RESEARCH ARTICLE

Development of HPLC Approach for Anticancer Drugs Combination Assay: Determination of Stability Indicating Quality and Stabilities of Anticancer Drugs Studied under the ICH Outlined Degrading Conditions

Kolapaneni A Kumar[1], S Aravind[2], Sri C Annam[3], Mannam S Rao[4*]

[1]*Department of Humanities and Sciences, DVR and Dr. HS MIC College of Technology, Kanchikacherla, Andhra Pradesh, India.*
[2]*Department of Chemistry, University College of Science, Telangana, India.*
[3]*Department of Humanities and Sciences, VNR Vignana Jyothi Institute of Engineering and Technology, Telangana, India.*
[4]*Department of Chemistry, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.*

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ABSTRACT

The combined use of tegafur (TAR), gimeracil (GAR), and oteracil (OAR) is often employed for the therapy of malignant tumors. A high-performance liquid chromatography (HPLC) approach was developed to consistently and precisely measure GAR, OAR, and TAR in a combined pharmaceutical formula that uses a shorter operation time. The separation followed by assessment of GAR, OAR and TAR is done with C18 (Waters, USA) column (250; 4.6 mm; temperature 25˚C) and the mobile phase ratio used included 60% vol (0.1 M $NaHSO_4$): 40% vol (methanol). Upon applying stress conditions, International Council for Harmonisation (ICH) recommended to GAR, OAR and TAR. The HPLC findings pointed out the nonexistence of any interference between the drugs under test and the degradation compounds. The stability indicating quality was established by the peak purity results for GAR, OAR, and TAR, which disclose that the test peaks (GAR, OAR, and TAR) were homogenous in all stress settings examined. From stability studies, we found that GAR showed significant degradation when rendered to dry heat, while TAR and OAR exhibited significant degradation after being exposed to acidic conditions. The suggested approach could potentially be implemented to assess the stability of GAR, OAR, and TAR under stress environments as well as to perform quality control checks on the three mentioned drugs in capsule doses.

Keywords: Anti-tumour agents, HPLC, Stability indicating, Peak purity, Quality check.

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INTRODUCTION

Treatment approval for the progressive or recurring head as well as neck carcinoma was granted for the combined use of tegafur (TAR), gimeracil (GAR), and oteracil (OAR).[1] The combined use of TAR, GAR, and OAR is often employed for the therapy of malignant tumors, including esophageal, gastric, colorectal, nonsmall cell lung and pancreatic carcinomas.[2-4] TAR is a prodrug of the antineoplastic substance fluorouracil, which stops cancerous cells from multiplying by obstructing the manufacture and operation of genetic material such as DNA and RNA. GAR helps sustain high doses of fluorouracil targeting cancer cells by preventing its degradation. The gastrointestinal sensitivity of fluorouracil is reduced by OAR.[5-7]

The kind of cancer is being treated and relevant variables, including general health and therapy response, might affect the required dose of TAR, GAR, and OAR. The TAR, GAR, and OAR combination, however, have probable side effects much like any drug. Hand-foot syndrome, exhaustion, nausea, vomiting, diarrhea, and lack of appetite are common adverse effects of TAR, GAR, and OAR combination.[8,9] Significant adverse effects might include cardiovascular events like erratic heartbeat or heart attack, as well as bone marrow repression, which may end up in anemia, infection, and bleeding problems.[8,9] Consequently, taking the right dosage of TAR, GAR, and OAR combination is important.

Analytical investigation of bulk drug substances, medicinal products, formulations of drugs, and compounds

**Author for Correspondence: mannamsubbarao39@gmail.com*

from degradation is crucial in the domain of pharmaceutical research.[10] With the intention of characterising the quality of bulk pharmaceutical materials by defining limitations regarding their active component content, analytical test techniques were added to the compendia monographs. The very efficient analytical technique, high-performance liquid chromatography (HPLC), is exploited to separate, identify, and ascertain chemical components in a variety of sample kinds, including blood, plasma, and urine, and in pharmaceutical formulation products.[11] comprehensive verification of the technique is required. Since HPLC is frequently applied to routinely analyze pharmaceuticals as well as raw materials at multiple stages, such as material approval, preformulation, quality assurance, and storage, comprehensive verification of the technique is required.[12]

One approach for TAR, GAR, and OAR utilizing HPLC was found to be the only one after reviewing the literature.[13] This approach requires a 12-minute runtime. Due to the extended duration, more solvent is used, raising the price of a single analysis. When deploying a lot of organic solvents, a lot of waste will have to be disposed of, which could negatively influence the environment and represent a risk to operator safety. Therefore, an approach to consistently and precisely measure TAR, GAR, and OAR in a combined pharmaceutical formula that uses a shorter operation duration was required to avoid solvent waste.

