Development of validated stability indicating HPLC method of gallic acid and GCMS study of degradation products

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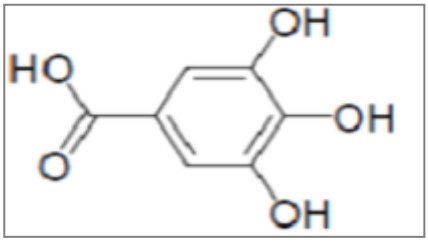
ABSTRACT:
The goal of this study was to create a validated specific stability indicating reversed-phase liquid chromatographic method for quantifying gallic acid in bulk samples in the presence of degradation products.
Forced degradation studies were performed on a bulk sample of Gallic acid using acid, base, oxidative, thermal stress, and photolytic degradation to demonstrate the method's stability and power.
During oxidative stress, significant degradation was observed, and the degradation product formed was identified using GCMS.
There was slight degradation in acidic and alkaline stress, but no degradation in other stress conditions. Good resolution between the peaks corresponds to degradation products from the analyte were achieved on C18 column 5µ (4.6 X 250 mm) using the mobile phase consists a mixture of Orthophosphoric acid (0.1%V/V), Acetonitrile and Methanol (50:35:15 V/V/V). The detection was carried out at 270 nm.
The stressed test solutions were assayed against the qualified working standard of Gallic acid indicating that the developed LC method was stability indicating. Validation of the developed LC method was carried out as per ICH requirements.
The developed LC method was found to be suitable to check the quality of bulk samples of Gallic acid at the time of batch release and also during its stability studies.

Keywords: Reversed phase chromatography, Forced degradation study, Gallic acid, GCMS

INTRODUCTION:

A polyphenolic substance with antioxidant properties is gallic acid. It is a 3, 4, and 5-trihydroxybenzoic acid chemically. It can be found both in a free state and as gallotannin, a component of tannins. Almost every component of the plant, including the bark, wood, leaf, fruit, root, and seed, contains gallic acid and its derivatives.

A class of naturally occurring polyphenol antioxidants called gallic acid and its derivatives have recently been proven to have potential health benefits. Antioxidant properties, neuroprotective benefits, and free radical scavenging properties are all present in gallic acid and its derivatives.



Structure 1. Gallic acid

Gallic acid (GA), a phenolic structure including carboxylic acid, is appealing for its expected high antioxidant capacity due to its three hydroxyl groups, while allowing for relatively straightforward copolymerization through its –COOH group.[1]

RP-HPLC method for simultaneous estimation of Ascorbic acid and Gallic acid in *Phyllanthus emblica* L [2] & RP HPLC method to determine gallic acid content of hydroalcoholic extract of dried flowers of *Nymphaea stellata* [3] have been reported.

Natural products have been a valuable source for such drugs, whether used as a natural resource or after chemical modifications. Natural phenolic compounds, in particular, have revealed interesting results concerning the inhibition of proliferation of several cancer cell lines.[4]

GA, as a natural plant secondary metabolite, has been proven to exert an anti-inflammatory role in neurodegenerative diseases, metabolic diseases, arthritis, cancer and other pathological conditions. GA can also reduce the incidence of inflammation-related diseases through anti-oxidative stress, anti-apoptosis and anti-viral mechanisms.[5] Gallic acid shows antimelanogenic and antioxidant properties. Gallic acid showed promising results as an anti-HSV-2 (Herpes simplex virus) agent. It has also been proposed to be as active moiety for treatment of brain tumours as it suppresses cell viability, proliferation, invasion, and angiogenesis in human glioma cells.[6]

Gallic acid has been shown to effectively suppress DSS-induced colitis in mice. Intriguingly, the anticolitis effect was attributed to the suppression of colonic NF-kB and STAT3 activation.[7]

