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Original Article

Development of a New Validated RP-HPLC Method for Simultaneous Estimation and Stability Study of Tegafur, Gimeracil and Oteracil in Combined Dosage Form

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ABSTRACT

Aim: This study developed a validated and stability-analyzing Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for simultaneous determination of selected drugs **Materials and Methods:** Separation, quantification and degradation of the selected drugs and their degradation study was performed on Waters Alliance-e2695, with X Bridge phenyl column (150x4.6 mM, 3.5 µ) column, mixture of Acetonitrile (ACN) and 0.1% v/v trifluoroacetic acid (50:50% v/v) as mobile phase, flow rate of 1.0 mL/min at 244 nm under ambient temperature using PDA detector. **Results:** Tegafur, Gimeracil and Oteracil were separated effectively with retention times of 2.8, 7.5 and 9.5 min respectively. The optimized method was validated in different aspects as per the ICH Q1A(R2) guidelines. The method showed linear responses at 50-300 µg/mL (r^2=0.99984), 14.50-87 µg/mL (r^2=0.99930) and 39.50-237.00 µg/mL (r^2=0.99980) for tegafur, gimeracil and oteracil, respectively. Results of inter- and intraday, robustness analysis in terms of %RSD were found to be less than 2.0%. Forced degradation analysis indicated that most of the forced degradation conditions induced impurities in the selected drugs, except for hydrolysis degradation. **Conclusion:** The results indicate that the method conditions are aligned with the ICH method validation limits. The forced degradation study results indicated that except in hydrolytic degradation, the drugs were degraded to produce impurities. Therefore, the developed method may be suitable for the simultaneous determination of the selected drugs in routine analysis.

Keywords: Gimeracil, Oteracil, RP-HPLC, Simultaneous, Stability studies, Tegafur.

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INTRODUCTION

Cancer is the deadliest and most lethal condition, occurs in many forms and is caused by a variety of factors.[1] Tegafur (TGF), Gimeracil (GMC) and Oteracil (ORC) are used in the combined therapy of advanced gastric cancer, breast cancer, non-small-cell lung cancer, head and neck cancer, pancreatic cancer, colorectal cancer and biliary tract malignancies along with cisplatin and other drugs.[2] Chemically, TGF[3] is known as 5-fluoro-1-(oxolan -2-yl) pyrimidine -2, 4-dione, used as a chemotherapeutic agent that has $C_8H_9FN_2O_3$ as molecular formula, molecular weight of 200.17 g/mol and its molecular structure was shown in Figure 1. The log P value of TGF is -0.3, soluble in water (28 mg/mL) and has a pKa value of 8.08. When TGF enters the biological system, it gets converted to fluorouracil a similar kind of pyrimidine. Due to its similarity, fluorouracil displaces the pyrimidine and interferes with the enzymes during the production of new DNA and RNA and prevents the growth of tumor cells and ultimately destroys them.[4]

GMC[5] is chemically known as 5-chloro-4-hydroxy-1H-pyridine-2-one, used as an antineoplastic agent, has $C_5H_4ClNO_2$ as molecular formula, molecular weight of 145.54 g/mol and its molecular structure was shown in Figure 1. The log *p* value of GMC is -0.1, soluble in water (14.2 mg/mL) and has a pKa value of 8.66. When combined with cisplatin, it was approved for the chemotherapy of people with early-stage gastric cancer. It works

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by reversibly inhibiting the Dihydropyrimidine Dehydrogenase (DPD) enzyme, which is responsible for the 5-Fluorouracil (5-FU) breakdown. The role of Gimeracil's is to safeguard 5-FU from breakdown, which leads to the maintenance of high concentrations of it and shows sustained effect against cancer cells.[6] ORC[7] is chemically called 4,6-dioxo-1H-1, 3, 5-triazine-2-carboxylate is an enzyme inhibitor has $C_4H_2HN_3O_4$ as molecular formula, molecular weight of 195.17 g/mol and its molecular structure was shown in Figure 1. The log P value of GMC is -1.1, soluble in water (7.15 mg/mL) and has a pKa value of 2.14. By preventing the enzyme orotate phosphoribosyl from working, it lowers the amount of 5-fluorouracil produced. Owing to its limited permeability, ORC primarily remains in the gut. Lower gastrointestinal toxicity is a result of reduced fluorouracil levels in the gut.[8]

