INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

AN EFFECTIVE AND SENSITIVE STABILITY INDICATING CHROMATOGRAPHIC APPROACH BASED ON RP-HPLC FOR TRIFLURIDINE AND TIPIRACIL IN BULK AND PHARMACEUTICAL DOSAGE FORM

Kusuma Jogi[1,2], Mandava Basaveswara Rao[2] and Rundraraju Ramesh Raju[1*]

[1]Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur(Dist), Andhra Pradesh, India.
[2]Department of Chemistry, KrishnaUniversity, Machilipatnam, Krishna(Dist), Andhra Pradesh, India.

ABSTRACT

A stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method with a high sensitivity was developed for the validation of stability of Trifluridine (FTD) and Tipiracil (TPI) in bulk and pharmaceutical dosage form. Chromatographic separation of FTD and TPI were successfully achieved on an Waters Luna C18 250x4.6 mm,5µ with an isocratic mobile phase composed of a mixture of orthophosphoric acid(OPA):acetonitrile(ACN) (50:50, v/v) at a flow rate of 1.00 mL min−1. The drugs were quantified using a photodiode array detector set at a wavelength of 292 nm. The reversed-phase HPLC method has been validated as per International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines to determine FTD and TPI in pharmaceutical dosage form. The proposed method showed a good linearity in the concentration range of 1.02–15.30 µg/mL for FTD and 0.41-_6.15 µg/mL for TPI under optimized conditions. The statistical performance of the HPLC method was fully validated and the performance results of the proposed HPLC method were considerably satisfactory with reference to the RSD values of validation parameters like linearity, system precision, method precision, robustness, ruggedness etc.,. The validated method was successfully applied to quantify the FTD and TPI in tablet form, and the corresponding recovery value was found to be 100 % for both FTD and TPI. The validated HPLC method is one of the promising alternative analytical tool for routine analysis of FTD and TPI in pharmaceutical samples.

Keywords: Trifluridine, Tipitacil, HPLC Determination and Validation.

Background

Trifluridine 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.

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 inhbiting the enzyme thymidinephosphorylase which metabolizes trifluridine.

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.

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[10].

Lonsurf® is a combination of trifluridine, a nucleoside metabolic inhibitor, and tipiracil, a thymidine phosphorylase inhibitor, indicated for the treatment of patients with metastatic colorectal cancer who have been previously treated with fluoropyrimidine-, oxaliplatin-and irinotecan-based chemotherapy, an anti-VEGF biological therapy, and if RAS wild-type, an anti-EGFR therapy

Based on animal studies and its mechanism of action, Lonsurf® can cause fetal harm when administered to a pregnant woman. Trifluridine/tipiracil caused embryo-fetal lethality and embryo-fetal toxicity in pregnant rats when orally administered during gestation at dose levels resulting in exposures lower than those achieved at the recommended dose of 35 mg/m^2 twice daily.

The other most common side effects of Lonsurf® include tiredness, vomiting, nausea, abdominal pain, decreased appetite, fever, diarrhea.

Trifluridine is described chemically as 2'-deoxy-5-(trifluoromethyl) uridine. It has a molecular formula $C_{10}H_{11}F_3N_2O_5$ and has the following structural formula.

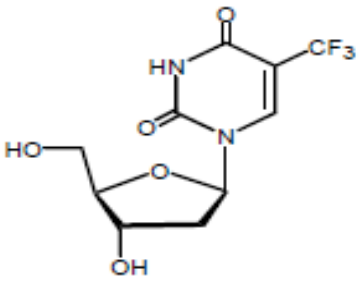

Fig. 1: Chemical structure of Trifluridine

Tipiracil is described chemically as 5chloro-6-[(2-iminopyrrolidin-1-yl)methyl]pyrimidine-2,4-(1*H*,3*H*)-dione mono hydro chloride. It has a molecular formula C $_9H_{11}$ Cl N_4O_2 and has the following structural formula

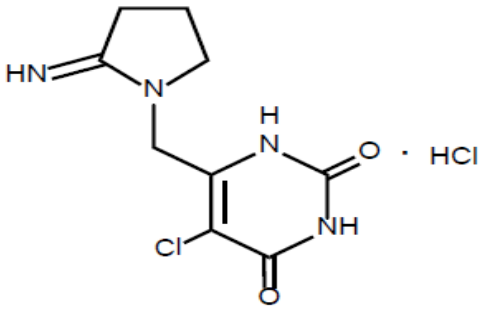

Fig. 2: Chemical structure of Tipiracil

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, Merk 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 fron local markrt Vijayawada, India.

