IJPSR (2020), Volume 11, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 20 August 2019; received in revised form, 26 January 2020; accepted, 03 March 2020; published 01 July 2020

METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATION OF TRIFLURIDINE IN HUMAN PLASMA BY USING LC-MS/MS TECHNIQUE

Abdul Saleem Mohammad [*1], Fazil Ahmad [2] and B. Jayanthi [3]

Department of Pharmaceutical Analysis and Quality Assurance [1], Department of Pharmacology [2], Nizam Institute of Pharmacy, Deshmukhi - 508284, Telangana, India.
Department of Pharmacy [3], Annamalai University, Annamalai Nagar, Chidambaram – 608002, Tamil Nadu, India.

Keywords:

Trifluridine, Antiviral, LC-ESI-MS/MS, Specificity, Accuracy and Matrix Effect

Correspondence to Author:
Abdul Saleem Mohammad

Assistant Professor,
Department of Pharmaceutical Analysis and Quality Assurance,
Nizam Institute of Pharmacy,
Deshmukhi - 508284, Telangana, India.

E-mail: abdulsaleemmohammad89@gmail.com

ABSTRACT: The main objective of the current study was to develop a highly sensitive LC-MS/MS (liquid chromatography-tandem mass spectrometry) technique for the quantification of trifluridine (TFD) in human plasma. Chromatography was processed on Phenomenex-RP-C18 (5 µm, 250 mm × 4.6 mm) column comprising acetonitrile, methanol and 5 mM ammonium formate (45:40:15, v/v) as a mobile phase, infused at a flow of 0.8 ml/min. TFD and internal standard β-thymidine (TMD) were separated from 150 ml plasma utilizing Phenomenex cartridges. Quantification of the [M+H] +ion of analyte was done with MRM (multiple reaction monitoring) modes in LC-MS/MS utilizing electro-spray ionization. The parent and product ions transitions were monitored atm/z 297-181 and m/z^2 41–42 for TFD and TMD respectively. The linearity was processed in the concentration range of 5.0–2000.0 ng/ml with regression coefficient (r^2) = 0.999. Method intra and inter-batch precision findings were <3.33% and intra and inter-batch accuracy findings at three different concentrations were within 97.34% to 103.74%. Matrix effects were calculated by determining the %CV values for High and Low QC samples and was found to be 1.65% and 0.73% respectively. The % change for all the re-injected QC solutions was ≤7.45. The method was specific, accurate, precise and stable for more time and the technique was successfully applied for the quantification of trifluridine in human plasma samples.

INTRODUCTION: Trifluridine chemically designated as 1- [4- Hydroxy- 5- (hydroxymethyl) oxolan- 2- yl] - 5- (trifluoromethyl)- (1H, 3H)- pyrimidine-2, 4-dione with molecular formula $C_{10}H_{11}F_3N_2O_5$ **Fig. 1** and molecular weight 296.2 g/mol. This drug belongs to the nucleoside of fluorinated pyrimidine and structurally similar to idoxuridine.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(7).3252-59

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(7).3252-59

In ophthalmic preparations, it acts against the virus to treat keratoconjunctivitis and also useful in epithelial keratitis caused by herpes simplex-virus type-I and type-II. This drug also useful in the treatment of metastatic colorectal cancer, when it combines with tipiracil (tablet) [1-3]. TFD acts by inhibiting viral replication.

The drug merges with DNA of the virus during the replication process and synthesis of an imperfect protein leads to an increase in the rate of mutations. TFD is also useful in cancer treatment through this mechanism only. The cancer cells will uptake and immediately phosphorylated by thymidine-kinase to active monophosphate. Subsequently, TFD-triphosphate was formed by phosphorylation and it

directly merges into the DNA of cancer cells in thymidine place leads to inhibition of DNA functioning, DNA production, and proliferation of cancer cells. TFD mono-phosphate also prevents the thymidylate synthetase enzyme reversibly, which is useful in DNA replication [4-6]. Several RP-HPLC methods [7-10] were reported in the literature of TFD for the quantification of TFD as single and with combined dosage forms. No single procedure was reported by utilizing LC-MS/MS technique. The goal of the present research was to develop a simple and highly sensitive bioanalytical LC-MS/MS technique for the quantification of TFD in human plasma samples.