Because these drugs are used in combinational therapy for cancer treatment the monitoring of these drug strengths or concentrations of these drugs in formulations and their purity is very essential to provide efficient treatment strategies for cancer patients. Monitoring of these drugs can be possible through the existence of highly effective, sensitive and accurate analytical methods. The extensive literature review revealed that few analytical methods like Ultraviolet (UV) Spectroscopy,[9,10] HPLC,[11-14] and Liquid Chromatography-Mass Spectrometry (LC-MS)[15,16] methods were reported for these estimation and stability indicating of selected drugs individually or combined with other. The systematic and extensive literature search deduced that there is no efficient and less expensive analytical method for the simultaneous determination of these selected drugs till date. Hence there is a great demand for a simple, robust, precise, accurate and efficient analytical technique for the simultaneous estimation of selected drugs. HPLC is an efficient technique for the separation and determination of compounds or analytes in a mixture.[17] The analysis by HPLC avail the advantages like specificity, selectivity, sensitivity, accuracy, robustness, rapidity and efficiency. HPLC can analyze a wide range of samples, requires less amount of sample and offers high throughput and automation, compatible with multiple detectors which will be utilized based on the type of analyte under investigation.[18] This work enumerates the development of validated, stability monitoring RP-HPLC process for simultaneous determination of TGF, GMC and ORC in capsule dosage form.

MATERIALS AND METHODS

The HPLC system of model Waters e 2695 ALLIANCE with a binary pump, rheodyne injector with 20 µL loop connected to UV detector, comprising of X Bridge Phenyl column (150x4.6 mm, 3.5 µ) and operated with Empower software 2.0 was used for separation, identification and determination. TGF, GMC and ORC were acquired from Hetero Pharmaceuticals, Hyderabad, Teysuno capsules (20, 5.8 and 15.8 mg of TGF, GMC and ORC) were purchased from Srushti Pharmacy, Karimnagar, Telangana. Acetonitrile and trifluoroacetic acid (all HPLC grade) were procured from Rankem Chemicals, Hyderabad. In-house production milli Q water was used.

The moving phase comprising acetonitrile: 0.1% v/v trifluoroacetic acid in water 50:50% v/v was prepared. Stock solutions of selected drugs were prepared by accurate transfer of 200.00. 58.00 and 158.00 mg of TGF, GMC and ORC respectively into 100 mL standard flasks and dissolved with enough of the mobile phase. Then it was diluted to the final volume with the mobile phase. Working standard solutions of TGF, GMC and ORC were prepared by transferring 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL of standard stock solution into separate 10 mL standard flasks and they were made up to the mark with mobile phase. Teysuno capsules were used for the preparation of the sample solution. Ten capsules were accurately weighed to determine their average weight, the homogeneous mixture of capsule content equivalent to 20 mg of TGF was transferred to 10 mL standard flask, dissolved with diluent by ultra-sonicated for 30 min and it was made up to the mark with diluent, then filter this solution through Whatman filter paper. 1 mL of the resultant filtrate was added to 10 mL standard flask and made up to the mark with diluent.

Method Development

The isosbestic point of the three selected drugs was determined by recording the overlay spectrum of three drug solutions in the Ultraviolet (UV) region and was used as the qualitative parameter in the simultaneous estimation of selected drugs. During the process of development and optimization of the chromatographic method, so many trails were carried out while injecting the diluted standard solution by changing the different method parameters like stationary phase, mobile phase composition, column temperature, flow rate, etc. The trial employed the chromatographic conditions given in Table 1 and the chromatograms with optimum resolution, shape, USP plate count and tailing factor for three drugs was selected as an optimized method.