Chromatographic conditions (instrumentation and analytical 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.

Analytical procedure

Preparation of standard solutions

10.2 mg of standard FTD and 4.0 mg of TPI powder were accurately weighed and dissolved in a 10-mL mixture of OPA:ACN (1:1, v/v) by sonication for 10 min. The FTD and TPI standard solutions were diluted by the mixture of OPA:ACN (1:1, v/v) OPA buffer to obtain the required working range concentrations for HPLC. Dilute 1mL of above solution to 10 mL with the diluents. Further dilute 1mL of above solution to 10 mL with the mixture of OPA:ACN (1:1, v/v). The solutions were filtered through a 0.45µm membrane filter before injection into the HPLC system.

Assay sample preparation

Ten tablets of Lonsurf® were carefully weighed and powdered to get a homogenous fine powder in a mortar. An appropriate weight of this powder equivalent to one tablet content was weighed, transferred into the calibrated flask and then dissolved in the mixture of OPA:ACN (1:1, v/v) in an ultrasonic bath. Further dilute 0.1 mL of above solution to 10 mL with the mixture of OPA : ACN (1:1, v/v). Filter through 0.45µ Nylon syringe filter to obtain the certain concentration in the linearity range of FTD and TPI for HPLC.

Validation procedure

Chromatographic separation was optimized in the aim to obtain a resolution above 1.5 between all components, with the respect of stationary and mobile phase compositions, flow rate, sample volume, detection wavelength and temperature.

The method was validated for linearity, range, precision (repeatability and intermediated precision), specificity, limit of quantization, limit of detection, robustness and forced degradation.

Linearity and range

Standard calibration curves were prepared with five calibrators over a concentration range of 1.02-15.30 mg mL^{-1} for Trifluridine and 0.41-6.15 mg mL^{-1} for Tipiracil. The data of peak area versus drug concentration were treated by linear least square regression analysis. The standard curves were evaluated for linearity.

Precision

The precision of the assay was studied with respect to both repeatability and intermediated precision. Repeatability was calculated from six replicate injections of freshly prepared solution in the same equipment on the same day. Repeatability for FTD and TPI was realized with a 102 and 41 µg mL^{-1} solution. The experiment was repeated by assaying freshly prepared solution at the same concentration on 2 additionally consecutive days to determine intermediate precision. Precision was expressed by the % of the relative standard deviation (R.S.D.) of the analyte peaks.

Specificity

Specificity of a method can be defined as absence of any interference at retention times of peaks of interest, and was evaluated by observing the chromatograms of blank samples and samples spiked with FTD and TPI. The variable number of excipient used in generic versions of FTD and TPI, as well as the lack of information in the composition of some generic formulations makes it difficult to assess selectivity by traditional analysis comparison with a placebo solution.

Limits of detection and quantization

Limits of detection (LOD) and limits of quantization (LOQ) were provided and calculation was made with the following equations:

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

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

When σ was the standard deviation of the response (estimated from the standard deviation of *y*- intercepts or regression lines) and S was the slope of the standard curve.

Sensitivity

The sensitivity (*6x*) of an analytical method is defined by the minimum

variation that requires to be applied to the magnitude measured in order to obtain a significant variation in the signal measured.

Robustness

Robustness of method was investigated by varying the chromatographic conditions such as change of flow rate(±20%), organic content in mobile phase (± 2%). Robustness of the developed method was indicated by the overall %RSD between the data at each variable condition.

Forced degradation

Forced degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principal peaks shall pass.