Herbs that contain Gallic acid as a major constituent are citrus fruits like *Phyllanthus emblica* (Amalaki), *Terminalia bellirica* (Bibhitaki), *Terminalia chebula* (Haritaki), etc. which is useful in common cold and fever, diuretic, laxative, liver tonic, refrigerant, stomachic, restorative, alterative, antipyretic, anti-inflammatory, hair tonic, to prevent peptic ulcer and dyspepsia, and as a digestive.[8]

Structural characterization of the major degradation products enables one to establish the degradation pathway under which the degradation products are formed. This further helps with the quantitative determination of the drug, in the presence of its process related impurities and degradation products. According to Current Good Manufacturing Practices, all drugs must be tested with a stability-indicating assay method before release. [9,10] To date, no stability indicating HPLC assay method for the determination of Gallic acid is available in the

literature. It was felt necessary to develop a stability-indicating liquid chromatography (LC) method for the determination of Gallic acid as bulk drug and separate the drug from the degradation products under the International Conference on Harmonization (ICH) suggested conditions (hydrolysis, oxidations, photolysis and thermal stress).[11]

Therefore, the principal objective of this study was to develop a new, simple, economical, precise, and reproducible stability indicating RP-HPLC method with a wide linear range and good sensitivity for assay of Gallic acid in the bulk drug.

Rational of the Study

The main objective of present research is to generate sufficient and fruitful stability data of selected moieties, which will be useful in future to resolve stability related issues of selected compounds. The prime rationale of present research is to find out reactive parts of the drug molecule that helps in order to alter the reaction condition and to reduce the quantity of impurity to an acceptable level. The ultimate goal is to utilize present research in future for obtaining more stable, least toxic and more effective drug therapy. The proposed study will also assist in the design of more stable analogues, development of stable in order to comply with the regulatory requirements for impurity limits. The proposed method will also be helpful to predict the storage conditions, selection of packaging material.

MATERIALS AND METHODS

Materials

Chemicals

HPLC grades Acetonitrile, Methanol and Distilled water were purchased from Fischer Scientific Pt. Ltd Mumbai. All other reagents including Orthophosphoric acid used in this study were of AR grade. Pure Gallic acid and excipients were collected from the store of CRPS Nanded Pharmacy College Nanded. Hydrogen peroxide solution (6% w/v) 20 VOL were purchased from retail medical shop.

Instruments & equipment

The chromatography was performed on Agilent 1120 LC instrument equipped with UV detector UV-20A with wavelength Range 190-700nm and Lab monitoring and EZ Elite chrome software. Agilent C18(2) (4.6 x 250mm, 5µ particle size) was used as stationary phase. Agilent manual injector with 20µL capacity loop was used. All weighing were done on single pan balance Dhona 200D. Ultrasonics bath was used in the study and pH meter LOBAL Digital pH meter were used.

Methods

Preparation of Mobile phase:

Solvent A – Ortho phosphoric acid (0.1%): An accurately measured 1.00 mL of OPA (85-88 %) transferred to 1000 ml volumetric flask and diluted up to mark with HPLC grade distilled water. The flask was kept in ultra sonicator for 30 minutes. Then it was filtered through the 0.45µ membrane filter. Filtrate was again sonicated for 30 minutes.

Solvent B- Acetonitrile: Acetonitrile also passes through the 0.45µ membrane filter and sonicate for 30 minutes.

Solvent A and solvent B were transferred to appropriate reservoirs attached to channel A and channel B respectively. Gradient program (Table 1) was followed for sufficient time to equilibrate stationary phase packed in column with mobile phase.

Laboratory mixture:

Laboratory mixture was prepared by mixing gallic acid and common excipients used in tablet formulation like talc, starch, calcium carbonate, lactose. 1000 mg prepared mixture contain 100 mg Gallic acid.

Linearity Study

Standard Stock Solution:

About 100 mg of Gallic acid STD was weighed into 100 mL volumetric flask and sonicate for 30 min. The volume was made up to the mark with diluent to make 1000 ppm stock solution.