Validation of the optimized method

The optimized method has been validated as stated in the ICH Q2(R1A) guidelines. The compliance of the results obtained in suitability, linearity, specificity, robustness, precision, sensitivity and degradation studies with ICH guidelines was determined.[19,20]

The specificity of the method, which indicates that the method must determine the selected drugs specifically, was validated by recording the chromatograms of blank, standard and sample solutions. The system suitability study was conducted by determining the Retention time (R_t), tailing factor, plate count and resolution. These results indicate the ability of the instruments for the efficient determination of selected drug

combination. The linearity was determined by preparing different subsequent concentrations of a standard solution in the range of 50.00-300.00, 14.50-87.00 and 39.50-237.00 µg/mL for TGF, GMC and ORC respectively. The linearity curve was plotted taking concentration on the abscissa and a peak area on ordinate. Precision was analyzed by taking 6 multiple injections of 200 µg/mL (TGF), 58 µg/mL (GMC) and 158 µg/mL (ORC), the acceptance criteria for precision study is the %RSD of replicate results should not be more than 2%. The accuracy or recovery study was performed through standard addition method at 50, 100 and 150% levels of analytes concentration, the acceptance criteria is the % recovery should be within 98 to 102% and %RSD of replicated results should not be more than 2%. Robustness was performed using a solution containing 200 µg/mL TGF, 58 µg/mL GMC, 158 µg/mL ORC, which was prepared and estimated at different flow rates (0.9, 1.0, 1.1 mL/min) and with different mobile phase compositions by changing organic phase ratio. Sensitivity was expressed in terms of the Limit of Detection (LOD) which indicates the minimum concentration of analyte required detecting and Limit of Quantification (LOQ) which indicate minimum concentration required to quantify the analyte in the sample. The following equations are used to determine the LOD and LOQ.

LOD=(3.3xSD)/S

LOQ=(10xSD)/S

Where SD is the standard deviation of y intercept, S is the mean of slopes of replicated calibration curves. Stability of the selected drugs under different forced degradation conditions by the developed method. The standard drug mixture was evaluated for its purity as per ICH guidelines under different stress degradation conditions such as exposure to water, 0.1 N hydrochloric acid, heat, light, 0.1 N sodium hydroxide, 3% w/v hydrogen peroxide and 10% sodium bisulfate for 24 hr.

RESULTS AND DISCUSSION

The isobestic point of the selected drugs was determined as 244 nm (shown in Figure 2) using overlay spectrum mode in a UV-visible spectrophotometer. The optimum separation of the selected drug with proper resolution and peak shape (shown in Figure 3) was achieved at 244 nm using the isocratic mode at ambient temperature with a run time of 12 min. The chromatographic method was optimized with Waters HPLC with an autosampler and UV detector on Waters X-Bridge Phenyl (150x4.6 mM, 3.5 µ) using a mixture of acetonitrile:0.1% v/v TFA (50:50% v/v), 10 µL injection volume, at wavelength of 244 nm, flow rate of 1 mL/min, runtime of 12 min through isocratic mode.

The system suitability results of the optimized method for the standard solution of three selected drugs were reported in Table 1 which indicates the method can have separated the three selected drugs with high resolution, optimum plate count and good peak shapes. The retention times of TGF, GMC and ORC were determined to be 2.86, 7.59 and 9.58 min respectively which indicated that the three drugs were separated, identified and quantified within 10 min.

Linearity concentrations in which the method showed a proportional increment in peak area against concentration were determined (given in Table 2) as 50-300 µg/mL, 14.50-87 µg/mL, 39.50-237.00 µg/mL with regression coefficients (R^2)

Table 1: **System suitability parameters for TGF, GMC and ORC.**

Sl. No.	Drug	Rt*	Peak Area*	Resolution*	Tailing Factor*	Plate Count*
1	TGF	2.862	3544621		1.09	7733
2	GMC	7.590	175138	23.50	1.08	29007
3	ORC	9.585	2125354	11.38	0.99	52190

* Mean of three replications.

