RESEARCH ARTICLE

Stability Indicating Reverse Phase Ultra Performance Liquid Chromatography Method Development and Validation for Amiloride and Hydrochlorothiazide Tablet in the Presence of Degradation Products and Application to Green Analytical Principles

Neetu Sabarwal[1*], Suman Jain[1], DD Agarwal[2]

[1]*School of Studies in Pharmaceutical Chemistry, Jiwaji University, Gwalior, Madhya Pradesh, India.*
[2]*School of Studies in Chemistry, Jiwaji University, Gwalior, Madhya Pradesh, India.*

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ABSTRACT

Amiloride (AML) and hydrochlorothiazide (HCTZ) combination is used alone or with other medicines to treat high blood pressure (hypertension). The present article provides the development and validation of an reverse phase ultra performance liquid chromatography (RP-UPLC) approach for the quantification of AML, HCTZ, and related impurities in pharmaceutical dosage forms. The analytical column used for the separation of impurities is the Waters X bridge C18 3.5 µm (50 mm X 4.6 mm). Used 0.1% formic acid and ethanol as MP in an isocratic mode with 0.3 mL/min as flow rate and 3 µL as injection volume, at 254 nm as detection in UV and 12 minutes as total run time. The samples were prepared specifically to undergo forced degradation, which involved subjecting them to hydrolysis, oxidation, thermal, and photolytic conditions. The technique underwent validation by the principles set forth by the ICH Q2 guidelines. The validation confirmed that the method possesses specificity, linearity, ruggedness, robustness and accuracy. The methodology employed exhibited a linear relationship extending from 10 to 150% for all impurities. The recovery analysis was conducted throughout a range of concentrations, starting from 10 to 150% concentration. The average recovery value was determined to be within acceptable limits. The evidence of degradation and the findings of the verified study suggest that the nature of the subject under investigation is stable. Hence, this approach might be employed within the domains of pharmaceutical research and development and quality control departments. Green analytical chemistry tools are used to assess the method's greenness and calculated using GAPI, AGREE and ecological-scale and found excellent green of >75%.

Keywords: Amiloride, Hydrochlorothiazide, Stability indicating assay method, Green analytical chemistry, ICH.

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INTRODUCTION

Amiloride (AML) is an antihypertensive and diuretic agent. That is taken orally and has the ability to spare potassium while exhibiting modest natriuretic and diuretic effects. The principal site of action of this substance is the distal tubule of the nephron, where it exhibits selective inhibition of sodium transport, consequently impeding sodium-potassium exchange. The substance under consideration is a solid of varying shades of yellow, ranging from light to dark, 229.63 g/mol as its molecular weight and its empirical formula is $C_6H_8ClN_7O$.[1–3] Amiloride chemical structure is depicted in Figure 1.

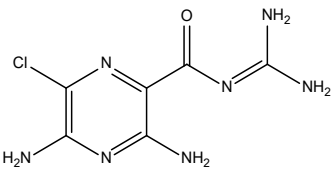

Figure 1: Representative chemical structure of amiloride

Hydrochlorothiazide (HCTZ) is classified as a thiazide-type diuretic, which functions by inhibiting the transfer of Na, Cl in the distal convoluted tubule. Subsequently, a greater amount of sodium is eliminated through renal excretion, along with a corresponding volume of fluid. It is a white crystalline powder. It has limited solubility in water but is readily soluble in sodium hydroxide solution.[4] The chemical structure of this substance is depicted in Figure 2.