Stability

Stability by preparing the analytical solution and injecting at periodic intervals of 24 hours to 48 hours at 3 to 4 hour intervals depending on the instrument utilization and sequence of injection.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

Firstly, HPLC conditions were optimized to obtain a desired peak with high purity and resolution. Therefore, the various parameters affecting the peak shape, retention time and resolution of FTD and TPI were investigated in detail. The separation efficiency of Waters Luna C18 (250x4.6 mm,5µ) was compared to the Phenylhexyl 250x4.6 mm,5µ for the determination of FTD and TPI under the same conditions, and the proposed column was chosen for the further optimization of parameters. During our preliminary experiments, the series of aqueous mobile phases containing buffer solutions with the different pH values in combination with different organic modifiers including the different ratios of acetonitrile, methanol and triethylamine were tested for obtaining the optimum separation conditions. Acetonitrile and *Ortho*-Phosphoric acid were selected as the eluents. The chromatographic analysis time of FTD and TPI was shortened with high organic solvent content, and also, the buffer solutions in the mobile phase ensured stable chromatographic retention times preventing broad peaks. The effect of the mobile phase pH on the retention time and peak shape of the analyte was studied especially in the acidic region. The best retention time and peak shape of FTD and TPI were achieved with OPA buffer. The best separation was achieved with the mobile phase consisting of ortho phosphoric acid:acetonitrile (50:50, v/v). The calibration curves of FTD and TPI for HPLC analysis were constructed by plotting the peak area aganist the concentration of the drugs.

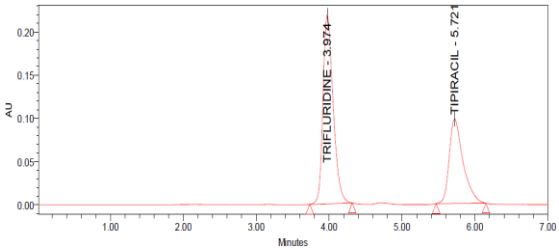

Fig. 3: Typical Chromatogram

METHOD VALIDATION

The method was validated for linearity, precision, accuracy, robustness, rugdness, forced degradation and stability of the FTD and TPI.

Linearity was prepared in the range of 1.02-15.30 µg/ml for Trifluridine and 0.41- 6.15 µg/ml solutions are analyzed through the high pressure liquid chromatographic technique. The peak area were plotted against concentration was subjected to linear plots shown in figures 4 & 5.

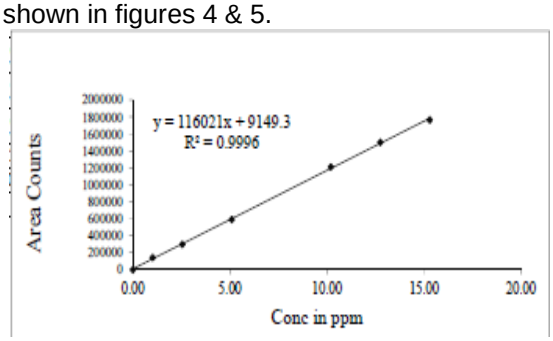

Fig. 4: Linearity plot for Tipiracil

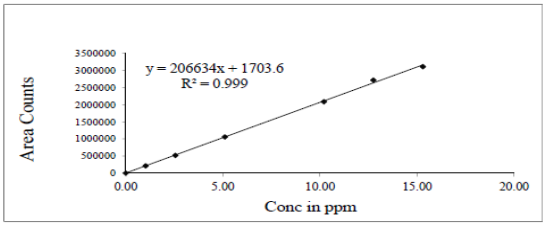

Fig. 5: Linearity plot for Trifluridine

Precision of this method was studied in inter day and intra day variation. The precision of intraday studies of six different concentration of the drug was repeated thrice in a day and in the inter day variation studies of six different concentration of the drug was repeated on three consecutive days. The developed method was found to be precise as

the percentage of RSD values for inter-day and intra-day precision studies were found to be less than 2%. Good recoveries (99 - 100%) of the drug were obtained at each added concentration, indicating that the method was accurate.

Table 1: Recovery of Trifluridine drug

Amount of Trifluridine drug mg/ml	Recovery Solution (area) mAU	% drug recovery
5.0	1070805	99.8
10.0	2152859	100.5
15.0	3102157	99.8

Table 2: Recovery of Tipiracil drug

Amount of Tipiracil drug mg/ml	Recovery Solution (area) mAU	% drug recovery
2.05	606836	100.7
4.1	1284689	100.7
6.15	1771250	100.3