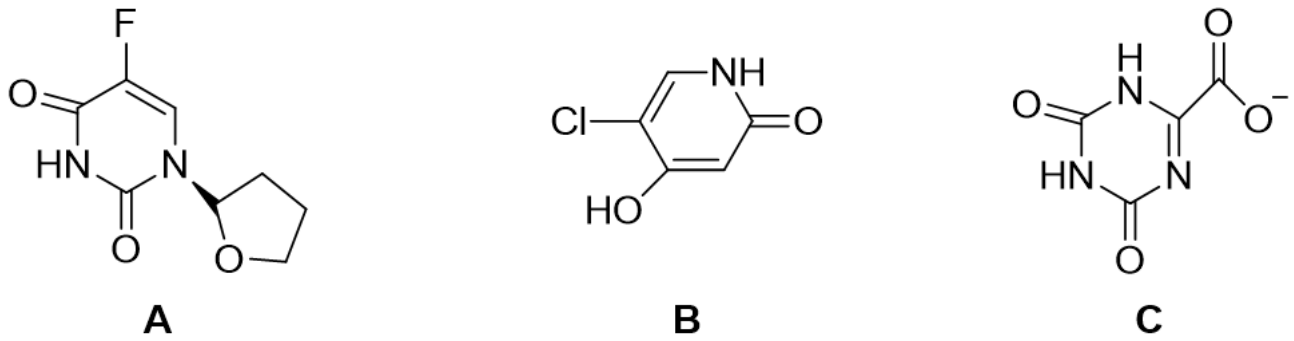

Figure 1: Chemical Structure of A) TGF, B) GMC and C) ORC potassium.

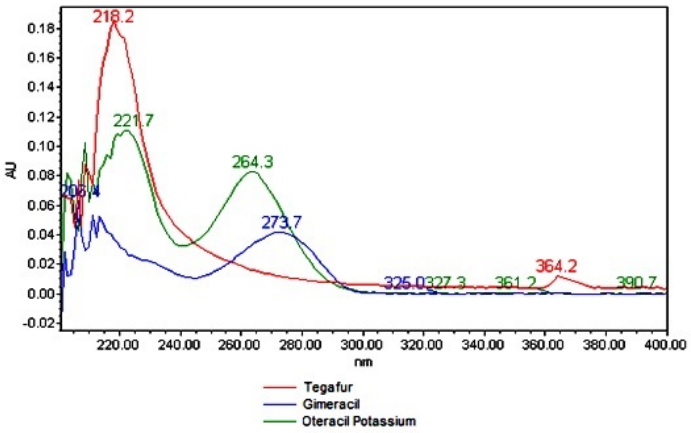

Figure 2: Overlay UV spectra of TGF, GMC, ORC.

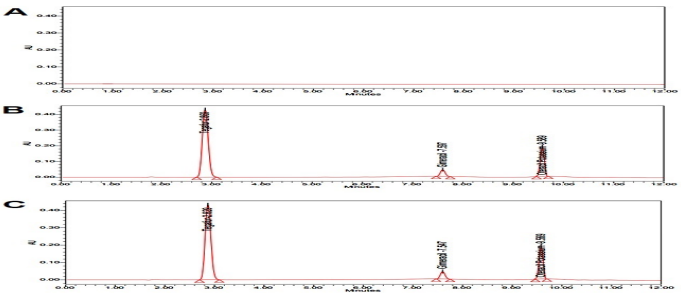

Figure 3: Typical chromatograms: A) Blank, B) Standard and C) Sample.

as 0.99984, 0.99930 and 0.99980 for TGF, GMC and ORC, respectively through construction of calibration curves by taking concentration on X-axis and peak area on Y-axis (depicted in Figure 4), which indicated that the developed method shown linear and proportional responses of selected drugs at microgram level.

The recovery of the selected drugs was ascertained at different levels using the standard addition method and the % amount recoveries (given in Table 3) at 50, 100 and 150% levels were determined as 98.60, 100.10 and 100.20 for TGF, 100.20, 100.00 and 101.42 for GMC and 101.13, 101.02 and 100.07 for ORC respectively. Moreover, the %RSD of the replications at different levels for three drugs were within 2%, which indicated that the developed method was highly accurate to quantify the selected drugs simultaneously.

The assay results of Teysuno capsules (20, 5.8 and 15.8 mg of TGF, GMC and ORC) given in Table 3 indicated that the average percentage amount found was determined as 100.11, 100.26 and 100.80 for TGF, GMC and ORC respectively and the %RSD values of three replications were within 2% which indicated that the method can quantify the selected drugs in formulations without any matrix effect of interference with the excipients.