**Author for Correspondence: neetubhalla1979@gmail.com*

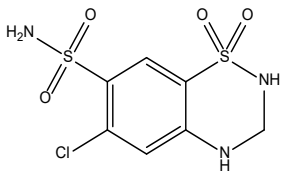

Figure 2: Representative chemical structure of hydrochlorothiazide

According to a literature review, several chromatographic techniques have been developed to quantify AML and HCTZ in pharmaceutical formulations individually. The earlier conducted reported research focused on the estimation of AML and HCTZ when present in a mixture among other medication combinations.[4-6]

The current study was to provide a simple, specific, linear, rugged, accurate, robust, and stability-indicating assay method (SIAM) for quantifying AML, HCTZ, and its related impurities in pharmaceutical formulations. The SIAM refers to a validated quantitative analytical procedure that often encompasses forced degradation and validation studies.[7–9] Based on the previously mentioned results, we conducted degradation studies and observed the appearance of a possible impurity in the pharmaceutical formulation when exposed to peroxide. The stability of the chemical is observed under both physical and hydrolysis degradation conditions, as evidenced by the validation parameters.[10-12] Therefore, this technique can be utilized to determine the presence of AML, HCTZ, and their related impurities, while also demonstrating cost-effectiveness.[13,14] The green analytical principles (GAP) were developed by Galuszka *et al*. These principles aim to minimize or remove the utilization of harmful substances. The objective of this study is to reduce the utilization of hazardous solvents while assessing the environmental sustainability of the existing approach. Several metric tools exist for evaluating the level of environmental sustainability, such the Analytical Eco-Scale, AGREE, NEMI and GAPI.[15-20]

The GAPI is a metric tool for assessing the level of environmental sustainability in relation to several factors such as sample preparation, procedures employed, and sample determination. The pictograph is represented utilizing a color scheme including of green, yellow, and red colors. The pictogram consists of six distinct sections.

Another metric tool to assess environmental sustainability is the Analytical Ecological scale. It achieves this by calculating penalty points associated with various aspects of the method's ecological impact. The eco-scale quantifies the cumulative penalty points derived from a comprehensive scale of 100. A method is deemed excellent if the cumulative penalty point exceeds 75%. The AGREE metric is a technique utilized to quantify the level of environmental sustainability of a particular method, drawing upon the 12 principles of GAP. The present study employed the AGREE, GAPI, the utilization of the analytical ecological scale tools for assessing the environmental sustainability of analytical approaches is advantageous due to their improved capabilities in evaluating both qualitative and quantitative aspects.

MATERIALS AND METHODS

Instrumentation

The experiment was executed using a Water Alliance UPLC 2695 UPLC system with PDA detector (Water Corporation, Milford, MA, USA). The experimental setup involved the utilization of an ultrasonication device, analytical balances, and a vacuum microfiltration machine equipped with 0.22 µm PVDF filters manufactured by Millipore.

Chemicals, Reagents and Standards

The AML drug substance was purchased from Anphar Laboratories Pvt. Ltd, located in Jammu. The drug substance of HCTZ was procured from Hetero Drugs Pvt. Ltd, located in Hyderabad. The AR-grade formic acid was procured from SD Fine Chemical in Mumbai, India, while the ethanol was obtained from Honeywell, also located in Mumbai, India. The Milli-Q water used in this study was acquired from Millipore. Samples of the finalized pharmaceutical tablets were obtained from a nearby drugstore.

Chromatographic Conditions

The buffer solution was prepared by including 0.1% formic acid and subsequently passing it through 0.22 µm membrane filter. The isocratic mode (55:45 v/v of Buffer: Ethanol) was used as the mobile phase. The total duration of the run was 12 minutes. The separation of impurities was achieved by employing the Waters Xbridge C18 column, 3.5µm (50 X 4.6 mm). 0.3 mL/min as the flow rate. The column temperature is 40 as and 25°C as the sample temperature. A total of 254 nm was used for the ultraviolet (UV) detection. The amount of injection is 3 µL and the overall duration of the run is 12 minutes, as shown by the isocratic mode of 55:45 v/v (0.1% Formic acid: Ethanol). The diluent is composed of a 500:500 volume/volume ratio mixture of ethanol and Milli-Q water.[21,22]

Preparation of the AML Standard Stock Solution

Prepared the standard stock solution for AML by measuring and transferring 100 mg into a 100 mL VF. In 70 mL of diluent was added to the substance, which was then subjected to sonication in order to aid in the process of dissolution. The flask was filled with the diluent and well mixed, resulting in a final solution of 250 µg/mL concentration. Furthermore, it is necessary to aliquot a 5 mL portion from the original solution and transfer it into a 25 mL VF. Proceed to completely fill the remaining volume of the flask with a diluent and ensure thorough mixing of the contents.