Working Standard Solution:

Appropriate aliquots were withdrawn from the stock solution and diluted up to10 mL with diluent to make standard solutions of concentrations 50, 75,100,125 and 150 ppm.

Calibration Curve:

Chromatogram was recorded for standard solutions with different concentration. Graph of concentration (as x-value) versus area (as y-value) were plotted (Fig.1). The correlation coefficient, y-intercept and slope of the regression were calculated and mentioned below (**Table 2 and 3**).

Formula:

Concentration of test solutions were calculated by using slope and intercept equation obtained from the calibration curve of Gallic acid.

Slope and intercept equation: $y = m x \pm c$

Where, y - y axis value i.e. Absorbance of test solution

x - x axis value i.e., Concentration of test solution

m - slope of linear curve

c - y intercept at zero concentration

Assay:

An accurately weighed Gallic acid was mixed with common excipients in definite proportion (1:4). This mixture was well triturated and uniformly mixed. Mixture was stored in a closed container. An accurately weighed sample i.e., LM equivalent to 100 mg Gallic acid was transferred to 100 ml volumetric flask and around 50 mL diluents added to it. This solution sonicates for 30 minutes. Excipients were filtered off and filtrate was diluted to 100 ml with diluents. Aliquot about 1 ml was taken in three different 10 ml volumetric flask and final volume made up to the mark with diluents. The results are shown in table 4.

The % content of drug was calculated by using following formula

$$\% \text{ Content} = \frac{At}{As} \times \frac{Ds}{Dt} \times \frac{Ws}{Wt} \times \frac{\text{Avg. wt.}}{LC} \times 100$$

Where,

At = Peak area of sample solution

As = Peak area of standard solution

Ds = Dilution factor for standard

Dt = Dilution factor for sample

Ws = Weight of standard (mg)

Wt = Weight of sample (mg)

Avg. wt. = Average weight

LC = Label claim

Validation of HPLC Method

The proposed RP-HPLC method was validated as per the International Conference on Harmonization (ICH)guidelines Q2 (R1). [12,13]

Forced degradation study: [14,15]

Standard Stock Solution: A standard stock solution (1000 ppm) was prepared by dissolving 100 mg of the GA in 100 ml of methanol.

Acid Solution: One mL of stock solution was diluted up to 10 mL with 1 M HCl and stored at room temperature for 24 hrs. 1 mL of the solution was taken in separate 10 mL volumetric flask and neutralized with 1 M NaOH. Final volume was adjusted to mark with diluent and analysed.

Alkaline Solution: To separate 10 ml volumetric flask, 1 mL of stock solution taken and diluted in 0.1 M NaOH to mark. Then solution stored at room temperature for 24 hrs. After the degradation time was achieved, 1 mL of the solution was neutralized with 0.1 M HCL and diluted to 10 mL with diluent. Final solution was used for the analysis.

Oxidative Solution: Aliquot about 1 mL of stock solution and 4 mL was taken in 10 mL volumetric flask. This mixture diluted to mark with 0.3% H_2O_2 solution and kept aside at room temperature for 24 hrs. After the degradation time was achieved, 1 mL of the solution was transferred to another 10 mL volumetric flask, diluted to mark and analysed.

Thermal Degradation: Analytically pure sample of GA was exposed in an oven at 110^0c for 12 hours and cooled to room temperature. Then, accurately weighed 100 mg sample was transferred to 100 ml volumetric flask and volume adjusted with methanol. Appropriate dilutions were done and final solution analysed.

Photo stability solution: A specific amount of compound was taken in a cleaned Petridish and exposed to radiations of wavelength 254 nm in UV chamber for 12 hrs. Accurately weighed 100 mg sample was transferred to 100 ml volumetric flask and diluted with methanol. Solution was further diluted by diluent taking appropriate aliquot.

Fresh standard stock solution was prepared for the study of each stress condition. All solutions were passed through a 0.45 µ Whatman filter paper and injected in the liquid chromatographic system and chromatograms were recorded.