The precision studies indicated that the method indicated that the replicated experimental results are within the precision results given in Table 4. The %RSD of system precision results was found

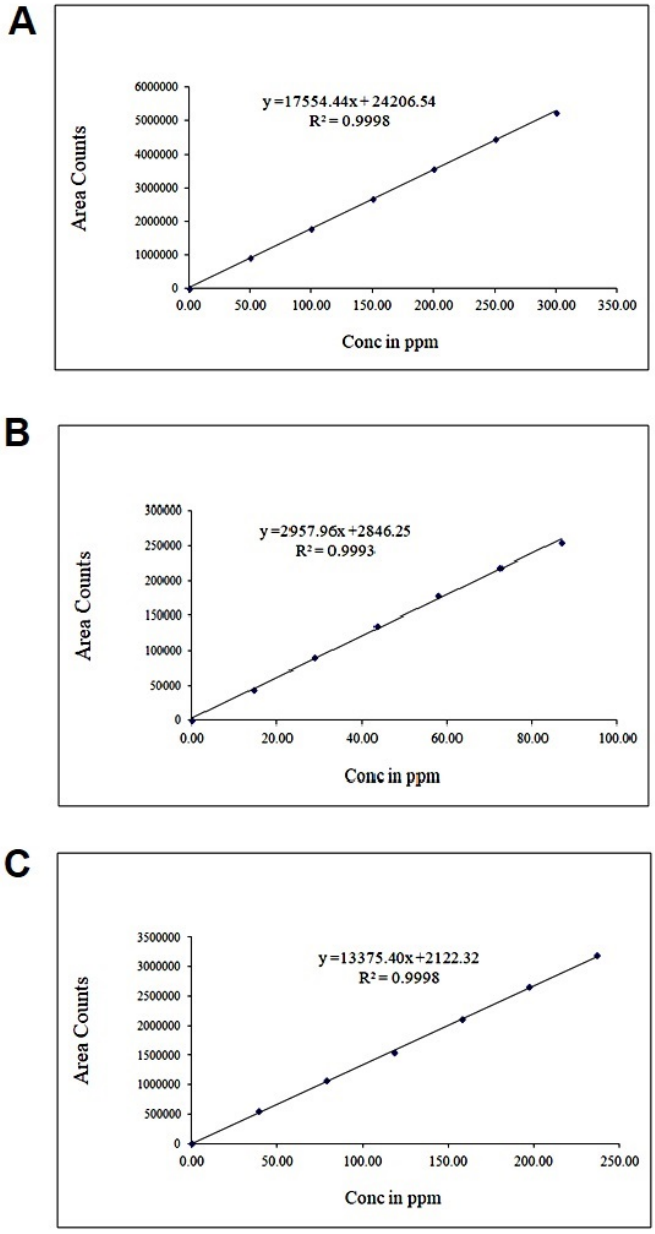

Figure 4: Calibration curves of A) TGF, B) GMC and C) ORC.

to be 0.36, 0.42 and 0.59 for TGF, GMC and ORC, respectively and method precision was found to be 0.378, 0.42 and 0.84% for TGF, GMC and ORC respectively. Similarly, the intermediate precision study results given in Table 4 indicate that the replication of the experiment on different days also shows results within the limits. The percentage RSD of results for two days indicated that the developed method proved precise and reproducible. The robustness which indicate the ability of the method to reproduce the results even in deliberate changes in method conditions, was determined by changing the method parameters like flow rate and mobile phase composition, the results given in Table 4 indicated that the method replication %RSD values are with 2%, in changed flow rate with %RSD ranging from 0.34-0.36%, 0.02-0.34 and

Table 2: Results for the linearity of TGF, GMC and ORC.

Sl. No.	TGF		GMC		ORC	
	Conc. (µg/mL)	Peak area*	Conc. (µg/mL)	Peak area*	Conc. (µg/mL)	Peak area*
1	50.00	911715	14.50	44301	39.50	553372
2	100.00	1763987	29.00	89653	79.00	1070451
3	150.00	2677973	43.50	135299	118.50	1542437
4	200.00	3564341	58.00	178191	158.00	2102224
5	250.00	4451487	72.50	218539	197.50	2655632
6	300.00	5232107	87.00	254639	237.00	3185632
Regression equation	y=17554.44x+24206.54		y=2957.96x+2846.25		y=14088.75x+2122.32	
Slope	17554.44		2957.96		13375.40	
Intercept	24206.54		2846.25		2122.32	
R^2	0.99984		0.99930		0.99980	

* Mean of three replications.