Preparation of the HCTZ Standard Stock Solution

Prepared the standard stock solution of HCTZ by weighing 100 mg of the HCTZ and transferring into a 100 mL VF. Added 70 mL of diluent to the flask, and subsequent sonication was employed to aid in the process of dissolution. The flask was filled to its maximum capacity with the diluent and well mixed, leading to the formation of a stock solution containing HCTZ at a concentration of approximately 250 µg/mL. Additionally, transferred 5 mL from the stock solution into 25 mL VF.

Proceed to fill the remaining volume of the flask with a diluent and thoroughly mix the contents.[23]

Preparation of the Sample Preparation

A sample solution containing AML and HCTZ was prepared for 250 µg/mL concentration. This was achieved by weighing a minimum of 10 tablets and calculating the average weight of the tablet. Pulverize the tablets using mortar and pestle into a finely homogenized powder. Measured equivalent to 100 mg of tablet powder and transferred into a 100 mL VF. A volume of 60 mL of diluent was added into the mixture, then subjected to sonication for a duration of 20 minutes, with occasional shaking. Further dilute the solution to its desired volume using the diluent and ensure thorough mixing. Centrifuge the solution at a speed of 5000 rpm for 10 minutes. In 5 mL was transferred from the supernatant stock solution into a 25 mL VF and diluted with a diluent and thoroughly mix the contents.

RESULT AND DISCUSSION

Method Development

The current aim of this study was to establish a chromatographic method (SIAM) for the successful separation of DPs in AML and HCTZ. The developed method employed liquid chromatography fitted with a diode array detector (LC-DAD) to assess the applicability of routine stability and quality control analyses. The method was optimized to separate degradation products obtained during the forced degradation studies (stress studies). The important criteria in developing this method are to achieve sufficient resolution of the impurities, peak asymmetry and total run time. To achieve the criteria several experiments were performed to optimise the stationary and mobile phases. The beginning of method development was the initiation of an isocratic method with mobile phase A as 20 mM ammonium acetate buffer adjusted to a pH of 4.0 with CH_3COOH and mobile phase B as ethanol in 60:40 v/v. 0.3 mL/min as the flow rate. The symmetry C8 column, with 50 X 4.6 mm and a particle size of 3.5 µm was used. The resolution between the analyte and DP was insufficient, resulting in a distorted peak shape of the analyte. In the optimized isocratic elution mode, a range of mobile phase compositions was assessed by employing varied concentrations of various buffers and ethanol. The most effective chromatographic separation was attained by employing an anisocratic mode utilizing an MP of 0.1% HCOOH and ethanol (55:45 v/v) using a Waters Xbridge C18 column (50 X 4.6 mm, 3.5 µm) for separating the analytes and DPs. The final optimized chromatograms are shown in Figure 3.

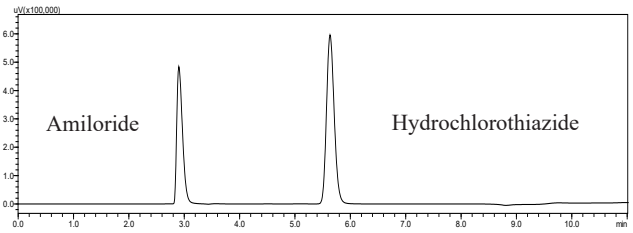

Figure 3: Representative chromatogram of standard

Method Validation

The method has undergone validation according to the ICH Q2 (R2) guidelines, encompassing many aspects such as specificity, system suitability, sensitivity, accuracy, precision, linearity, range, solution stability and ruggedness.[24–26]

System Suitability Testing

The evaluation of system suitability testing (SST) was involved by injecting six replications of the standard solution following the guidelines provided in the United States Pharmacopeia (USP). The calculations were performed to determine the peak asymmetry, theoretical plates, and %RSD for the regions of the main peaks. Table 1 illustrates the results of the SST. Figure 3 illustrates the representative chromatogram of the standard.