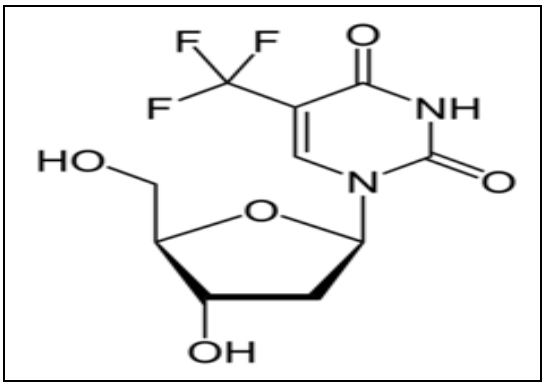

FIG. 1: STRUCTURE OF TRIFLURIDINE

MATERIALS AND METHODS:

Reagents and Chemicals: Blank plasma samples in K3EDTA were acquired from Supratech Labs, Ahmedabad, India. Phenomenex (StrataXC-33 m) RP-extraction cartridges were acquired from Phenomenex, India. Trifluridine reference material of 99.62% and β-thymidine internal standard (IS) of 99.89% purity were acquired from Biophore, Hyderabad, India. HPLC-grade methanol and acetonitrile and ammonium formate were procured from Sisco Laboratories, India.

Instruments: An LC-Shimadzu united with Shimadzu-LCMS triple quadrupole mass spectro-meter detection system (Shimadzu, Japan) was utilized. Chromatography was processed on Pheno-menex-RP-C_{18} (5 µm, 250 mm × 4.6 mm) column comprising acetonitrile, methanol and 5 mM ammonium formate (45:40:15, v/v) as a mobile phase, infused at a flow of 0.8 ml/min. Analytical column-oven and auto-sampler temperatures were kept at 40 °C and 5 °C respectively. The electrospray ionization source was processed in positive mode of ionization.

Calibration Standards: TFD 1mg/ml stock solution was processed freshly by diluting 10mg drug in 10 ml of 70% methanol. Calibration standards of eight different concentration levels were processed by spiking blank plasma with TFD standard solution to get the concentrations of 5.0, 20.0, 100, 250, 500, 1000, 1500 and 2000 ng/ml.

Quality Control Standards: These solutions were processed at three different levels of Lowest Quality Control (LQC), standards, Median Quality Control (MQC) standards, and Highest Quality Control (HQC) standards. Quality control (QC) samples were processed according to calibration standards to get the concentrations of 20, 500 and 1800 ng/ml for LQC, MQC and HQC respectively. Processed solutions were kept in the freezer at-20 ºC till further analysis.

Mass Instrument Conditions: The mass instrument was operated in positive (+) ionization (PI) mode. The mass conditions were optimized based on tuning the instrument for TFD and IS by injecting the 500 ng/ml solution of both drugs at a flow of 10 µl/min into the LC mobile phase (0.20 ml/min). Optimized mass instrument settings for specific TFD and IS where: curtain gas (CG) was 21 psi, ISG (ion source gas) 1 was 40 psi, ISG (ion source gas) 2 was 40 psi, an ion-spray voltage was 4500 V, turbo-spray temperature at 550 ℃. Quantitative analysis was achieved by MRM of the transition pairs of transition sm/z 297–181 for TFD and m/z 241–42 for TMD, with 145 ms per transition as dwell time. TFD and IS precursor ions were formed by de-clustering potential (DP) s of 150 and 160 V respectively and precursor ions of TFD and IS were converted into fragments at collision energies (CE) of 25 and 25 EV with nitrogen (N_2) gas at a pressure of 5 arbitrary units.

Protocol for Sample Separation: 25 ml of TMDIS working standard solution was mixed with 150 ml of spiked plasma samples and sonicated for 2 min. 500 ml of water was mixes to the resultant solution and subjected to vortex. The resulting solution was filled in Strata XC-33 m separation cartridge, which was conditioned by utilizing 1 ml of methanol and subsequently by 1 ml water. Sample washing was processed by 1.0 ml of water, subsequently drying of the cartridge by N_2 gas (1.72×10^5 Pa) for 2.0 min.