Table 3: Accuracy and assay results for TGF, GMC and ORC.

Sl. No.	Parameter		TGF		GMC		ORC	
			% Amount	%RSD	% Amount	%RSD	% Amount	%RSD
1	Accuracy	50%	98.60	0.52	100.20	0.08	101.13	1.05
		100	100.10	0.46	100.00	0.46	101.02	1.27
		150	100.20	0.94	101.42	0.73	100.07	0.44
2	Assay		100.11	1.15	100.26	0.86	100.80	1.09

Table 4: Precision and robustness results for TGF, GMC and ORC.

Sl. No.	Parameter	Change in the parameter		TGF		GMC		ORC	
				Peak Area	%RSD	Peak Area	%RSD	Peak Area	%RSD
1	Precision	System precision		3556677	0.36	176033	0.42	2127474	0.59
		Method Precision		3557245	0.78	176033	0.42	2135541	0.84
		Intermediate Precision	Day 1	3555484	0.90	175697	1.06	2147043	0.57
			Day 2	3564316	0.76	175880	1.05	2144627	0.85
2	Robustness	Flow Rate (mL/min)	0.9	3652641	0.34	185623	0.34	2304269	0.10
			1.0	3544621	0.90	175138	0.02	2125354	0.30
			1.1	3356982	0.36	166298	0.03	1985361	0.42
		Mobile phase composition	45:55	3794138	0.03	207542	0.10	2494268	0.16
			50:50	3552861	0.76	177048	0.11	2112874	0.31
			55:45	3204138	0.05	148502	0.63	1848257	0.22

The LODs of TGF, GMC and ORC were found as 0.17, 0.60 and 0.47 µg/mL respectively and the LOQ of TGF, GMC and ORC were found as 0.58, 2.01 and 1.58 µg/mL respectively these results indicating that the method is highly sensitive to identify and simultaneously quantify the selected drugs in microgram or even pictogram level.

Table 5: Forced degradation results for TGF, GMC and ORC.

Forced degradation condition	TGF				GMC				ORC Potassium			
	% Assay	% Deg	Purity Angle	Purity Threshold	% Assay	% deg	Purity Angle	Purity Threshold	% Assay	% Deg	Purity Angle	Purity Threshold
Control	100	0	0.49	4.06	100	0	0.948	1.22	100	0	1.056	2.222
Acid	85.90	14.11	0.40	4.03	89.80	10.20	0.95	1.25	86.91	13.14	1.02	2.22
Alkali	86.41	13.63	0.40	4.02	87.11	12.97	0.96	1.26	87.47	12.60	1.03	2.23
Peroxide	83.94	16.10	0.47	4.05	85.34	14.75	0.96	1.29	84.80	15.21	1.04	2.28
Reduction	88.15	11.95	0.42	4.05	99.50	0.53	0.94	1.22	98.13	1.93	1.08	2.27
Hydrolysis	99.73	0.33	0.45	4.09	98.53	1.50	0.95	1.24	99.22	0.84	1.06	2.26
Thermal	89.78	10.03	0.47	4.04	97.99	2.14	0.96	1.20	86.27	13.81	1.01	2.20
Photolytic	85.50	14.52	0.46	4.07	87.07	13.05	0.97	1.22	89.60	10.44	1.02	2.21