GC-MS analysis

GC-MS analysis of GA stressed at acidic medium, alkaline medium and oxidative stress conditions was carried out to characterize degradation products. Separated degradation products and pure compound was achieved through gas chromatographic method. Separated compounds were characterized from their mass spectra. [10,13]

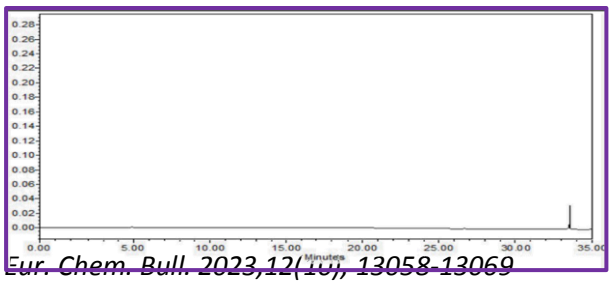
RESULT AND DISCUSSION:

Optimization of mobile phase

Several chromatographic runs were taken on C18 column 5µ (4.6 X 250 mm) with varying composition of mobile phase at flow rate 1 mL/min and detection wavelength 280 nm. As per the solubility of Gallic acid, a mixture of orthophosphoric acid (OPA) (0.1%V/V) and methanol with different compositions were tried as a mobile phase. But no satisfactory peak was obtained with former mobile phase. Then, methanol was replaced with acetonitrile and varying composition of orthophosphoric acid (0.1%V/V) and acetonitrile was tried for isocratic and gradient mode (Table 1). After several run, gradient program as given below was finalised for HPLC method of Gallic acid as it gave well resolved symmetric peak with reproducible retention time Fig. 2.

TABLE 1 DETAILS OF GRADIENT PROGRAM

Time (minute)	Flow (mL/minute)	% Solvent A	% Solvent B
0	1.0	99	1
20	1.0	99	1
21	1.0	5	95
30	1.0	5	95
31	1.0	99	1
35	1.0	99	1



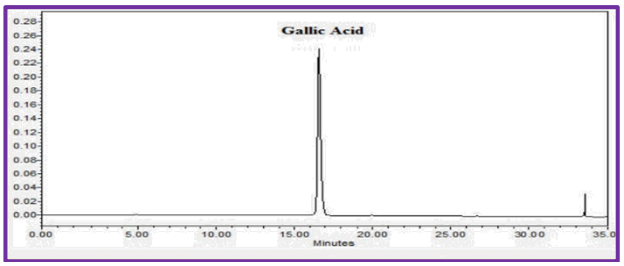


Figure 2 HPLC Chromatogram of blank solution

Figure 3 HPLC Chromatogram of std. solution

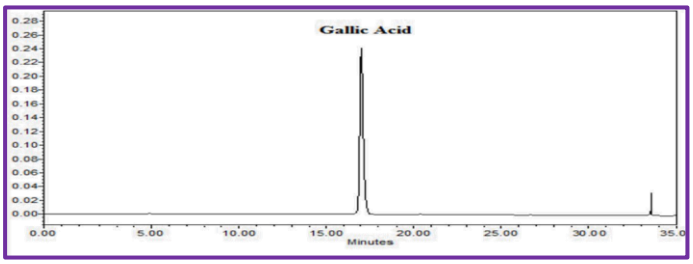


Figure 4 HPLC Chromatogram of sample solution

Assay

Proposed method was used for the estimation of GA from its tablet dosage form. Results of assay are summarized in table 4.