Specificity and forced degradation studies

The method specificity was assessed by analyzing the blank, reference, and sample solution at a 250 µg/mL concentration. The approach demonstrated specificity, as there was no observable interference in the blank chromatograms during the primary peak and impurity retention time. Figures 4 and 5 illustrate the representative overlay chromatograms of the blank, standard and sample. Table 2 presents the values for the individual RTs, RRTs, PA, and PT. The assessment of the method's specificity is carried out using forced degradation studies in accordance with the recommendations outlined in ICH Q1A guidelines. The degradation of the sample was conducted according to the experimental conditions outlined below.[27–31]

- *Acid degradation*

Weighed and transferred the equivalent to 50 mg of the sample powder which is ground using the motar and pestle into a

Table 1: SST results

Injection No.	*Area of AML peak*	*Area of HCTZ peak*
1	63535	85351
2	65217	84366
3	65339	86152
4	65535	85349
5	65634	85172
6	65598	86255
Mean	65143	85441
SD	803.90	695
%RSD	1.2	0.8
Theoretical plate count	14107	9899
Tailing factor	1.0	1.2

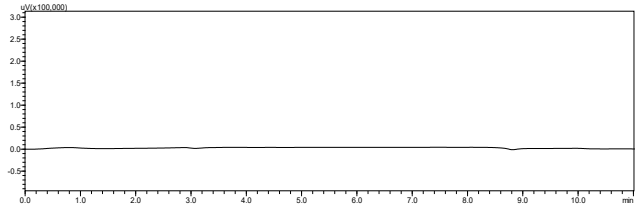

Figure 4: Representative chromatogram of blank

Table 2: Specificity and peak purity summary

Peak name	*Retention time (minutes)*		*Relative retention time*	*Purity angle*	*Purity threshold*
	Standard solution	*Sample solution*			
Amiloride	2.98	2.96	1.00	0.150	0.427
Hydrochlorothiazide	5.65	5.65	1.90	0.082	0.366

Table 3: Stress conditions and results

Stress condition	*%Assay of AML*	*%Assay of HCTZ*	*%Degradation*	*Total (%w/w)*	*%Mass balance*	*Purity angle*	*Purity threshold*
As Such	98.52	99.26	NA	0.0	NA	0.028	0.222
Acid degradation	97.22	98.91	0.0	0.0	98.51	0.028	0.225
Base degradation	98.01	99.17	0.0	0.0	98.54	0.027	0.226
Thermal degradation	98.54	99.23	0.0	0.0	97.72	0.028	0.239
Photolytic degradation	98.52	98.90	0.0.	0.0	98.43	0.026	0.224
Humidity degradation	98.45	99.32	0.0	0.0	99.27	0.026	0.223
Peroxide degradation	89.95	97.2	8.57	8.57	95.21	0.027	0.537

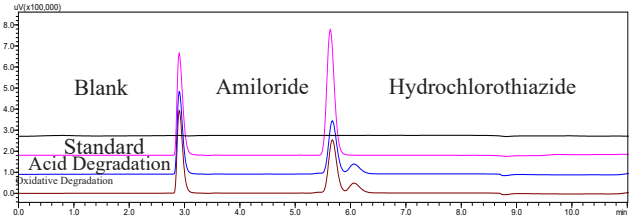

Figure 5: Representative overlay chromatogram of blank, standard, API, marketed formulation