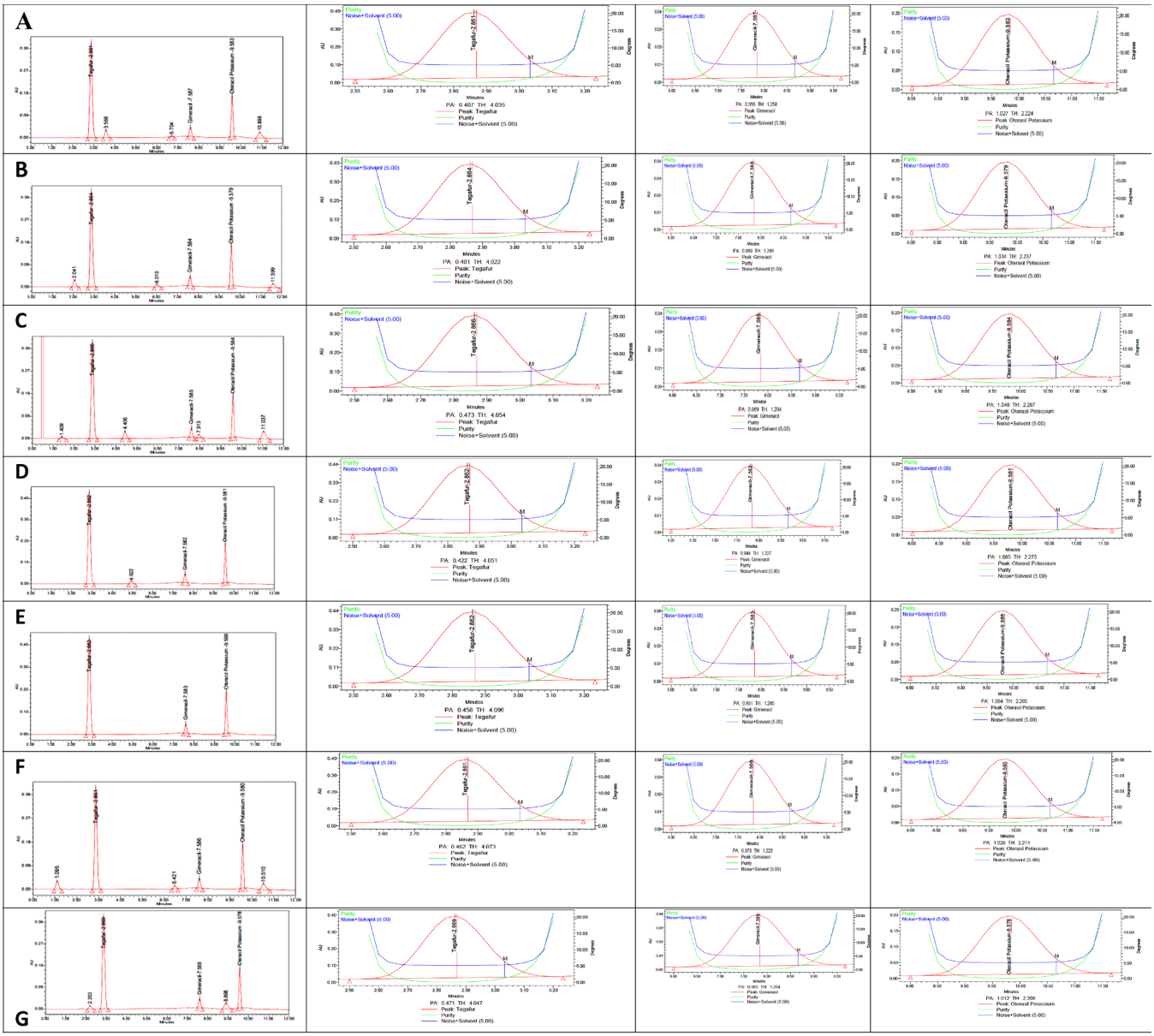

Figure 5: Chromatograms of forced degradation by: A) acid, B) Alkali, C) peroxide, D) Reduction, E) Hydrolysis, F) Photolytic, G) Thermal.

0.10-0.42 for TGF, GMC and ORC respectively and in change in mobile phase condition with %RSD ranging from 0.03-0.76, 0.10-0.63 and 0.16-0.31% for TGF, GMC and ORC respectively, which indicated that the developed method was robust and can reproduce the results accurately and precisely in any deliberate changes in the method conditions.

The forced degradation study results given in Table 5 shown in Figure 5 indicated that the developed method identified the impurities in all types of forced conditions except in hydrolysis. The peak purity and threshold value of the selected drugs in a chromatogram of different degradation conditions were analyzed using a PDA detector. The peak areas of the selected drugs differed when compared with the in-degradation conditions in same concentration which confirms that the degradation of selected drugs. The method identified the impurities in the acid degradation study with retention times 3.56, 6.70 and 10.05 min in alkali degradation with retention times 2.04, 6.01 and 11.30 in the peroxide degradation study with retention times 1.40, 4.40, 7.91 and 11.07 min in the reduction degradation study with retention times 6.29, in thermal degradation study with retention times 1.09, 6.42 and 10.50 min. Characterization of the identified impurities during this study through spectral analysis might be required to elucidate the mechanism of degradation of selected drugs.