Separation of TFD and TMD was processed by utilizing 500 ml of mobile phase into pre-labeled vials. From the resultant solution, 10.0 ml was utilized for infusing into the LC-system.

Method Validation: Complete TFD-method validation in human K3EDTA-plasma was processed, as per USFDA and EMEA guidelines. The technique was validated for specificity, matrix effect, precision, linearity, sensitivity, process efficiency, accuracy, reinjection reproducibility and dilution integrity and stability study of TFD [11-14].

Selectivity and Sensitivity: Analytical technique selectivity concerning matrix metabolites, constituents, and associated medicaments were evaluated after analyzing 10 lots (lipemic-2, haemolyzed-2 and normal-6) of human K3EDTA-plasma. The resulting processed samples were extracted with SPE and analyzed for TFD at LOQ level. The peak response of all the matrix constituents in the blank sample at TFD and IS retention times should be <20 and 5% of the average peak response of TFD and IS at LOQ level respectively. The sensitivity was estimated by assessing signal to noise-(S/N) ratio in 10 batches of screened and spiked LOQ-samples. The S/N was measured by the formula:

S/N ratio = (Signal: noise ratio of LOQ) / (average signal: noise ratio of blanks) > 5

Dilution Integrity: It was processed by making the sample concentration nearly two times the 90% ULOQ concentration. The resultant solution was made dilution (2 and 4 times) with blank plasma to get the solution concentration of solution within linearity range.

Then the samples were evaluated against a fresh calibration standard solution. The acceptable norms were the same as precision and accuracy parameter.

Linearity: Calibration standards (Non-zero) of eight different concentrations of 5.0, 20.0, 100, 250, 500, 1000, 1500 and 2000 ng/ml were freshly processed and quantified in 3 individual runs.

Linear plots (Peak area ratio of analyte and IS peaks versus nominal concentration) were plotted by the least-squares linear regression and reciprocal of the squared concentration ($1/x^2$) used as a weighting factor.

Method Precision and Accuracy: The inter-day and intraday precision and accuracy were processed for TFD in human plasma. Within a day intra-run and between days, inter-run accuracy was analyzed for six replica samples of Quality control at LLOQ, Low, median and high QCs. Method precision was evaluated by determining the % Coefficient of variation for all control samples. The percentage deviation should be <15.0. In the same way, the average accuracy should be ± 15 [15-18].

Matrix Effect: Each of LQC and HQC samples after extraction in 6 different blank matrix lots (post-extraction spiked samples) were prepared. Simultaneously, SIX replicates of equivalent aqueous/neat QC samples were prepared and analyzed [19-21]. Matrix factor for the analyte and IS in each lot were evaluated using the formula:

Matrix Factor = Peak response in the presence of matrix ions / Average peak response in aqueous samples

Reinjection Reproducibility: This parameter was processed by re-injecting QC-samples from accepted precision and accuracy lot during validation. The re-injected sample concentrations were evaluated by comparing with calibration standard solutions of the same accuracy-precision lot which were estimated 48 h before. The percentage difference between re-injected and original values was measured by the formula:

% difference = Actual concentration-reinjected concentration / (Actual concentration) × 100

Stability: Each six low and high QCs were regained from the freezer after 3 freeze-thaw phases. Samples were kept at -30 °C in three cycles of 24.0, 48.0, 72.0 h. Bench-top stability was assessed for a 6.5 h period with standard concentrations. For the long-term stability quality control standards were determined by analysis after 121 days kept at -50 °C. Stability solutions were prepared and separated along with freshly spiked CSs. The accuracy and precision of the stability solutions should be ± 15% of their nominal concentrations. The auto-sampler stability esti-mated after 72 h under auto-sampler (at 10 °C) condition. The freeze-thaw stability was performed by storing the QC solutions at -50 °C (frozen) and thawed at room conditions for 3 times. The change in analyte concentration was less (<15%) then the compound said to be stable [17].

Ruggedness: Method ruggedness was assessed by processing QC standards for one precision and accuracy batch utilizing different columns of same composition by different analysts. The % RSDs for LQC, MQC and HQCs should be ≤ 20% for LLOQ and ≤ 15% for the remaining QC standards.