50 mL VF, and added 5 mL of a 1N HCl solution to the flask. The sample was then subjected at 80°C temperature on a water bath for 6 hours. The sample was allowed to undergo cooling until it reached the ambient temperature, after which it was further neutralized by adding 5 mL of 1N NaOH solution, 30 mL of diluent was added made sonication for 10 minutes, in order to facilitate dissolution. Finally, the desired volume was adjusted to by adding a diluent and thoroughly agitated. Centrifuge the sample at a speed of 5000 rpm for 10 minutes. Furthermore, transferred 5 mL of the clear solution into a 25 mL VF and make up to the volume with diluent and mixed well before being injected into the UPLC analysis. No significant degradation was shown in the chromatogram obtained when the sample was subjected to acidic conditions. The obtained mass balance, percentage assay, percentage degradation and peak purity of AML and HCTZ in the study are tabulated in Table 3.

- *Base degradation*

The amount of the sample powder, weighing 50 mg, was carefully transferred to a 50 mL VF after being ground using a mortar and pestle. Subsequently, a 5 mL of a 1N NaOH solution was introduced into the volumetric flask. The specimen was subjected to a water bath maintained at a temperature of 80°C for three hours. The sample was subjected to a cooling process until it equilibrated with the surrounding temperature. Subsequently, it was neutralized by the introduction of 5 mL of a 1N HCl solution. A volume of 30 mL of diluent was introduced, and then, sonication was performed for a period of 10 minutes to aid in the process of dissolution. Following this, the volume was increased by adding a diluent and the mixture was well agitated. The above solution was made centrifugation at 5000 rpm for 10 minutes. The above solution obtained was subsequently employed in the following operations. Further transferred 5 mL from the above solution and subsequently placed into a 25 mL VF. The remaining volume required to reach the desired level was achieved by adding a diluent, followed by thorough mixing. and the contents were thoroughly mixed before being injected into the UPLC analysis. The chromatogram obtained indicates that there is no significant degradation observed when subjected to basic conditions. The results pertaining to the percentage assay, percentage degradation, mass balance, and peak purity of AML and HCTZ were observed in Table 3.

- *Thermal degradation*

The tablets for the study were subjected to thermal exposure in a controlled environment, namely a hot air oven, where a constant temperature of 120°C was maintained for a period of 24 hours. The exposed samples were accurately weighed to a mass of 50 mg and subsequently transferred to a 50 mL VF. In 30 mL of diluent was added to the volumetric flask to dissolve the sample, and the resulting mixture was subjected to sonication for a duration of 10 minutes. The sample was let to undergo cooling until it reached the ambient temperature. Then, the diluent was added up to the mark and thoroughly mixed. The sample solution was centrifuged at 5000 rpm speed for a duration of 10 minutes, and the resulting supernatant solution was utilized. Additionally, 5 mL from the above stock solution was transferred into a 25 mL VF. The remaining volume should be filled with a diluent, thoroughly mixed, and then injected into the UPLC analysis. The chromatogram shows that there was no significant degradation under the thermal stress conditions. The results to the mass balance, percentage assay, percentage degradation, and peak purity of AML and HCTZ in the study are illustrated in Table 3.

- *Photo degradation*

The sample was exposed to a photostability chamber intended to provide an exposure of 1.2 mLux hours and 200 watts hours per sq. mts of UV-visible. The exposed samples were weighted and transferred 50 mg of equivalent sample powder into a 50 mL VF. In 30 mL of a diluent was added and subjected to sonication for a duration of 10 minutes in order to facilitate dissolution and add up to the mark with diluent and mixed well. The sample was centrifuged at 5000 rpm speed for 10 minutes of duration, 5 mL of the supernatant stock solution was transferred into a 25 mL VF and made upto the volume with diluent, thoroughly mixed, and subsequently injected for UPLC analysis. The chromatogram obtained does not exhibit any significant degradation when subjected to the Photo condition. The results to the percentage assay, percentage degradation, mass balance, and peak purity of AML and HCTZ are in Table 3.