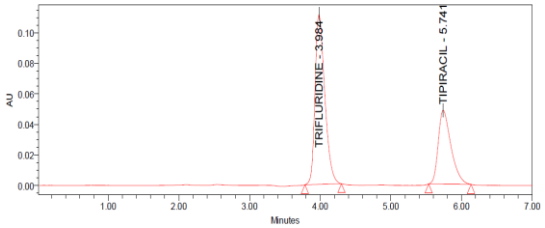

Fig. 6: Chromatogram for Accuracy 50%

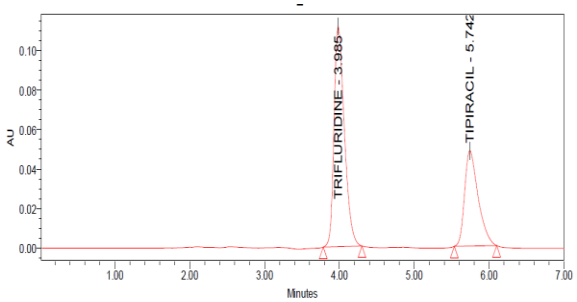

Fig. 7: Chromatogram for Accuracy 100%

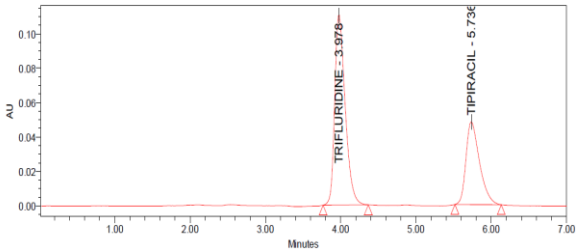

Fig. 8: Chromatogram for Accuracy 150%

The limit of detection (LOD) for FTD and TPI were found to be 0.255 and 0.1025 µg/mL calculated from related equation (S/N = 3). The similar study claimed that a narrow working range(LOQ) such as 0.51– 0.205 µg /mL for FTD and TPI were obtained at the excitation wavelength of 292 nm.

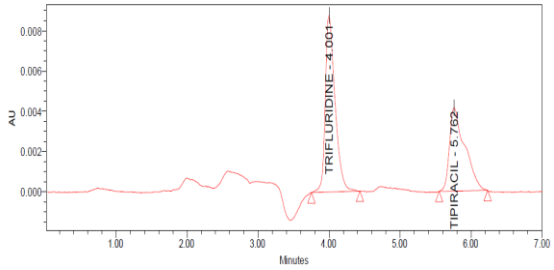

Fig. 9: Chromatogram for LOD

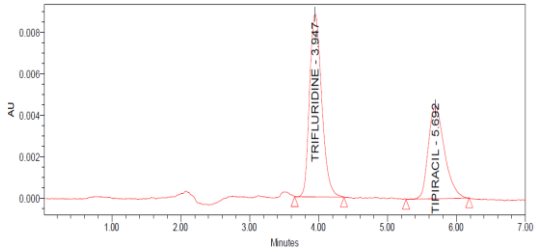

Fig. 10: Chromatogram for LOQ

Forced degradation experiments were also performed to evaluate the stability and specificity of the proposed HPLC method in different mediums. The acidic, alkaline and oxidative degradation of FTD and TPI were studied by treating with strengths of base (0.05 N and 0.5 N NaOH), acid (0.05 N, 0.5 N and 1 N HCl), 30 % H2O2 solutions at 80 °C for 1 h, respectively. The thermal degradation of FTD and TPI were also studied by heating the FTD and TPI solution at 80 °C for 3 h and photolytic degradation was studied by exposing FTD and TPI solution to sunlight for 6 h. The whole degradation products were observed at approximately 3.90 and 5.70 min for FTD and TPI, respectively, in all proposed stress conditions as shown in figures 11-17. The stress studies showed no significant difference in terms of retention times of drugs, and no interfering peaks were observed within the retention time under alkaline, acidic, oxidative, thermal and pyrolytic degradation conditions. Considering all these data, FTD and TPI were successfully separated from all the degradation products which were confirmed by the resolution values calculated from each chromatogram (Rs > 1.5)

Table 3: Results of forced degradation studies

Stress Condition/ duration/solution	Degradation %	
	Trifluridine	Tipiracil
Acid degradation (0.5 N HCl, 1 hr)	20.4%	26.2%
Alkaline degradation (0.5 N NaOH, 1 hr)	21.8%	25.6%
Oxidative degradation (30 % H2O2,80°C for 10 min)	23.2%	27.8%
Reduction Degrdation (10% Sod.Bisul, 1hr)	23.0%	24.5%
Thermal degradation (Solid sample, 80°C, 3 hr)	22.2%	25.6%
Photolytic Degradation (sample expose sun light 6hr)	28.2%	24.2%
Hydralysis Degradation	21.8%	28.4%