TABLE 2 OBSERVATIONS OF LINEARITY STUDY

Sr. No	Conc. of Gallic acid (ppm)	Average Peak Area (for n = 3)
1	50	66758
2	75	98569
3	100	132659
4	125	165753
5	150	198569

TABLE 3 REGRESSION LINE PARAMETER FOR ANALYTICAL PROCEDURE

Linearity Parameter	Results
Linearity Range	50-150 PPM
Correlation coefficient (r^2)	0.999
Slope	1323.2
y-intercept	139.2

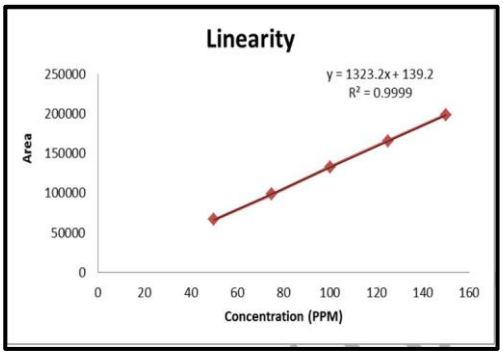


Figure 1 Calibration curve of GA

TABLE 4 RESULT OF ASSAY OF GA

Sr. No.	Wt. of standard in mg	Wt. of sample in mg	Peak area of standard	Peak area of sample	% GA content
1.	10	50.15	132439	132754	100.24
2.		50.05		131542	99.12
3.		50.1		132581	99.91
4		50.00		131582	99.35
5		50.25		132074	99.62
6		50.3		134520	100.96
Statistical values for n=6				**Mean**	**99.60**
				SD	**0.287**
				%RSD	**0.288**

Method validation

Specificity:

The specificity of the method for Assay was demonstrated by detecting Peak of STD, blank and sample solutions by developed HPLC method for rectification of specificity of method. Results of specificity study are shown in table 5.

Precision

System precision

System precision was evaluated from five replicate injections of standard as per proposed method. The Peak area, average and % RSD were calculated and tabulated in the Table 5.

Method Precision

The six sample solutions were prepared separately. Each sample solution was injected in HPLC system and analysed as per proposed procedure. The % assay, average and % RSD were calculated and tabulated in the Table 5.

Intermediate precision

The Intermediate precision was determined by comparison of two independent analyses on different days. The data of the 1st day analysis was taken from the analysis of "Method precision". Results of Intermediate precision study were shown in table 5.

Linearity

The linearity of peak area response for GA HCL was determined from 50 % to 150 % level of test concentration. Accurately weighed quantity of tablet powder was diluted to obtain concentration in the range of 50-150 % of test concentration. Graph of concentration (as x-value) versus peak area (as y-value) was plotted as shown in fig 6 and observation in table 5.

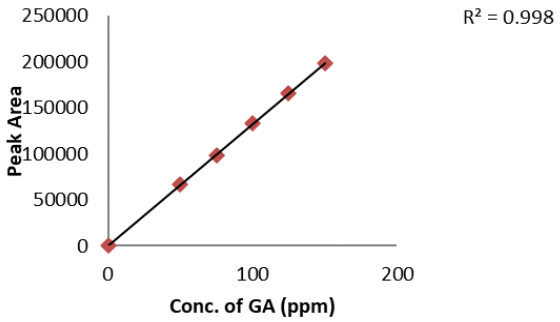


Figure 5 Linearity Graph for LM Gallic acid

Robustness

The influence of slightly changed parameters of the chromatographic conditions was tested according to ICH guidelines to demonstrate sufficient robustness of the method. The tests were carried out by injecting standard solution by changing column temperature ($\pm 5^{0}$**c**) and flow rate of mobile phase (± 0.1 mL / minute). Results are as shown in table 5.

Accuracy

The accuracy was determined by standard addition method. A known but varying amount of Standard GA was spiked into pre-analysed Drug sample solution at 80%, 100% and 120% recovery levels of working concentration in triplicate. The spiked sample solution was injected in HPLC system and analysed by the proposed method. The percentage recoveries were calculated against respective levels and mentioned in Table 5.

Limit of detection (LD) & Limit of Quantification (LQ)

LD and LQ were mathematically determined through the calibration curve of predicated linearity. LD and LQ were calculated using the following equation as per ICH guidelines.

$$LD = 3.3 \times \sigma/S; \qquad LQ = 10 \times \sigma/S$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve. Results are shown in table 5.

