**Development and validation of a RP-HPLC-PDA method for simultaneous determination of trifluridine and tipiracil in pure and pharmaceutical dosageform**

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**Abstract**

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Tipiracil and Trifluridine, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Hypersil C18 (4.6×250mm) 5µ column using a mixture of Acetonitrile: Water:Methanol (60:20:20v/v)as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 230nm. The retention time of the Tipiracil and Trifluridine was 2.8, 3.8±0.02min respectively. The method produce linear responses in the concentration range of 10-50µg/ml of Tipiracil and 66.6-330µg/ml of Trifluridine. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

**Keywords** Tipiracil, Trifluridine, RP-HPLC, validation.

**INTRODUCTION**

Trifluridine is an anti-herpesvirus antiviral drug, used primarily on the eye. It is an antiviral derivative of thymidine used mainly in the treatment of primary keratoconjunctivitis and recurrent epithelial keratitis due to herpes simplex virus [1-4]. Tipiracil is a Nucleoside Analog Antiviral and Nucleoside Metabolic Inhibitor. The mechanism of action of trifluridine is as a Nucleic Acid Synthesis Inhibitor. The chemical classification of trifluridine is Nucleoside Analog.

Trifluridine is a fluorinated thymidine analog with potential antineoplastic activity. Trifluridine is incorporated into DNA and inhibits thymidylatesynthase, resulting in inhibition of DNA synthesis, inhibition of protein synthesis, and apoptosis. This agent also exhibits antiviral activity.

Tipiracil is a ThymidinePhosphorylase Inhibitor. The mechanism of action of it is as a Thymidine Phosphorylase Inhibitor [5-8].

Tipiracil is a drug used in the treatment of cancer. In Japan, it is approved for use in combination with trifluridine (as the drug TAS-102 or Lonsurf) for the treatment of un-resectable advanced or recurrent colorectal cancer. Tipiracil helps to maintain the blood concentration of trifluridine by inhibiting the enzyme thymidinephosphorylase which metabolizes trifluridine [9].

Colorectal cancer (CRC) is the fourth most common cause of cancer-related mortality [1]. The main treatments for patients with advanced metastatic colorectal cancer (mCRC) include systemic combination chemotherapies [2-7]. Although the standard therapies are initially effective, many patients relapse due to the onset of drug resistance and are subsequently placed on salvage chemotherapy [10].

Lonsurf® is a novel oral nucleoside antitumor agent that consists of trifluridine (FTD) and tipiracil (TPI) at a molar ratio of 1:0.5. FTD is the antitumor component of Lonsurf®, whereas TPI prevents degradation of FTD through a first-pass effect as a thymidine phosphorylase inhibitor. FTD is a well-known anti proliferative agent with two mechanisms of action; it inhibits thymidylate synthase (TS) and is also incorporated into DNA [8,9].

Recently, Lonsurf® was found to significantly improve overall survival of mCRC patients in whom systemic chemotherapy is either ineffective or not tolerated. Fig 1&2.

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Fig 1. Chemical structure of Trifluridine

Fig 2. Chemical structure of Tipiracil

Tipiracil is described chemically as 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]pyrimidine-2,4-(1H,3H)-dione mono hydro chloride. It has a molecular formula C9H11ClN4O2 and has the following structural formula.

EXPERIMENTAL METHODS
CHEMICALS AND REAGENTS

All the reagents used in the experimental work were of analytical grade. HPLC grade water was prepared by Milli-Q reverse osmosis (Millipore, Bedford, USA) and meets European Pharma-copoeia requirements. ACN and OPA (Sigma–Aldrich, Merck and Rankem) were used for preparing the mobile phase. Mobile Phase was used as solvent.

Working standards of FTD and TPI were provided by Glenmark Pharmaceuticals (Mahape, Navi Mumbai). FTD and TPI was checked by comparison with European Pharmacopoeia CRS standards. Lonsurf® containing 15mg of FTD and 6.14mg of TPI were purchased from local market Vijayawada, India.