- *Humidity degradation*

The samples were kept in a controlled chamber maintained at 25°C temperature and 80% RH for 7 days. The exposed samples were measured equivalent to 50 mg of sample powered which is ground using mortar and pestle and subsequently transferred to a 50 mL VF. Added 30 mL of diluent to the VF and subjected to sonication for 10 minutes in order to facilitate dissolution. The sample was let to undergo cooling until it reached ambient temperature. Subsequently, the diluent was added to the designated level and thoroughly mixed. The sample at a speed of 5000 rpm for the duration of 10 minutes. The resulting solution was subsequently utilized for experimentation. Transferred 5 mL from the supernatant solution into a 25 mL VF, and made up to the volume with diluent, and mixed well. Finally, the prepared solution was analyzed into the UPLC system for analysis. The obtained chromatogram shows no significant degradation under the humidity condition. The results of the percentage assay, percentage degradation, mass balance and peak purity of AML and HCTZ in Table 3.

- *Peroxide degradation*

Weighed and transferred 50 mg of equivalent sample powdered was weighed which is grinded using a motor and pestle into a 50 mL VF. Then, 5 mL of 10% H_2O_2 solution to the flask. The sample was then kept onto a water bath maintained at 80°C temperature for 5 hours. The sample was allowed to reach ambient temperature. About 30 mL of diluent was added and sonicated for a duration of 10 minutes to facilitate dissolution. Subsequently, the diluent was made up to the mark and mixed well. The sample solution is centrifugation at 5000 rpm speed for a duration of 10 minutes, and the resulting supernatant solution was utilized. Transferred 5 mL of the clear solution into a 25 mL VF and with diluent makeup to the mark and mixed well before being injected for UPLC analysis. The obtained chromatogram shows significant degradation under the oxidative condition. From the above results, the drug is more susceptible to oxidative stress conditions The results of percentage assay, percentage degradation, mass balance and peak purity of AML and HCTZ in Table 3.

Linearity

The linearity was assessed by injecting the reference solutions of AML and HCTZ at concentrations covering five levels, ranging from 10% to 150% (n=5). The calibration curve was constructed by plotting a graphical representation that illustrates the relationship between the peak areas and the concentrations of AML and HTCZ individually. The calibration curve that was obtained showed a correlation coefficient above 0.999 for both AML and HCTZ, indicating a strong linear relationship. Thus, the approach employed can be considered to exhibit linearity. The tabulated results are shown in Table 4.

Precision and IP

- *System precision*

The system precision (instrument) was assessed by carrying out six replicate injections of reference solutions containing AML and HCTZ on the instrument. The resulting relative standard deviations (% RSD) were determined to be 0.53 and 0.63 for AML and HCTZ, respectively. The results obtained from the system precision study concluded that the system is precise for determining AML and HCTZ in tablets by UPLC and results shown in Table 4.

- *Method precision*

The method precision was assessed by injecting six different sample solutions at the specification limit of 100%. This analysis involved determining the sample concentration and calculating the %RSD for the areas for each analyte. The results are shown in Table 4 and confirm that the method is rugged for the determination of AML and HCTZ.

- *Intermediate precision*

The assessment of intermediate precision involved the injection of six distinct preparations of sample solutions at the 100% specification limit on various days, utilizing different equipment. The %RSD values for the individual samples of AML and HCTZ were computed. The findings are presented in Table 4, which provides confirmation of the method's ruggedness for the determination of AML and HCTZ.

Accuracy

The method's accuracy was assessed by utilizing the standard addition method (API). The study was conducted in triplicate at various concentration levels, specifically 10, 50, 100, and 150%. The percentage recoveries were subsequently determined. The percentage recovery values for each impurity, namely AML and HCTZ, fell within the range of 100.0 to 106.0, which met the acceptability standards. The %RSD values for the analyte recoveries at each level were found to be less than 5.0. The findings are presented in Table 4.

Solution stability

The sample solution stability of AML and HCTZ were determined by storing the samples in tightly capped volumetric flasks at 25°C and 2 to 8°C for 24 and 48 hours, respectively.