RESULTS AND DISCUSSION:

Internal Standard Selection: Identification and selection of IS was a very important thing in an LC-MS/MS technique. The IS should have similar mass and chromatographic behaviour with an analyte to be determined. Therefore, TMD chosen for TFD internal standard [18, 20].

Optimization of Chromatography: To get a good resolution between TFD and TMD different phenyl and C18-columns like Ascentis, Zorbax SB-C18, Hypurity C-18, Poroshell-EC-C18, Sunshell-C18, Kinetex-C18, Discovery C18, Luna-RP-C18 and ACE-RP-C18 were utilized. TFD and IS were well separated using Phenomenex-RP-C18 (5 µm, 250 mm × 4.6 mm) column. However, a mobile phase comprising acetonitrile, methanol and 5 mM ammonium formate (45:40:15, v/v) was established optimal.

Method Validation: Method was validated as per the EMEA and USFDA-guidelines and around was no nosiness noticed at the RT (retaining time) of TFD and TMD in the lots of plasma.

The blank, LLOQ, Low, Medium and High QC chromate-grams were represented in **Fig. 2** and **3**. The S/N-ratio during the method validation was found to be more than 25, which was acceptable in accordance with the USFDA and EMEA-guidelines.

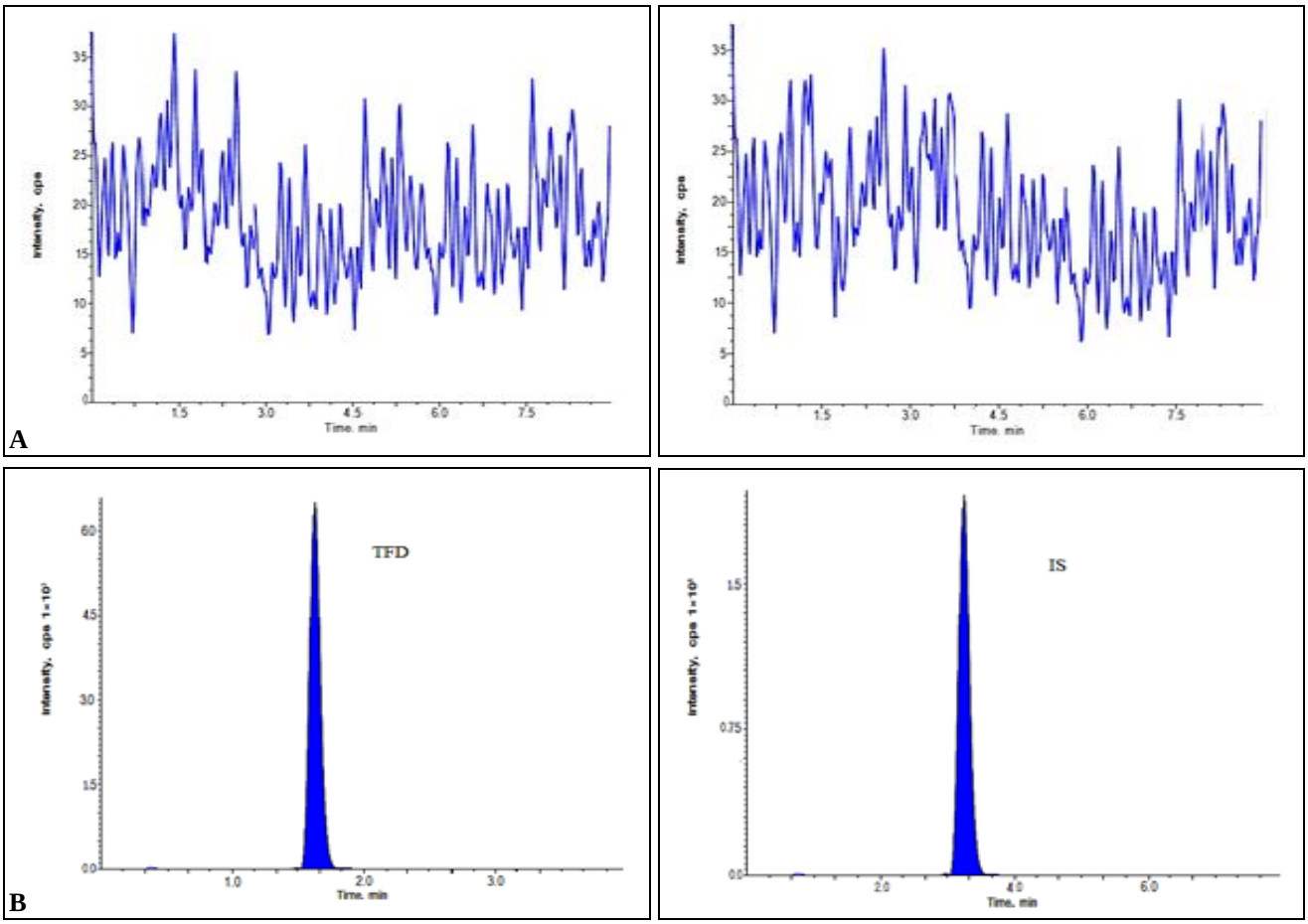

FIG. 2: REPRESENTATIVE CHROMATOGRAMS OF BLANK (A) AND SPIKED LLOQ-SAMPLE (B) OF TRIFLURIDINE