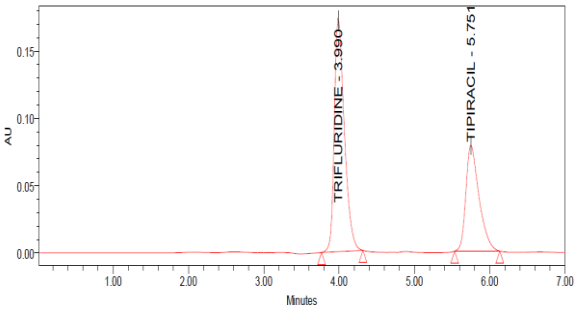

Fig. 11: Chromatogram for Acid Degradation

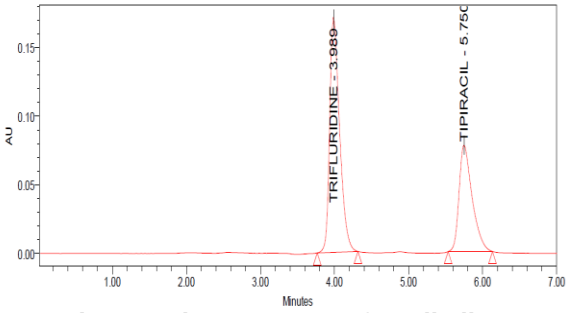

Fig. 12: Chromatogram for Alkali Degradation

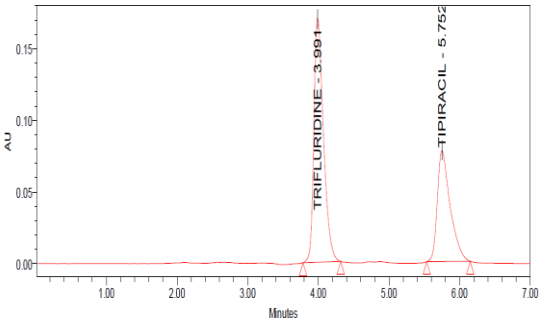

Fig. 13: Chromatogram for Peroxide Degradation

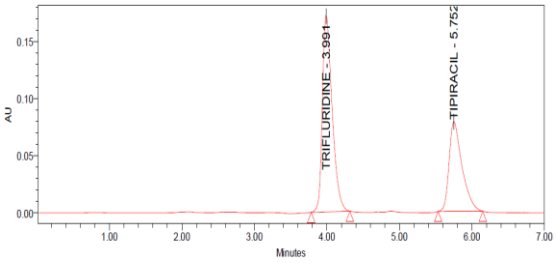

Fig. 14: Chromatogram for Reduction Degradation

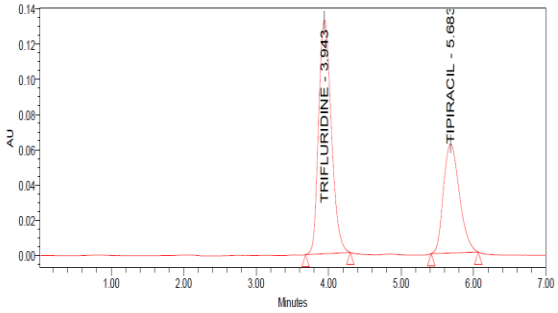

Fig. 15: Chromatogram for Thermal Degradation

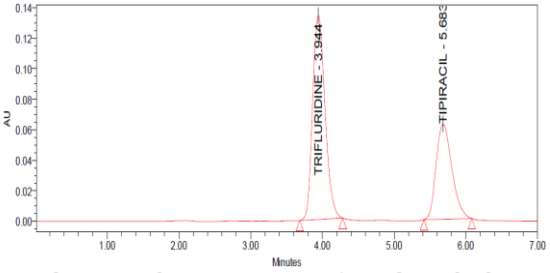

Fig. 16: Chromatogram for Photolytic Degradation

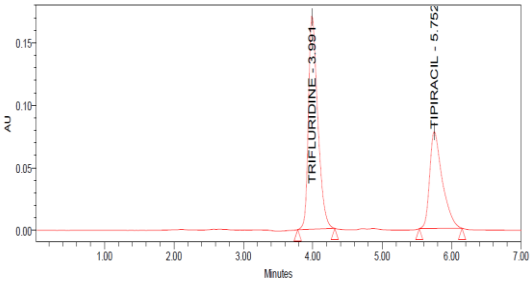

Fig. 17: Chromatogram for Hydralysis Degradation

Robustness of the method includes small changes in chromatographic conditions such as change in flow rate (± 20%), organic content in mobile phase (± 2%), pH (±0.2) and wavelength of detection (± 5%) . To determine the robustness of the method for the analysis of FTD and TPI the above mentioned changes has been undertaken and the RSD values were found to be reliable(RSD<1.5%).

The influence of changes in

chromatographic parameters was shown in table 4. The chromatographic data has been shown in figures 18 to 21.

Table 4: Results for Robustness study

Change in parameter	% RSD
Flow plus (1.2 ml/min)	0.65
Flow minus(0.8 ml/min)	0.92