Chromatographic conditions

An Alliance 2695 (Waters, USA) chromatographic system was used, equipped with a Quaternary pump, and waters 2996 photo diode array detector, Luna C18 250x4.6 mm, 5μ, auto sampler thermostat and degasser. Chromatographic software Empower was used for data collection and processing. Separations were performed using Luna C18, analytical column, 250x4.6 mm packed with 5 μm particle size. A 1m long steel capillary with 0.25 mm internal diameter, was inserted between the injection system and the entrance of the column, and injection volume was 10μL. Separations and simultaneous determination of FTD and TPI were performed using the mixture of ACN: OPA (0.1%) (50:50, v/v) as a mobile phase. Mobile phase was filtered through a 0.45 μm Millipore filter. The flow rate was 1.0 mL min−1 and the UV detection was performed at 292 nm.

Optimized chromatographic condition
Mobile Phase Optimization

Initially the mobile phase tried was Methanol: Orthophosphoric acid and Phosphoric acid (pH 3): Acetonitrile and Methanol: ACN and Methanol: Ammonium acetate Buffer with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Water in proportion 70:30 v/v respectively.

Optimization of Column

The method was performed with various columns like C18 column, X-bridge column, Xterra and C8 column. Hypersil C18 (4.6 x 150mm, 5μm, Make: Waters) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow. fig.3&4

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Instrument used: Waters HPLC with auto sampler and PDA detector 996 model.
Temperature: Ambient
Column: Hypersil C18 (4.6 x 150mm, 5μm, Make: Waters)
Mobile phase: Acetonitrile: Water: Methanol (60:20:20v/v)
Flow rate: 1 ml per min
Wavelength: 230 nm
Injection volume: 10 μl
Run time: 7 min.

Optimized chromatogram, blank, System suitability parameters is shown in the figure and the results are shown in Table.

PREPARATION OF MOBILE PHASE
Preparation of mobile phase
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Accurately measured 700 ml (70%) of Acetonitrile and 300 ml of Water (30%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

**Diluent Preparation**
The Mobile phase was used as the diluent.

**VALIDATION PARAMETERS**

**Method Precision**

**Preparation of Standard Solution**
Accurately weigh and transfer 10 mg of Tipiracil and 10 mg of Trifluridine working standard into a 10 ml of clean dry volumetric flasks individually and add about 7ml of Diluents to each volumetric flasks and sonicate to dissolve it completely and make up the volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml and 1.98ml of the above Tipiracil, Trifluridine stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

**Preparation of Sample Solution**
Accurately weigh 10 combination tablets crush in mortar and pestle and transfer equivalent to 10 mg of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 25 mg, Dose of Trifluridine is 50 mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.98ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. The standard and sample solutions of containing concentrations were 30µg/ml of Tipiracil and 198µg/ml of Trifluridine.

**Procedure**
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

**Accuracy**

**Preparation of Standard stock solution**
Accurately weigh and transfer 10 mg of Tipiracil and 10 mg of Trifluridine working standard into a 10 ml and 10 ml of clean dry volumetric flasks add about 7ml and 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml and 1.98ml of the above Tipiracil, Trifluridine stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

**Preparation of Sample solutions**
For preparation of 50% solution (With respect to target Assay concentration)
Accurately weigh 10 combination tablets crush in mortar and pestle and transfer equivalent to 10 mg of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 3mg, Dose of Trifluridine is 20mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.99ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. The standard and sample solutions of containing concentrations were 50%.

**For preparation of 100% solution (With respect to target Assay concentration)**
Accurately weigh 10 combination tablets crush in mortar and pestle and transfer equivalent to 10 mg of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 3mg, Dose of Trifluridine is 20mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.99ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. The standard and sample solutions of containing concentrations were 50%.

**Intermediate Precision/Ruggedness**
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

**Preparation of stock solution**
Accurately weigh 10 combination tablets crush in mortar and pestle and transfer equivalent to 10 mg of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 3mg, Dose of Trifluridine is 20mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1.98ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. The standard and sample solutions of containing concentrations were 30µg/ml of Tipiracil and 198µg/ml of Trifluridine.
of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 3mg, Dose of Trifluridine is 20mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.98ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. The standard and sample solutions of containing concentrations were 100%.