TABLE 5 SUMMARISED RESULTS OF VALIDATION STUDY

Sr. No.	Parameters	Acceptance Criteria	Result
1	**Specificity**	No interference for Analyte peak	Specific
2	**Linearity**	$R^2 > 0.98$	0.999
3	**Precision**		
3.1	System precision RT, TF, PC, PA	% RSD <2.0	1.36, 1.401, 0.574, 0.50
3.2	Method precision	% RSD <2.0	0.3038
3.3	Intermediate precision	% RSD <2.0	0.276
4	**Accuracy**		
4.1	80 %	Average % Recovery should be in the range of 98% - 102 %	100.66
4.2	100 %		100.58
4.3	120 %		101.46
5	**Robustness**	System suitability parameters should comply	Robust

Forced degradation study

Various Conditions used for stability study of GA. The forced degradation was carried out to achieve enough degradation. The following degradation behaviour of the drug was observed during the stress degradation studies. Summarised results of forced degradation study are shown in table 6.

Acidic condition

Two impurities are found to be generated in acidic solution. Those impurities were separated from the GA peak by proposed HPLC method and the RT of impurity I is found to be at 11.51 and impurity II at 18.56 along with GA peak at 16.86 minutes as observed in fig 6.

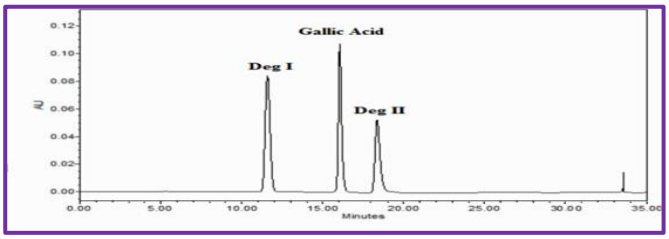


Figure 6 Chromatogram of acid hydrolytic sample (1 N HCL)

Alkaline condition

Two impurities are found to be generated in alkaline solution. Those two impurities were separated from peak of GA given HPLC method and the RT of Impurity I is found to be at 18.96 and RT of Impurity II is found to be at 20.87 minutes. GA peak was obtained at 16.83 minute as observed in fig 7.

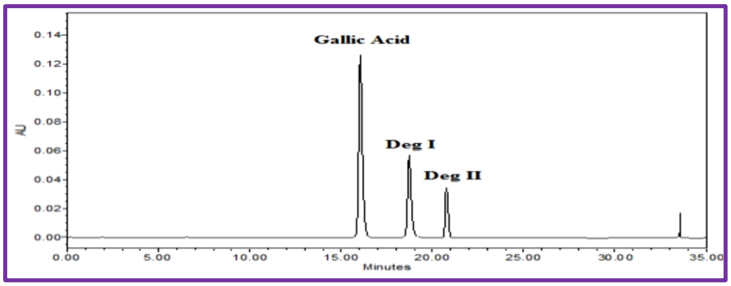


Figure 7 Chromatogram of alkaline hydrolytic sample (0.1 N NaOH)

Oxidative condition

One impurity is found to be generated in H_2O_2 solution. This impurity was resolved well by the developed HPLC method. Impurity was observed at RT Impurity I – 23.16 and GA at 16.83 min as seen in fig 8.

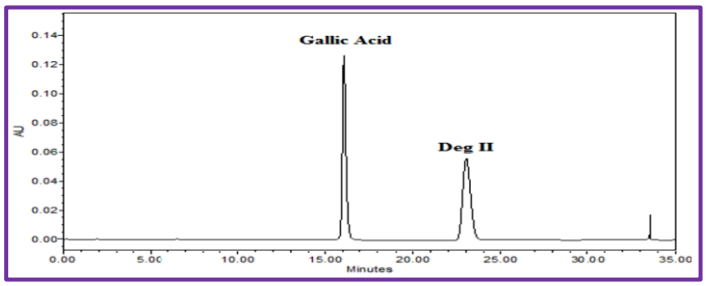


Figure 8 Chromatogram of oxidative stressed sample (0.3 % H_2O_2)

Thermal degradation

Gallic acid was found thermally stable. Impurities were not found at this stress condition as confirmed from fig 9.