Wavelength plus (220 nm)	0.74
Wavelength minus (210 nm)	0.56
Organic phase composition (+2%)	0.24
Organic phase composition (-2%)	0.22
pH Variation (+0.2)	0.36
pH Variation (-0.2)	0.48

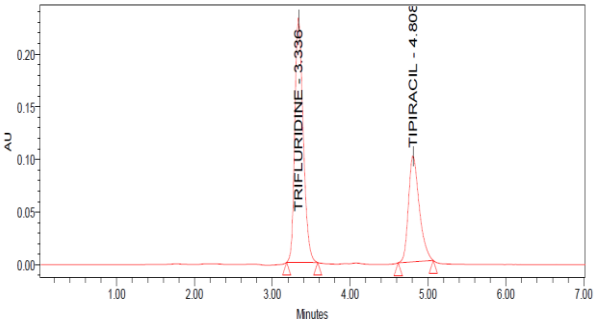

Fig. 18: Chromatogram for Flow Plus

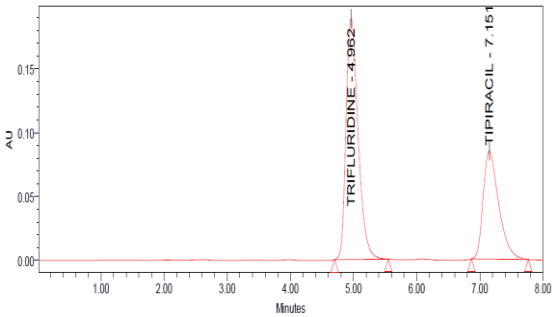

Fig. 19: Chromatogram for Flow Minus

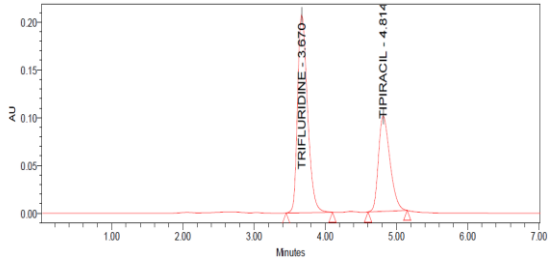

Fig. 20: Chromatogram for organic Plus

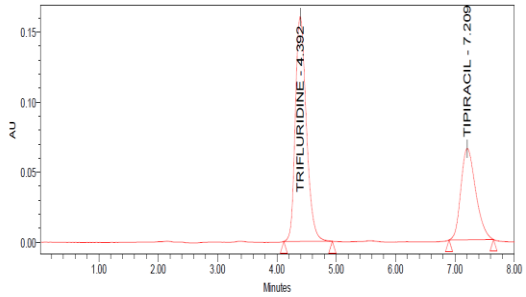

Fig. 21: Chromatogram for organic Minus

CONCLUSIONS

A highly sensitive and effective validated reversed-phase HPLC method was successfully developed with a low LOD value for FTD and TPI assay. The FTD and TPI were subjected to forced degradation under several stress conditions. The satisfactory results were achieved from degradation studies, which revealed that the method was stability indicating. Besides This method was validated for linearity, accuracy, precision, robustness of FTD and TPI drug. The RSD values for all parameters were found to be less 2, which indicates the validity of method and results obtained by this method are in fair agreement. Finally this method can be used as better analytical tool for pharmaceutical formulations of FTD and TPI drug.