Table 4: Summary of method validation data

Parameters	*Amiloride*	*Hydrochlorothiazide*
System Suitability n = 6		
%RSD, Theoretical plates, tailing factor	1.2, 14107, 1.0	0.8, 9899, 1.2
Linearity		
Correlation coefficient	0.9999	1.0000
Accuracy (% of recovery) n = 3 (Mean)		
10, %RSD	106.033, 4.08	97.6, 4.06
50, %RSD	104.233, 2.41	100.5, 3.66
100, %RSD	104.298, 0.12	102.1, 3.86
150, %RSD	104.006, 0.11	NA
Repeatability %RSD (n = 6)		
SP, %RSD	0.53	NA
MP, %RSD	0.70	0.82
IP, Overall %RSD	0.82	3.20
Solution Stability		
In Sample at 25°C after 24 hours, %Difference	0.97	1.28
In Sample at 25°C after 48 hours, % Difference	1.40	2.26
In Sample at 2–8°C after 24 hours, % Difference	0.37	1.13
In Sample at 2–8°C after 48 hours, % Difference	0.20	2.16
Robustness		
Temperature as such (40°C), RRT	1.00	1.20
Temperature minus (35°C), RRT	1.00	1.20
Temperature plus (45°C), RRT	1.00	1.20
Wavelength as such (254 nm), RRT	1.00	1.20
Wavelength minus (250 nm), RRT	1.00	1.20
Wavelength plus (260 nm), RRT	1.00	1.20
Flow rate as such (0.3 mL/Min)	1.00	1.21
Flow minus (0.2 mL/Min)	1.00	1.21
Flow plus (0.4 mL/Min)	1.00	1.20

Abbreviations: NA: Not Applicable, RSD: Relative Standard Deviation, RRT: Relative Retention Time.

The %difference in the area of samples was calculated against freshly prepared sample solution. The results were shown in Table 4 and found that AML and HCTZ were stable at 2 to 8°C after 24 hours.

Robustness

The robustness is the deliberate change in the chromatographic conditions and it was evaluated through variations observed in system suitability parameters. The study was evaluated considering several variables, including column temperature ranging from 35 to 45°C, flow rate ranging from 0.2 to 0.4 mL/min, and wavelength ranging from 250 to 260 nm. The results obtained were found to meet the specified acceptance criteria, as shown in Table 4.

Green Analytical Metric Assessment

The Analytical Eco-scale, a metric tool, is employed to measure penalty points. The total penalty points amount to 18, thus the current analytical method of analysis obtains a score of 82, indicating excellent performance. For detailed results; please refer to Table 5. The AGREE software employs

Table 5: Represents the Ecological scale metric to the analytical method

Analytical Ecological Scale		
S. No	*Name*	*Penalty Points*
Chemicals or reagents		
1	Formic acid	4
2	Ethanol	4
Instruments		
1	Energy- 1.5kWh of energy per sample for UPLC and LC-MS	1
2	Occupational Waste- Procedure releases vapours into the environment	3
Waste		
1	Total Amount of waste generated (Waste Generated >10mL)	5
2	Management (The generated waste has a degradation process)	1
Total Penalty Points		18
Total Score (100-Total Penalty Points)		82
Greenness Evaluation		Excellent

Table 6: Represents the AGREE metric to the analytical method

AGREE tool for assessing the analytical method on UPLC and LC-MS/MS		
S. No	*Green analytical chemistry principles*	*Sample procedure*
1	Direct analytical techniques (online/off-line/at-line/in-line)	Off-line analysis
2	Achieved minimal number of samples	1 g
3	Performed *in-situ* measurement	At-line
4	Avoid integration processes and operations	3 distinct steps involved in the sample preparation procedure
5	Applied fully automated/Semi-automated and miniaturized in the method	Semi-automatic and miniaturized
6	Avoid Derivatization in the method	No derivatization
7	Avoid generation of a large volume of analytical waste, and provide the proper management	1 g
8	Multi-analyte or parameters are analyzed at a time	4 analytes were determined in single run; 3 samples were analysed per 1 hour.
9	Used the energy-saving technique and estimate the total power consumption in kWh for a single analysis	UPLC, LC-MS, 0.15 kWh
10	Preferred renewable source of reagents	All reagents are bio-based
11	Eliminate Toxic reagents or replace	Used reagents or solvents are non-toxic
12	Increase operator's safety.	The threats that are not avoided are a. Bio-accumulative b. Highly flammable c. Explosive