The drug has a limit of quantitation value of 5.0 ng/ml and the precision and accuracy values were found to be 8.62% and 98.12% at LLOQ concentration level. The linearity graph was linear over the concentration range of 5.0–2000.0 ng/ml for TFD. Linearity graph was made using the peak response ratio of drug to IS and the 'R^2' value was estimated and the value was more than 0.99 and findings were shown in **Table 1**. Accuracy and precision were processed, and the findings were

tabulated in **Table 2**. The inter-day and intra-day precision were measured in % CV and the values were found between 0.42% to 3.33% and the inter-day and intra-day accuracy findings were present between 97.34% to 103.74%. Matrix effect was estimated by determining the % CV values for High and Low QC samples and was found to be 1.65% and 0.73% respectively. The findings were shown in **Table 3**.

Stability studies of TFD were processed for Auto-sampler stability (10 °C, 76.90 h), Benchtop stability (ice-cold water, 6.5 h), Freeze and thaw stability (three freeze-thaw cycles), Long-term stability (−50 °C, 121 days) and all the findings were shown in **Table 4**. The detected average nominal concentrations of TFD were within ± 15% of their particular nominal concentration. There was no change in the concentration of TFD drugs in human K3EDTA for 2.0 h. Method ruggedness for TFD was processed and estimated. The % RSD findings were calculated for the same and were represented in **Table 5**.

Method Re-injection reproducibility was proven by re-injecting quality control solutions of precision and accuracy lot three and quantified against the actual estimated linear graph of precision and accuracy lot three. The % change for all the re-injected QC solutions was ≤7.45.

