml)	RT	Area
1.	50	2.682	1890875
2.	75	2.688	2853836
3.	100	2.692	3810719
4.	125	2.697	4770756
5.	150	2.704	5736227
Correlation coefficient (r^2)			1.000



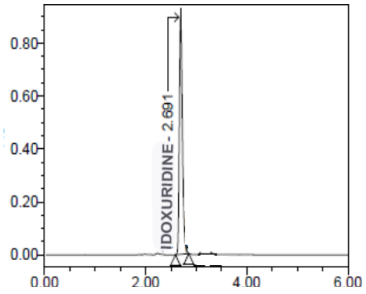
Linearity



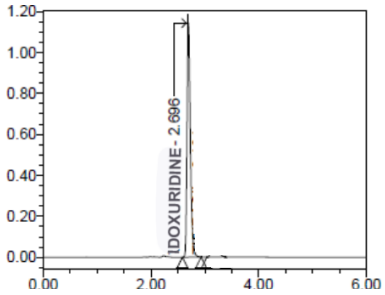
Chromatogram representing linearity 1



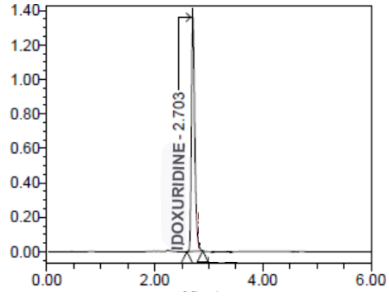
Chromatogram representing linearity 2



Chromatogram representing linearity 3



Chromatogram representing linearity 4



Chromatogram representing linearity 5

RESULT

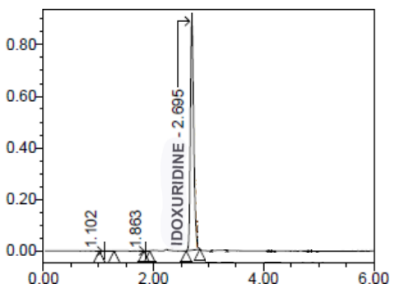
A linear relationship between peak areas versus concentrations was observed for Idoxuridine in the range of 50% to 150% of nominal concentration. Correlation coefficient was 1.000 and 1.000 for both Idoxuridine which prove that the method is linear in the range of 50% to 150%.

ROBUSTNESS

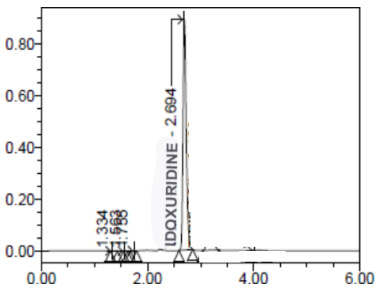
Parameter	RT	Theoretical plates	Asymmetry
Decreased flow Rate (0.8ml/min)	2.224	9184	1.28
Increased flow Rate (1.2ml/min)	3.420	9669	1.37
Decreased Temperature (200c)	2.434	9248	1.32
Increased Temperature (300c)	3.013	9555	1.35
Decreased comp Rate (5%)	2.857	9715	1.27
Increased comp Rate (5%)	3.979	10148	1.31
Decreased pH (0.2)	2.695	9322	1.36
Increased pH (0.2)	2.697	9383	1.36
Decreased Nm (2)	2.699	9248	1.36
Increased Nm (2)	2.697	9384	1.36

RESULT

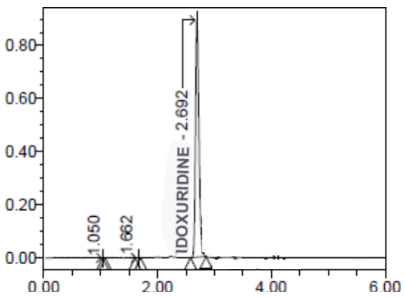
The results of Robustness of the presentmethod had shown that changes made in the Flow and Temperature did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is Robust.



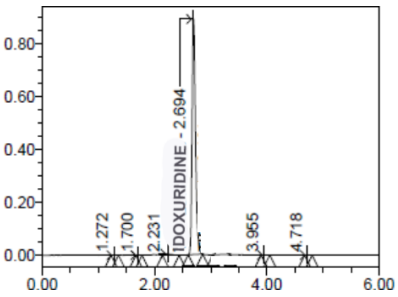
Acid degraded sample chromatogram Graph : 1



Base degraded sample chromatogram Graph : 2



Oxidant degraded sample chromatogram Graph : 3



Thermal degraded sample chromatogram Graph : 4.

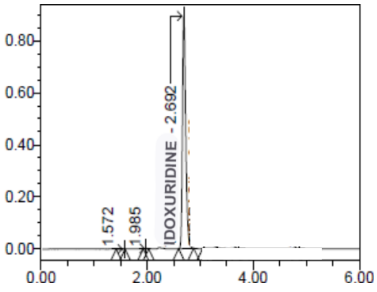
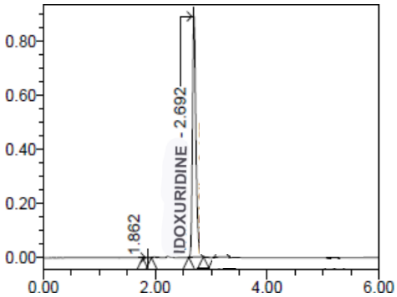


Photo degraded sample chromatogram Graph : 5



Water degraded sample chromatogram Graph : 6

Idoxuridine degradation data

Condition	Percent assay	Percent degradation
	Idoxuridine	Idoxuridine
0.1 N HCl	89.53	10.49
0.1N NaOH	92.49	7.53
30% H2O2	94.41	5.61
105oC	91.59	8.43
Sunlight	93.48	6.52
Water	98.38	1.64

SUMMARY

- A RP-HPLC method for Idoxuridine was developed and validated in tablet dosage form as per ICH guide lines. The results of this validation are as summarized in the report. The results are found to be complying with theacceptance criteria for each of the parameter.
- Waters Alliance RP-HPLC (Empower software with PDA detector) with Hypersil ACE C18 (250 x 4.6 mm, 5 µ) column, Injection volume of 10 µl is injected and eluted with the Mobile phase (OPA and Methanol in the ratioof 60:40) which was pumped at a flow rate of 1.0 ml at 270 nm. The peak of Idoxuridine was found well separated at 6 min.
- As per ICH guidelines, system suitability was found to be 0.2%.
- As per ICH guidelines, accuracy was to be 100%.
- As per ICH guidelines, precision was found to be 99%.
- As per ICH guidelines, linearity was found to be 1.000.
- As per ICH guidelines, limit of detection was found to be 2.673.
- As per ICH guidelines, solution stability was found to be 0.2.
- As per ICH guidelines, specificity was found to be 2.696.

Hence it is concluded that the assay method is found to be valid in terms of reliability,

precision, accuracy and specificity and hence it is suitable for routine analysis as well as for stability analysis.

CONCLUSION

▸ The study is focused to develop and validate RP - HPLC methods for estimation of Idoxuridine in tablet dosage form. For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developed methods were specific for Idoxuridine.

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