Table 7: Represents the GAC tool GAPI to assess the Greenness analytical method

GAPI tool for assessing the analytical method on UPLC and LC-MS/MS		
Sample sourcing		
1	Sample collection	Off-line
2	Sample storage	None
3	Sample preservation	Physical or Chemical
4	Sample transport	None
Method type and sample preparation		
5	Method type	Simple procedure
6	Extraction scale	Micro-extraction
7	Solvent or reagent used	Green solvents/ Reagents used
8	Additional treatment	Simple treatment
Reagents and solvents		
9	Amount	<10 g used
10	Health hazard	Formic acid: NFPA score of 0 to 1. Slightly toxic, slight irritant.
11	Safety hazard	Ethanol: NFPA score of 0 or 1, flammability. No Special hazards.
Instrumentation		
12	Consumed Instrument Energy	</= 0.1 kWh per sample
13	Occupational hazard	Hermetic sealing of the analytical process
14	Instrument Waste generated	< 1 g
15	Waste treatment	Degradation, Passivation
Quantification/Qualification		
1	Symbol O	Procedure for qualification and quantification

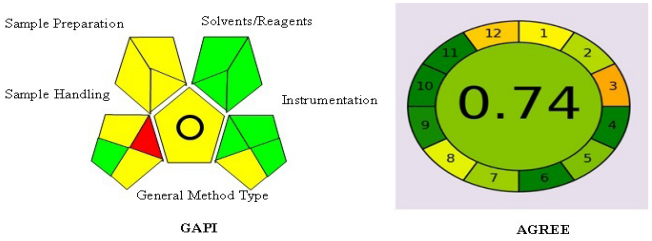

Figure 6: Representative pictograms of GAPI and AGREE

a framework comprising 12 GAP components. Each principle is granted a numerical value ranging from 0.1 to 1.0, indicating the extent to which it adheres to sustainability criteria. The method's overall AGREE score of 0.74 is depicted in Table 6 and Figure 6. The GAPI tool displays a collection of 15 pictograms and 5 pentagrams. Among the several pictograms utilized, it is significant that the pictogram denoted by the number 8 is depicted in the color red, symbolizing the specific sample treatment used. Conversely, the pictograms about sample preparation and sample handling are illustrated in yellow. The reagents and instruments utilized in the study are indicated by a green pictogram. The results obtained from the GAPI are presented in Table 7 and Figure 6.

CONCLUSION

Experimental results and method validation confirm that the proposed procedure is capable of determining AML and HCTZ accurately in the formulated drug product. Furthermore, it effectively differentiates all contaminants through the appropriate selection of stationary and mobile phases. The efficacy of the suggested method has been validated in accordance with the ICH Q2 guidelines. During

the degradation process of the peroxide sample, we identified a single possible impurity. The drugs AML and HCTZ were observed to be sensitive to degradation when exposed to peroxide, while they demonstrated stability when subjected to acid, basic, heat, humidity, and photo-stability conditions. The observed degradation and subsequent validation of the approach indicate that it possesses specific, sensitive, linear, accurate, range, rugged, precise, robust, and SIAM. The utilization of this method has the potential to provide an estimation of degradation products with AML and HCTZ in the pharmaceutical dosage form and active pharmaceutical ingredients (API). The optimized analytical method was evaluated with GAP metric tools like Analytical Ecological scale, AGREE, GAPI and found that the method is excellent green according to the GAP principles.

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