CONCLUSION

An efficient, accurate, precise, robust, cost-effective and validated method was developed by RP-HPLC for the simultaneous estimation of TGF, GMC and ORC using Waters Alliance-e2695 and an X Bridge phenyl column (150x4.6 mM, 3.5 µ) column employing ACN and 0.1% v/v Trifluoroacetic acid (50:50% v/v) as mobile phase, with flow rate 1.0 mL/min. The system suitability conditions proved that the method was sufficient to separate, identify and quantify the selected drugs with high accuracy, resolution and specificity. The method was validated because all validation parameters results are in line and within the specifications of ICH guidelines. Moreover, the developed method was applied for stability studies of selected drugs, and it was proved to identify the possible impurities in different types of forced degradation conditions. Hence, the developed method was a validated method as per ICH guidelines and may be used for the routine analysis of the selected drugs and their capsule formulations.

The study focuses on the simultaneous estimation of three chemotherapeutic agents-Tegafur (TGF), Gimeracil (GMC) and Oteracil (ORC)-using a robust RP-HPLC method. These drugs, often used in combination for treating various cancers, require precise monitoring of their concentrations in formulations to ensure efficacy and safety. The study aimed to develop a validated, cost-effective and efficient analytical method due to the absence of existing methods for simultaneous estimation of these compounds.

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ABBREVIATIONS

RP-HPLC: Reverse Phase-High-Performance Liquid Chromatography; **API:** Active pharmaceutical ingredients; **RSD:** Relative standard deviation; **SD:** Standard deviation; **mL:** Milliliter; **µg:** Microgram; **%:** Percentage; **v/v:** Volume by volume; **mg:** Milligram; **hr:** Hour; **nm:** Nanometer; **Rt:** Retention time; **µM:** Micrometer; **mm:** Millimeter; **min:** Minutes; **PDA:** Photodiode array; **UPLC:** Ultra High-Performance Liquid Chromatography; **LC-MS/MS:** Liquid chromatography-Tandem mass spectrometry; **HCl:** Hydrochloric acid; **NaOH:** Sodium hydroxide.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The developed HPLC method for TGF, GMC and ORC was optimized using a Waters X-Bridge Phenyl column with acetonitrile: 0.1% TFA (50:50% v/v) as the mobile phase at 244 nm, achieving retention times of 2.86, 7.59 and 9.58 min, respectively. System suitability results showed excellent resolution, peak symmetry and high plate counts. Linearity was observed over wide concentration ranges with R^2>0.999 for all drugs. Accuracy studies demonstrated recovery rates within 98.60-101.42% and % RSD<2%, confirming reliability. The assay of Teysuno capsules showed no interference from excipients, with % amounts around 100%. Precision studies (% RSD<1%) confirmed reproducibility and robustness tests validated consistency under varying conditions. LOD and LOQ values indicated high sensitivity for microgram-level detection. The forced degradation study was conducted to assess the stability of TGF, GMC and ORC Potassium under various stress conditions, including acidic, alkaline, oxidative, reductive, hydrolytic, thermal and photolytic environments. Control samples showed no degradation for all three compounds, with a % assay at 100%. TGF was most susceptible to oxidative (peroxide) and photolytic stress, exhibiting significant degradation with % assay reducing to 83.94% and 85.50%, respectively. GMC demonstrated good stability under reductive and hydrolytic conditions, with minimal degradation observed. However, it showed moderate susceptibility to acidic, alkaline and oxidative stress. ORC Potassium was relatively stable under hydrolytic and reductive conditions, with % assay remaining above 98%, but was vulnerable to oxidative

and thermal stress. Across all stress conditions, purity angles for TGF, GMC and ORC remained below their respective thresholds, indicating no interference from degradation products. This data is vital for understanding the degradation behavior and optimizing storage conditions for these compounds. Overall, the method is accurate, precise, robust and suitable for simultaneous drug estimation.

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