In this paper, we described an affordable HPLC method with a shorter duration of operation to determine the TAR, GAR, and OAR concentrations in a mixed pharmaceutical formula (capsule) and raw TAR, GAR, and OAR materials. The entire process has been validated to be consistent with ICH norms. Additionally, utilizing this newly developed HPLC approach, the stabilities of TAR, GAR, and OAR under the ICH outlined degrading conditions were also determined.

MATERIALS AND METHODS

Materials

The "Gold fish Pvt. Ltd", (India) supplied the reference TAR, GAR, and OAR materials. The "Merck Lifesciences Ltd", (India) provided $NaHSO_4$, NaOH, H_2O_2, HCl and methanol. Every chemical is of analytical or HPLC quality. Milli Q Millipore Ultrapure equipment was used to process, deionize, and even distill the water. The "BDR Pharmaceuticals Pvt" (India) manufactured furmecil capsules with strength 20 mg TAR, 5.8 mg GAR and 15.8 mg OAR were used.

Conditions and Procedure for TAR, GAR, and OAR Assay

The C18 (Waters, USA) column (250; 4.6 mm; temperature 25˚C) was equilibrated with mobile phase 0.1 M $NaHSO_4$, pH-4.6: methanol. The mobile phase ratio was 60% vol (0.1 M $NaHSO_4$): 40% vol (methanol). Kept the isocratic flow rate constant at 1-mL/min. An PDA detection device operating at 275 nm wavelength was utilized for monitoring the eluents. For a single injection, a 10 µL quantity of sample was deployed. Ten minutes was the whole run time. Software called Empower Version 2 was the tool utilized for processing the data. At 275 nm, the peak areas of TAR, GAR, and OAR were all measured. Peak area measurements were then used to calculate samples' TAR, GAR, and OAR amounts.

Standards Preparation

The mixture was thoroughly shaken to dissolve 20 mg TAR, 5.8 mg GAR and 15.8 mg OAR in 25 mL of diluent (0.1 M NaHSO4 of 60% vol: methanol of 40% vol). The volume was thereafter adjusted to 100 mL, including 85 mL of diluent. This stock TAR:GAR:OAR solution has 200 µg/mL TAR, 58 µg/mL GAR and 158 µg/mL OAR concentrations. A 5 mL of that stock TAR:GAR:OAR solution was drawn out and using diluent, the volume was increased to 50 mL. This working TAR:GAR:OAR solution has 20 µg/mL TAR, 5.8 µg/mL GAR and 15.8 µg/mL OAR concentrations.

Capsule Furmecil Solution

A 100 mL dry and dirt-free volumetric flask was added with capsule content containing 20 mg TAR, 5.8 mg GAR and 15.8 mg OAR by weight. 25 mL of diluent (0.1 M $NaHSO_4$ of 60% vol: methanol of 40% vol) was subsequently included, and it was adequately shaken via sonication to extract TAR, GAR and OAR. The volume was eventually adjusted to 100 mL through the inclusion of 85 mL of same diluent water. This stock fumeracil solution has 200 µg/mL TAR, 58 µg/mL GAR and 158 µg/mL OAR concentrations. A 100 mL dry and dirt-free volumetric flask was filled with 1-mL of the solution that was extracted from capsule fumeracil. The volume was raised with same diluent until it reached 10 mL. This working fumeracil solution has 20 µg/mL TAR, 5.8 µg/mL GAR and 15.8 µg/mL OAR concentrations.

TAR, GAR and OAR Content Determination in Fumeracil Solution

The working fumeracil solution was infused (10 µL) into C18 (Waters, USA) column (250; 4.6 mm; temperature 25˚C) and elution was performed using mobile phase 0.1 M $NaHSO_4$, pH 4.6: methanol. The peak areas of TAR, GAR, and OAR in fumeracil sample were all assessed at 275 nm, which were then used for calculating the amounts of TAR, GAR, and OAR in Fumeracil samples.