ACKNOWLEDGEMENTS

The authors are thankful to the Acharya Nagarjuna University, Guntur and Krishna University, Machilipatnam for providing the facilities and timely support for carrying out this research work. The authors thank the Glenmark Pharmaceuticals for gifting the drug.

Authors' contributions

All authors equally contributed in experimental design, analysis results, writing and proofing and approved the final manuscript. Experimental work was performed by Jogi Kusuma.

Competing interests

The authors declare that they have no competing interests.

REFERENCES

1. Costin D, Dogaru M, Popa AS, Cijevschi I. [Trifluridine therapy in herpetic in keratitis] Rev Med Chir Soc Med Nat Iasi. 2004;108(2):409-12.
2. Hong DS, Abbruzzese JL, Bogaard K, Lassere Y, Fukushima M, Mita A, Kuwata K and Hoff PM. Phase I study to determine the safety and pharmacokinetics of oral

administration of TAS-102 in patients with solid tumors. Cancer. 2006; 107(6):1383-90.
3. Temmink OH, Prins HJ, van Gelderop E and Peters GJ. The Hollow Fibre Assay as a model for in vivo pharmacodynamics of fluoropyrimidines in colon cancer cells. Br J Cancer. 2007;15;96(1):61-6. Epub 2006 Dec 19.
4. Tsunoda N and Yoshimura H. Metabolic fate of noscapine. III. Further studies on dentification and determination of the metabolites. Xenobiotica. 1981;11(1):23-32.
5. Lenz EM, D'Souza RA, Jordan AC, King CD, Smith SM, Phillips PJ, McCormick AD and Roberts DW. HPLC-NMR with severe column overloading: fast-track metabolite identification in urine and bile samples from rat and dog treated with [14C]-ZD6126. J Pharm Biomed Anal. 2007;43(3):1065-77.
6. Sweetman SC. Martindale: the complete drug reference. 36th ed. London: The Pharmaceutical Press; 2009.
7. Tanaka N, Sakamoto K, Okabe H, Fujioka A and Yamamura K. Repeated oral dosing of TAS-102 confers high trifluridine incorporation into DNA and sustained antitumor activity in mouse models. Oncology reports 2014-12-01
8. Nannizzi S, Veal GJ, Giovannetti E and Mey V. Cellular and molecular mechanisms for the synergistic cytotoxicity elicited by oxaliplatin and pemetrexed in colon cancer cell lines. Cancer chemotherapy and pharmacology. 2010-08-01
9. Nozomu Tana KA, Ka zuki Sakamoto, Hir oyuki Okabe, Akio Fujioka, Keisu ke Ya mamura, Fumio Nakagawa, Hideki Nagase, Tatsushi Yokogawa, Kei O guchi,b Keiji Ishida, Akiko Osada, Hir omi Ka zun o, Yu kari Ya mada 1 and Kenichi Matsu o. Repeated oral dosing of TAS-102 confers high trifluridine incorporation into DNA and sustained anti tumor activity in mouse models. Oncology Reports. 2014; 32: 2319-2326.
10. ICH Q2 (R1). International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceutical for Human Use. Validation of analytical procedures: text and methodology. Geneva, Switzerland; 2005.
11. VA Pharmacy Benefits Management Services, Medical Advisory Panel, and VISN Pharmacist Executives : Trifluridine - tipiracil (LONSURF®). National Drug Monograph February 2016.
12. FDA approves new oral medication to treat patients with advanced colorectal cancer. 2015
13. Burness CB and Duggan ST. "Trifluridine/Tipiracil: A Review in Metastatic Colorectal Cancer". Drugs. 2016;76(14):1393–402. doi:10.1007/s40265-016-0633-9.
14. Lonsurf EPAR – Summary for the Public. European Medicines Agency. April 2016.
15. A novel combination antimetabolite, TAS-102, exhibits antitumor activity in FU-resistant human cancer cells through a mechanism involving FTD incorporation in DNA". Sep 2004.
16. Matsushita, Shigeto. The Effect of a Thymidine Phosphorylase Inhibitor on Angiogenesis and Apoptosis in Tumors. Cancer Research. 15 April 1999.
17. Peters, Godefridus. TAS-102: more than an antimetabolite. The Lancet Oncology. 2012.