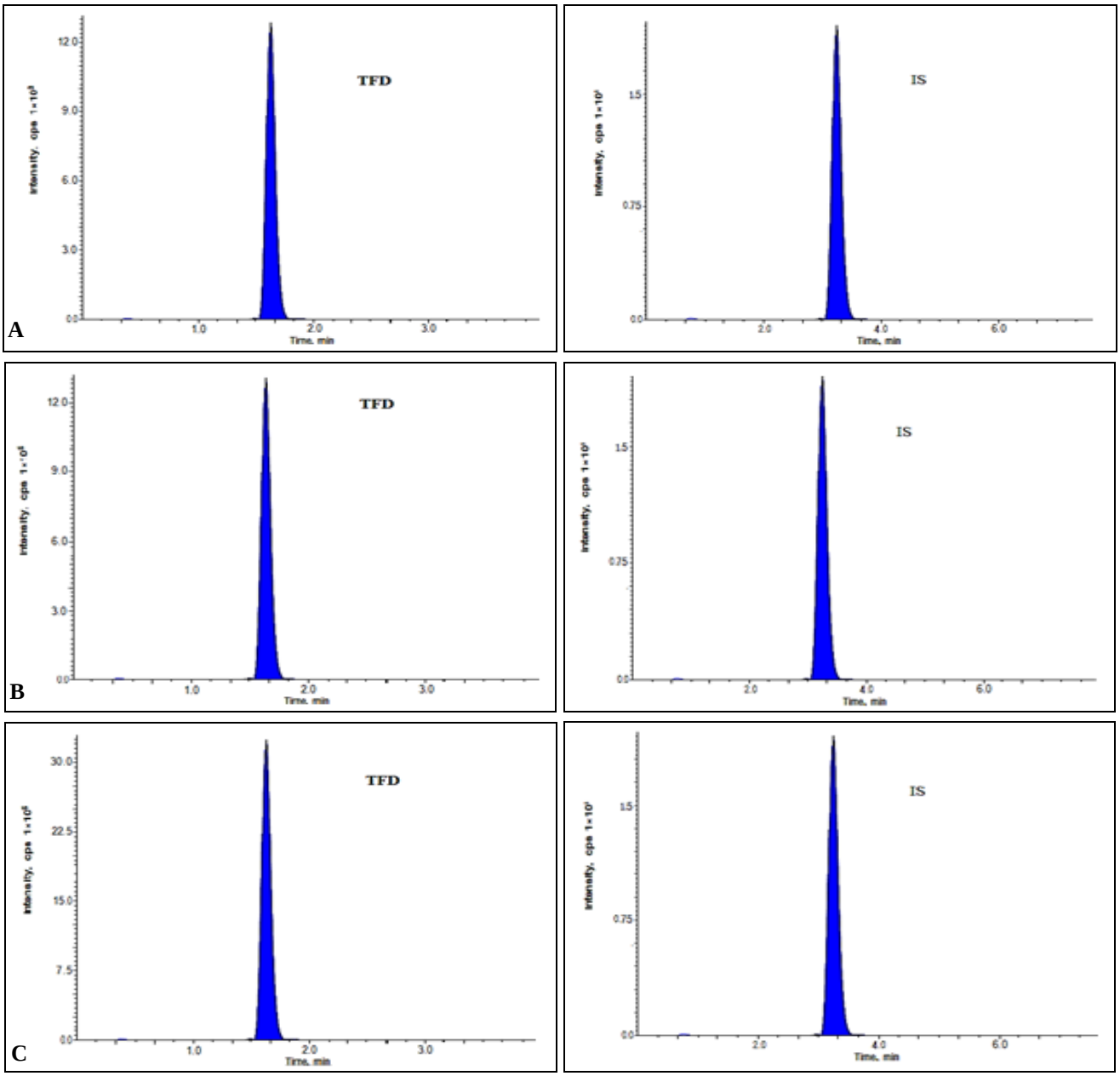

FIG. 3: CHROMATOGRAMS OF TRIFLURIDINE SPIKED (A) LQC, (B) MQC AND (C) HQC SAMPLES

TABLE 1: LINEARITY OF TRIFLURIDINE

Actual conc. (ng/ml)	5	20	100	250	500	1000	1500	2000	Slope	Intercept
1	4.73	18.3	99.111	247.241	501.5	997.5444	1547.5	1955	0.995	2.635
2	4.84	19.33	101.00	249.83	502.67	991.11	1577.50	1962.50	1.002	2.351
3	4.97	19.67	101.78	246.17	493.83	984.44	1535.00	1940.00	0.986	3.000
Mean	4.85	19.10	100.63	247.75	499.33	991.03	1553.33	1952.50	0.994	2.662
± SD	0.12	0.71	1.37	1.88	4.80	6.55	21.84	11.46	0.008	0.325
% CV	2.39	3.73	1.36	0.76	0.96	0.66	1.41	0.59		
% Accuracy	97.00	95.50	100.63	99.10	99.87	99.10	103.56	97.63		