Stability Studies

Stress tests were executed out with TAR, GAR, and OAR in Fumeracil samples at a starting concentration of 200 µg/mL TAR, 58 µg/mL GAR and 158 µg/mL OAR to reveal the stability-indicating property, specificity property, and stabilities of the drugs (TAR, GAR, OAR) under study.[14] To test the developed new method's potential to separate TAR, GAR, OAR from its degradation products, intentional process degradation was attempted using the succeeding stress conditions: Light (samples left under sunlight around a duration of 6 hours), dry heat (samples left open to 80ºC over duration of 6 hours), acid (by applying 0.1 N HCl over duration of 30 minutes with boiling in water bath at 60ºC), base (by applying 0.1N NaOH over duration of 30 minutes with boiling in water bath at 60ºC), hydrolytic (by applying water for over

duration of 30 minutes with boiling in water bath at 60ºC), and oxidation (by applying 3% H_2O_2 over duration of 30 minutes with boiling in a water bath at 60ºC). A diode array detecting device was used to conduct a peak purity assessment on the Fumeracil-stressed samples. Also, TAR, GAR, and OAR assay investigations were conducted using stress Fumeracil samples.

RESULTS AND DISCUSSION

Parameters for HPLC- Optimization

For TAR, GAR, and OAR to be successfully evaluated by HPLC, the following criteria had to be fulfilled: the technique should indicate stability, be unaffected by excipient as well as degradant interference, meet appropriate ICH validation specifications, and be simple enough for regular usage in a quality monitoring lab. All above criterias were fulfilled with C18 (Waters, USA) column (250; 4.6 mm; temperature 25˚C), with mobile phase 0.1 M $NaHSO_4$, pH-4.6 (60% vol) methanol (40% vol) $NaHSO_4$):40% vol (methanol), with isocratic flow rate constant at 1-mL/min, with single injection quantity of 10 µL. An PDA detection device operating at 275 nm wavelength was utilized to monitor the eluents because the peak area was good enough for TAR, GAR, and OAR and was suitably selective. Under such optimized conditions, the analysis took an overall of six minutes, during which GAR, OAR and TAR eluted at times of 1.916, 2.832 and 4.297 minutes (Figure 1).

Validation

We validated our newly developed methodology by applying the ICH Guidance for industrial analytical validation strategy.[15]

LoD and LoQ

The least detectable quantities of GAR, OAR and TAR within a sample matrix that have a signal-to-noise of 3 and 10, respectively, were designated as their limit of detection (LOD) and limit of quantitation (LOQ). The GAR, OAR and TAR had LoDs of 0.040, 0.173 and 0.135 µg/mL. While their LoQs, respectively, were 0.134, 0.577 and 0.450 µg/mL. These values guarantee an adequate and sensitive GAR, OAR, and TAR assessment with new HPLC- GAR/OAR/TAR assay procedure.

Linearity

Our current investigation assessed the linearity for GAR, OAR and TAR. For GAR, the linearity varied from 2.9 to 8.7 µg/mL, for OAR, it varied from 7.9 to 23.70 µg/mL and for TAR, it varied from 10 to 30 µg/mL. The calibration curve of analysis (Figure 2), created for GAR, OAR and TAR showed a satisfactory linear relation for with the new HPLC- GAR/OAR/TAR assay procedure. The correlation coefficient was good, with 0.99997 for GAR, 0.99991 for OAR and 0.99999 for TAR.

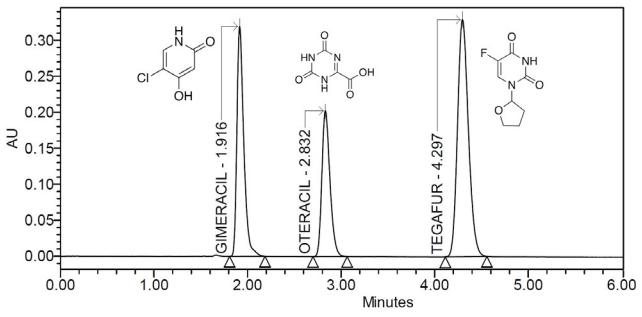

Figure 1: Structures and typical chromatogram of GAR, OAR and TAR

System suitability

Following six assessments of working TAR:GAR:OAR solution with new HPLC- GAR/OAR/TAR assay procedure, Table 1 lists the system suitability findings. The values found out fall in line with ICH guidance's authorization requirements.