**For preparation of 150% solution (With respect to target Assay concentration)**

Accurately weigh 10 combination tablets crush in mortar and pestle and transfer equivalent to 10 mg of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 3mg, Dose of Trifluridine is 20mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.97ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. The standard and sample solutions of containing concentrations were 150%.

**Procedure**

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Tipiracil, Trifluridine and calculate the individual recovery and mean recovery values. These solutions were filtered through 0.45µ membrane and then each concentration, three replicate injections were made under the optimized conditions. Recorded the chromatograms and measured the peak responses.

**LINEARITY**

**Preparation of stock solution**

Accurately weigh 10 combination tablets crush in mortar and pestle and transfer equivalent to 10 mg of Tipiracil, Trifluridine (marketed formulation-dose of Tipiracil is 3mg, Dose of Trifluridine is 20mg in combination tablet) sample into a 10mL clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

**Preparation of Level – I (10ppm of Tipiracil & 66.6ppm of Trifluridine)**

0.66ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

**Preparation of Level – II (20ppm of Tipiracil & 132ppm of Trifluridine)**

1.32ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

**Preparation of Level – III (30ppm of Tipiracil & 198ppm of Trifluridine)**

1.98ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

**Preparation of Level – IV (40ppm of Tipiracil & 264ppm of Trifluridine)**

2.64ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

**Preparation of Level – V (50ppm of Tipiracil & 330ppm of Trifluridine)**

3.3ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent. The results are shown on Fig 5&6, Table 1 & 2)

**Procedure**

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

**ROBUSTNESS**

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

**Preparation of sample solution (75µg/ml of Tipiracil & 90µg/ml of Trifluridine)**

Accurately weigh and transfer 10 mg of Tipiracil and 10mg of Trifluridine working standard into a 10 mL and 10 ml of clean dry volumetric flasks add about 7mL and 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml and 1.98ml of the above Tipiracil, Trifluridine stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

**Effect of Variation of flow**

The sample was analyzed at 0.8 ml/min and 1.0 ml/min instead of 0.9 ml/min, remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded.
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Effect of Variation of mobile phase organic composition
The sample was analyzed by variation of mobile phase i.e. Acetonitrile: Water was taken in the ratio and 60:40, 80:20 instead 70:30, remaining conditions are same. 10µl of the above sample was injected twice and chromatograms were recorded. The results are shown Table 3 & 4.

Table 1. Linearity data of Tipiracil

<table>
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<th>S.No</th>
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<th>Area</th>
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<td>491862</td>
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<tr>
<td></td>
<td></td>
<td>Correlation Coefficient</td>
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Table 2. Linearity data of Trifluridine

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<tr>
<td></td>
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<td>Correlation Coefficient</td>
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Table 3. System suitability results for Tipiracil

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<th>Change in Organic Composition in the Mobile Phase</th>
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<td>2</td>
<td>*Actual</td>
<td>2423.3</td>
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<tr>
<td>3</td>
<td>10% more</td>
<td>2423.52</td>
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Table 4. System suitability results for Trifluridine

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<tr>
<td>3</td>
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</table>

Fig 3. Typical chromatogram of Trifluridine and tipiracil
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CONCLUSION
High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Tipiracil and Trifluridine was done by RP-HPLC. The mobile phase was optimized with consists of Acetonitrile: Water. Acetonitrile mixed in the ratio of 70:30 % v/ v. A Hypersil C18 column (4.6 x 150mm, 5μm, Make:
Waters) or equivalent chemically bonded to porous silica particles was used as stationary phase. The solutions were chromatographed at a constant flow rate of 1ml/min. the linearity range of Tipiracil and Trifluridine were found to be from 10-50ppm, 66.6-330ppm respectively. Linear regression coefficient was not more than 0.999, 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery of Tipiracil is 99.6 and Trifluridine is 99.8%. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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CONFLICT OF INTEREST
No Conflict of Interest.

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