CV- coefficient of variance; SD- standard deviation

TABLE 2: TRIFLURIDINE INTRA AND INTER-DAY QUALITY CONTROL STANDARDS

Trifluridine				
Intra-batch	**LLOQ (5.0 ng/ml)**	**LQC (20.0 ng/ml)**	**MQC (500.0 ng/ml)**	**HQC (1800.0 ng/ml)**
Mean	5.93	20.10	489.38	1844.12
SD	0.05	0.67	11.24	17.47
% CV	0.88	3.33	2.30	0.95
Mean	6.18	17.03	503.18	1887.87
SD	0.05	0.56	10.79	43.88
% CV	0.84	3.31	2.14	2.32
Mean	5.65	19.35	499.85	1830.65
SD	0.05	0.60	9.84	38.09
% CV	0.92	3.08	1.98	2.07
Inter-batch	**LLOQ (5.0 ng/ml)**	**LQC (20.0 ng/ml)**	**MQC (500.0 ng/ml)**	**HQC (1800.0 ng/ml)**
Average	5.76	19.84	506.45	1798.98
SD	0.05	0.15	5.68	7.61
% CV	0.90	0.78	1.12	0.42

SD- standard deviation; CV- coefficient of variance

TABLE 3: MATRIX EFFECT RESULTS FOR TFD

S. no.	LQC	HQC
	20 ng/ml	1800 ng/ml
1	19.752	1806.541
2	19.834	1795.937
3	19.673	1782.301
4	20.0731	1783.808
5	19.916	1731.893
6	19.982	1810.095
Mean	19.884	1784.283
± SD	0.141	29.272
% CV	0.73	1.65
% Accuracy	99.43	99.18

SD- standard deviation; CV- coefficient of variance

TABLE 4: TRIFLURIDINE STABILITY DATA

Stability type	Concentration level	Comparison sample (ng/ml)	% CV	Stability sample concentration	% CV	% Change
Benchtop stability	LQC	20.0	1.46	20.528	2.6	2.64
(ice-cold water for 6.5 h,)	HQC	1800	1.82	1810.44	1.85	−0.58
Auto-sampler stability	LQC	20.0	7	19.648	1.57	−1.76
(10 °C for 76.90 h)	HQC	1800	1.27	1823.58	1.38	1.31
Long-term stability	LQC	20.0	2.35	20.12	2.43	0.6
(−50 °C for 121 days)	HQC	1800	0.63	1857.24	0.96	3.18
Freeze and thaw stability	LQC	20.0	1.46	20.414	2.75	2.07
	HQC	1800	1.82	1815.48	1.85	0.86

CV: Coefficient of variation

TABLE 5: TRIFLURIDINE RUGGEDNESS DATA

Nominal concentration (ng/ml)	Analyst-1 and column-1		Analyst-2 and column-2	
	Accuracy (%)	% RSD[n]	Accuracy (%)	% RSD[n]
5	107.2	4.9	105.9	7.1
20	97.6	6.1	96.5	4.2
500	104.5	4.3	95.1	2.9
1800	102.9	5.8	104.3	5.2

n- 6 replicates; RSD- Relative standard deviation

CONCLUSION: A simple and sensitive bioanalytical LC-MS/MS technique for the quantification of TFD in human plasma was developed and validated as per the USFDA and EMEA guidelines. The drug has a limit of quantitation value of 5.0 ng/ml and the precision and accuracy values were found to be 8.62% and 98.12% at LLOQ concentration level. The % change for all the re-injected QC solutions was ≤7.45. Matrix effect was estimated by determining the % CV values for High and Low QC samples and was found to be 1.65% and 0.73% respectively. The inter-day and intra-day precision were measured in % CV and the values were found between 0.42% to 3.33% and the inter-day and intra-day accuracy findings were present between 97.34% to 103.74%. The drug has more stability under different stability conditions. This developed analytical technique was useful in the routine quality control of TFD in human plasma samples.

AUTHORS' CONTRIBUTIONS: All authors contributed equally to this manuscript.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTERESTS: There are no conflicts of interest.

REFERENCES:

1. Matsuoka K, Nakagawa F, Kobunai T and Takechi T: Trifluridine/tipiracil overcomes the resistance of human gastric 5-fluorouracil-refractory cells with high thymidylate synthase expression. Oncotarget 2018; 9: 13438-450.
2. Burness CB and Duggan ST: Trifluridine/Tipiracil: A review in metastatic colorectal cancer. Drugs 2016; 76: 1393-02.
3. Kirk WR: Antiviral treatment and other therapeutic interventions for herpes simplex virus epithelial keratitis. John Wiley & Sons, Ltd, edition 1st 2015.
4. Carmine AA, Brogden RN, Heel RC, Speight TM and Avery GS: Trifluridine: a review of its antiviral activity and therapeutic use in the topical treatment of viral eye infections. Drugs 1982; 23: 329-53.
5. Sarah LS, Larry PK and Charles PG: principles and practice of pediatric infectious disease. Elsevier Health Sciences Edition 4th 2012.
6. Pavan-Langston D and Nelson DJ: Intraocular penetration of trifluridine. Am J Ophthalmol 1979; 87: 814-18.
7. Hazra BB, Vageesh NM, Kistayya C and Shahanaz M: Analytical method development and validation for simultaneous estimation of trifluridine and tipiracil in pure and pharmaceutical dosage form. Innovat international Journal of Medical and Pharma Sciences 2018; 3: 55-58.
8. Sahu SK and Akula G: Development and validation of a RP-HPLC-PDA method for simultaneous determination of trifluridine and tipiracil in pure and pharmaceutical dosageform. International Journal of Novel Trends in Pharmaceutical Sciences 2017; 7: 145-51.
9. Prathap B, Baskar HV, Kumar B, Raghu PS and Reddy BKS: Method development and validation for the simultaneous estimation of trifluridine and tipiracil in tablet dosage form by RP-HPLC method. J Global Trends Pharm Sci 2017; 8: 4514-21.
10. Mainaaz, Rizwan SH, Bhameshan KM and Sultana A: Analytical method development and validation for the simultaneous determination of tipiracil and trifluridine in bulk and capsule dosage form by RP-HPLC method. Int J of Innovative Pharma Sciences and Res 2017; 5: 32-42.
11. Tijare LK, Rangari NT and Mahajan UN: A review on bioanalytical method development and validation. Asian J Pharm Clin Res 2016; 9: 6-10.
12. ICH: Q2B. Harmonized Tripartite Guideline, Validation of Analytical Procedure: Methodology, IFPMA, in: Proceedings of the International Conference on Harmonization, Geneva; 1996.
13. ICH: Q2 (R1), Validation of analytical procedures: text and methodology; 2005.
14. US FDA, Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, Maryland, USA, 2001.
15. Christian S, Patel R and Shivprakash: A rapid and sensitive LC-MS/MS assay for the determination of clobazam in human plasma using electro spray ionization technology. Int J Pharm Sci Res 2018; 9(6): 2369-77.
16. Yatha R and Rajkamal B: An improved LC-MS/MS method development and validation for the determination of trandolapril and verapamil in human plasma. International J Pharm Pharmaceu Sci 2019; 11: 91-95.
17. Mishra TD, Kurani H, Singhal P and Shrivastav PS: Simultaneous Quantitation of HIV-Protease Inhibitors Ritonavir, Lopinavir and Indinavir in Human Plasma by UPLC–ESI-MS-MS. J of Chroma Sci 2012; 50: 625-35.
18. Puram SR, Batheja R and Vivekanand PA: Rapid, sensitive method for the determination of cinnarizine in plasma by LC-MS/MS and its application to pharmacokinetic study. Int J Pharm Sci Res 2017; 8(12): 5264-69.
19. Zhao M: Specific method for determination of gefitinib in human plasma, mouse plasma and tissues using high performance liquid chromatography coupled to tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 819:73-80.

20. Rezk MR, Basalious EB and Karim IA: Development of a sensitive UPLC-ESI-MS/MS method for quantification of abacavir and its metabolite, GS-331007, in human plasma: application to a bioequivalence study. J Pharm Biomed Anal 2015; 114:97-104.

21. Udhayavani S, Sastry VG, Rajan RG, Srinivas A and Tejaswi JKD: Bio-analytical method development and validation of Ethinyl Estradiol with Ethinyl Estradiol-D4 as internal standard in human k2-EDTA plasma BY LC-MS/MS. Int J Pharm Sci & Res 2017; 8(7): 2996-03.

How to cite this article:
AS Mohammad, Ahmad F and Jayanthi B: Method development and validation for the quantitation of trifluridine in human plasma by using LC-MS/MS technique. Int J Pharm Sci & Res 2020; 11(7): 3252-59. doi: 10.13040/IJPSR.0975-8232.11(7).3252-59.