Precision

Six working TAR:GAR:OAR solution replicates had been evaluated in a single day with new HPLC- GAR/OAR/TAR assay procedure to establish its precision. The precisions were verified and represented as a RSD percentage (Table 2). GAR, OAR, and TAR fell between 0.195 and 0.471%, all of which remain within the ICH acceptable ranges.

Accuracy

Six working TAR:GAR:OAR solution replicates were injected to examine the accuracy new HPLC- GAR/OAR/TAR assay procedure. The accuracies were verified and represented as an assay percentage for GAR, OAR, and TAR (Table 2). They fell between 98.50 and 99.08%, for GAR, OAR, and TAR. All of these remain within the acceptable ICH ranges.

Recovery

The GAR, OAR, and TAR were spiked in three different doses to fumeracil solutions, which were eventually injected in triplicate onto the HPLC to conduct the recovery test. For every one of the three spiked fumeracil solutions, the GAR, OAR, and TAR values are depicted as their recovery percentages (Table 2). The recovered injected concentration (GAR: 99.51–100.30%; OAR: 100.41–101.29%; TAR: 99.95–100.54%) fell within the permissible limits.

Robustness

Determining the impact of minute changes in various chromatographic-optimized experimental settings is helpful. By altering the column's temperature (varied values – 23 and

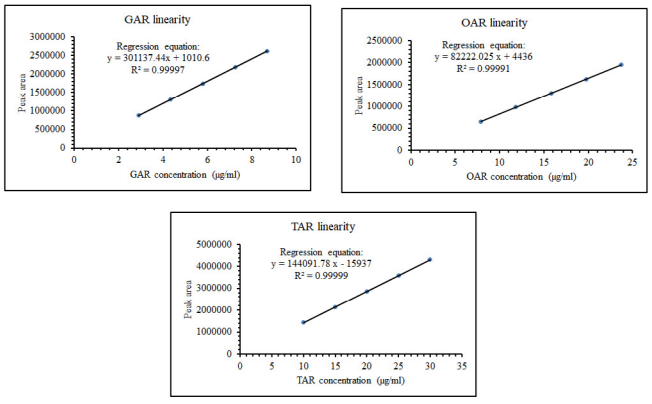

Figure 2: Regression equations and linearity curves for GAR, OAR and TAR

Table 1: GAR, OAR and TAR findings in system suitability

Analyte (µg/mL)	*Value*	*Retention time*	*Plate count*	*Area*	*Tailing*	*Resolution*
GAR (5.8)	Mean (n = 5 experiments)	1.913	3450	1761947	1.418	-
	± SD/RSD%	± 0.0036/ 0.186%	± 66.8379/ 1.937%	± 10124.9906/ 0.575%	± 0.0130/ 0.919%	-
OAR (15.8)	Mean (n = 5 experiments)	2.815	5103	1313855	1.276	6.078
	± SD/RSD%	± 0.0100/ 0.354%	± 100.3180/ 1.966%	± 3298.0019/ 0.251%	± 0.0114/ 0.894%	± 0.0581/ 0.955%
TAR (20.0)	Mean (n = 5 experiments)	4.261	6497	2880530	1.190	7.644
	± SD/RSD%	± 0.0223/ 0.524%	± 90.1904/ 1.388%	± 10406.9215/ 0.361%	± 0.0071/ 0.594%	± 0.0677/ 0.885%

27°C; Actual value – 25°C), flowing rate (varied values – 0.9 and 1.1 mL/min; Actual value – 1.0 mL/min), wavelength (varied values – 273 and 277 nm; Actual value – 275 nm), pH (varied values – 4.4 and 4.8; Actual value – 4.6) and methanol ratio (varied values – 35 and 45% vol; Actual value – 40% vol), the new HPLC- GAR/OAR/TAR assay procedure's robustness was examined. After injecting the sample (working TAR:GAR:OAR solution) under each circumstance, the drug's area, tailing factor, and plate counts were computed. The mean for each parameter, their corresponding standard deviation, and the percentage RSD of the data were also estimated (Table 3). According to the findings, there were no predominant variations (%RSD = >2.0%) among the values obtained for GAR peak, OAR peak, and TAR peak under the aforementioned conditions.

Stabilities of GAR, OAR and TAR

Stress tests were executed out with GAR, OAR and TAR in fumeracil samples to reveal the stabilities of the drugs (TAR, GAR, OAR) under study. When rendered to dry heat, GAR shown significant degradation (10.72%), while after being exposed to acidic conditions, TAR (12.5%) and OAR (11.38%) exhibited significant degradation (Table 4). Under the neutral degradation settings, all three drugs (GAR; OAR; TAR) exhibited extremely slight degradation (Table 4). all three drugs showed very mild degradation (GAR – 1.58%); OAR – 0.90%; TAR – 1.09%) under neutral conditions of degradation (Table 4).

Stability indicating feature

Stability-indicating test are applied in the forced degradation examination of pharmaceutical goods including active

Table 2: GAR, OAR, and TAR findings in precision, accuracy and recovery tests

Parameter	*GAR (5.8 µg/mL)*		*OAR (15.8 µg/mL)*		*TAR (20 µg/mL)*	
	Precision (peak area)	*Accuracy (assay%)*	*Precision (peak area)*	*Accuracy (assay%)*	*Precision (peak area)*	*Accuracy (assay%)*
Mean (n = 6 experiments)	1742505	98.50	1299649	98.62	2859632	99.08
± SD/RSD%	± 7712.8816/ 0.443	± 0.398/ 0.404	± 6120.0262/ 0.471	± 0.460/ 0.466	± 5570.6948/ 0.195	± 0.175/ 0.177
Recovery test						
Parameter	*GAR added (µg/mL)*	*GAR (%) recovered*	*OAR added (µg/mL)*	*OAR (%) recovered*	*TAR added (µg/mL)*	*TAR (%) recovered*
Mean (n = 3 experiments)	2.871	100.30	7.742	101.29	9.90	99.95
± SD/RSD%		± 0.0924/ 0.092		± 0.0513/ 0.051		± 0.5160/ 0.516
Mean (n = 3 experiments)	5.742	99.51	15.484	100.41	19.80	100.15
± SD/RSD%		± 0.4562/ 0.459		± 0.1931/ 0.192		± 0.1762/ 0.176
Mean (n = 3 experiments)	8.613	99.71	23.226	101.14	29.70	100.54
± SD/RSD%		± 0.1626/ 0.163		± 0.4757/ 0.470		± 0.0404/ 0.404

Table 3: GAR, OAR and TAR findings in robustness

Drug (µg/mL)	*Parameter studied*	*Value*	*Tailing*	*Area*	*Plate count*
GAR (5.8)	Temperature	Mean (n = 3 experiments)	1.4	1761211.3	3428.0
		± SD/RSD%	± 0.01/0.7	± 23005.2/1.3	± 33.8/1.0
	Wavelength	Mean (n = 3 experiments)	1.4	1758669.7	3413.3
		± SD/RSD%	± 0.01/1.2	± 19052.6/1.1	± 52.9/1.5
	Flow rate	Mean (n = 3 experiments)	1.4	1752891.3	3414.3
		± SD/RSD%	± 0.01/0.7	± 32165.2/1.8	± 62.0/1.8
	pH	Mean (n = 3 experiments)	1.1	1783188.3	3459.6
		± SD/RSD%	± 0.01/1.4	± 31675.4/1.8	± 63.7/1.8
	Methanol ratio	Mean (n = 3 experiments)	1.4	1754067.7	3440.7
		± SD/RSD%	± 0.01/1.1	± 30491.2/1.7	± 48.4/1.4
OAR (15.8)	Temperature	Mean (n = 3 experiments)	1.3	1309671.0	5127.3
		± SD/RSD%	± 0.01/1.2	± 24466.0/1.9	± 64.6/1.3
	Wavelength	Mean (n = 3 experiments)	1.3	1334944.0	5095.7
		± SD/RSD%	± 0.01/0.5	± 24503.7/1.8	± 49.7/1.0
	Flow rate	Mean (n = 3 experiments)	1.3	1311413.0	5085.7
		± SD/RSD%	± 0.01/1.7	± 19446.3/1.5	± 35.2/0.7
	pH	Mean (n = 3 experiments)	1.3	1334944.0	5112.3
		± SD/RSD%	± 0.02/0.5	± 24503.7/1.8	± 49.7/1.0
	Methanol ratio	Mean (n = 3 experiments)	1.3	1315538.3	5091.0
		± SD/RSD%	± 0.01/1.2	± 23618.2/1.8	± 44.2/0.9
TAR (20.0)	Temperature	Mean (n = 3 experiments)	1.2	2861038.3	6626.3
		± SD/RSD%	± 0.02/1.3	± 49775.5/1.7	± 98.2/1.5
	Wavelength	Mean (n = 3 experiments)	1.2	2874494.0	6514.3
		± SD/RSD%	± 0.01/0.5	± 42005.1/1.5	± 25.0/0.4
	Flow rate	Mean (n = 3 experiments)	1.2	2876828.3	6603.7
		± SD/RSD%	± 0.01/1.8	± 32114.9/1.1	± 86.0/1.3
	pH	Mean (n = 3 experiments)	1.2	2926827.7	6539.3
		± SD/RSD%	± 0.01/0.5	± 51443.5/1.8	± 44.7/0.7
	Methanol ratio	Mean (n = 3 experiments)	1.2	2876709.7	6526.3
		± SD/RSD%	± 0.01/1.3	± 32282.5/1.1	± 50.3/0.8

medicinal components. This process can detect changes in the amounts of essential pharmaceutical components in pharmaceutical goods and monitor their degradation. The chromatographic patterns of GAR, OAR, TAR, and degradation compounds following exposure of the prepared fumeracil samples to many stress conditions are put on show in Figure 3. The chromatogram showed no interference amongst the peaks for GAR, OAR, or TAR, or any additional degradation compounds. This evidenced that new HPLC-GAR/OAR/TAR assay procedure's ability to assay GAR, OAR, or TAR in stability samples.

Peak purity/specificity

Using the Empower version II software's standard settings and without manually entering additional data, a peak purity analysis on stressed fumeracil samples was performed. The results of this test indicate whether the approach could be exploited to resolve the pure GAR, OAR, or TAR peaks throughout the degradation study. For the GAR, OAR, and TAR peaks, the purity angle along with thresholds (Table 5) were established. The results confirmed the high purity of the GAR, OAR, and TAR peaks in every stressed fumeracil sample. This also confirms the specificity of the new HPLC-GAR/OAR/TAR assay procedure.

Selectivity

Since our relevant GAR, OAR, and TAR samples are obtained from capsules, it's critical that the excipients found in these capsules don't affect the quantification of GAR, OAR, and TAR. In order to check that the new HPLC- GAR/OAR/TAR assay procedure can produce "true results" with no excipient influence, further selectivity examinations were attempted. None of the experimental solutions (working TAR:GAR:OAR solution, working fumeracil solution, and

Table 4: Stabilities of GAR, OAR and TAR

Condition	*GAR (%) assay*	*GAR (%) degraded*	*OAR(%) assay*	*OAR (%) degraded*	*TAR(%) assay*	*TAR (%) degraded*
Acid	90.99	9.01	88.62	11.38	87.85	12.15
Base	92.98	7.02	91.38	8.62	90.42	9.58
Peroxide	94.99	5.01	93.29	6.71	91.19	8.81
Heat	89.28	10.72	90.42	9.58	89.73	10.27
Sunlight	92.02	7.98	94.51	5.49	93.66	6.34
Water	98.44	1.56	99.1	0.9	98.91	1.09

Table 5: GAR, OAR and TAR findings in peak purity/specificity

Condition	*GAR peak purity*	*GAR peak threshold*	*OAR peak purity*	*OAR peak threshold*	*TAR peak purity*	*TAR peak threshold*
Acid	0.287	0.783	0.301	0.664	0.403	0.891
Base	0.280	0.891	0.322	0.664	0.496	0.789
Peroxide	0.342	0.789	0.310	0.863	0.280	0.889
Heat	0.296	0.590	0.499	0.863	0.388	0.689
Sunlight	0.349	0.877	0.204	0.562	0.404	0.686
Water	0.257	0.877	0.322	0.762	0.297	0.688

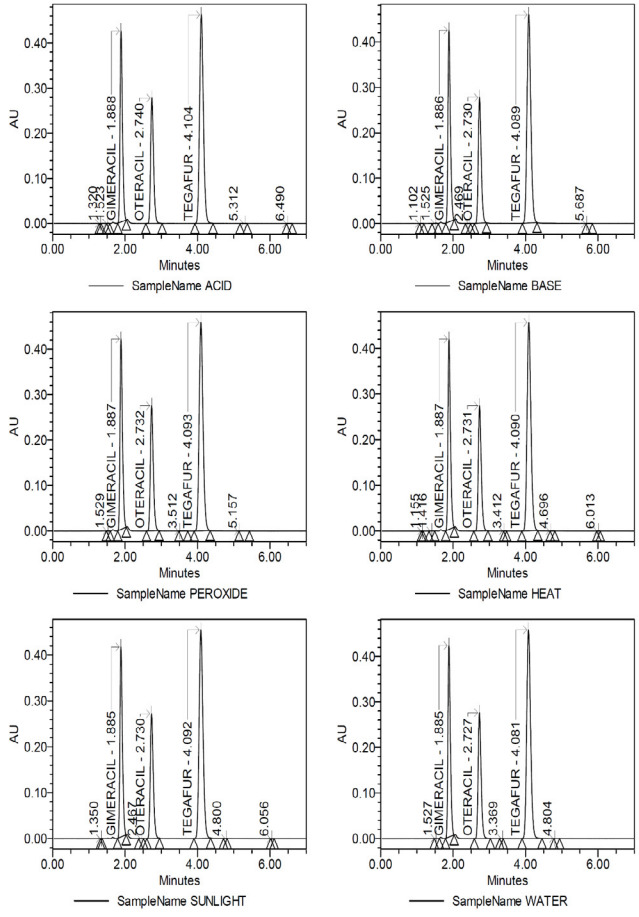

Figure 3: Chromatograms of stressed fumeracil samples

diluent blank) showed a peak around the GAR (1.917 and 1.896 minutes), OAR 2.828 and 2.758 minutes), or TAR (4.288 and 4.144 minutes) retention times, according their respective HPLC spectra (Figure 4). This confirms the selectivity of the new HPLC- GAR/OAR/TAR assay procedure.

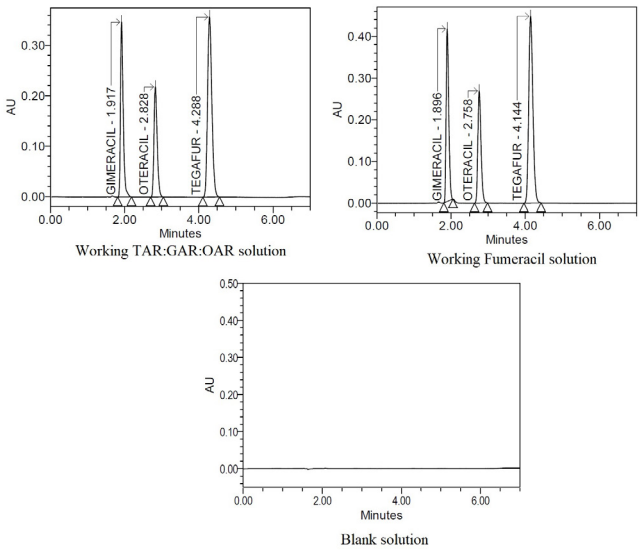

Figure 4: Chromatograms of samples from selectivity

CONCLUSION

To ascertain the TAR, GAR, and OAR concentrations in a combination pharmaceutical formula (capsule) and raw TAR, GAR, and OAR materials, we presented an economical HPLC approach with a reduced operating time. The high recovery and the small relative standard variance validate the suggested method's applicability. The well separated peaks of TAR, GAR, and OAR, and a few other peaks in all stressed fumeracil samples demonstrated the stability-indicating quality. Using this newly developed HPLC approach, the stabilities of TAR, GAR, and OAR under the ICH outlined degrading conditions can also be determined.

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