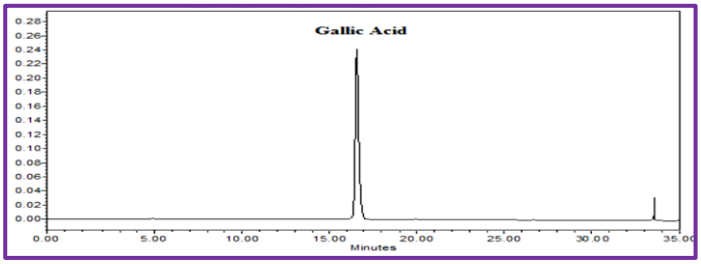


Figure 9 Chromatogram of thermally degraded sample

Photo Stability condition

In photolytic solution of Gallic acid was found stable. Impurities were not found at this stress condition and confirmed from fig 10.

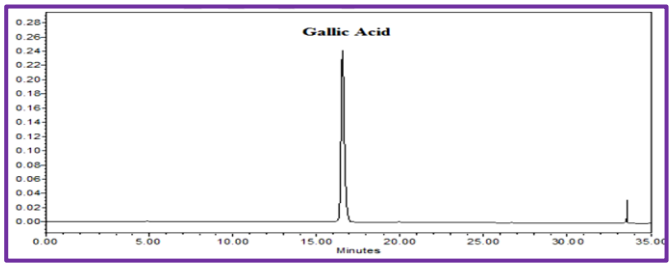


Figure 10 Chromatogram of photo degraded sample

GC-MS analysis

Reference mass spectrum of Gallic acid contains parent peak at m/z 310 and major fragments peak at m/z 294, 280, 266,245,230, 217,108 and 41(Fig 11).

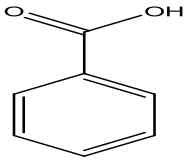
Acidic Hydrolytic Sample

Two additional peaks were observed in chromatogram at RT 2.246 and 2.413 referred as Degradation product –I (DP-I) and Degradation product-II (DP-II) peak respectively.

Mass spectrum of DP-I (Fig 12) contains parent peak at m/z 120. Major fragments were observed at m/z 105,73 and 59. A mass spectrum of DP-II (Fig 13) contains parent peak at m/z 84. Major fragments were observed at m/z 69,56 and 41.

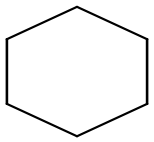
Mass spectrum of degradation products revealed enough information about the structure. From m/z value of parent peak and fragment peak, degradation products were characterised as Benzoic acid (DP-I) & Cyclohexane (DP-II)

Predicted structures of degradation products were given as below



benzoic acid

Structure 2. DP-I



cyclohexane

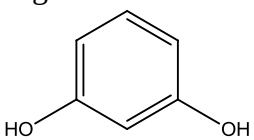
Structure 3. DP-II

Alkaline Hydrolytic Sample

Two additional peaks were observed in chromatogram at RT 1.980 and 4.031 referred as Degradation product –I (DP-III) and Degradation product-II (DP-IV) peak respectively.

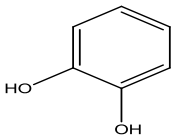
Mass spectrum of DP-III (Fig 14) contains parent peak at m/z 105. Major fragments were observed at m/z 75 and 59. A mass spectrum of DP-IV (Fig 15) contains parent peak at m/z 105. Major fragments were observed at m/z 91, 75, 61 and 45.

Mass spectrum of degradation products in alkaline medium revealed enough information about the structure. From m/z value of parent peak and fragment peak, degradation products were characterised as Resorcinol (DP-III) & Pyrocatechol (DP-IV) Predicted structures of degradation products were given as below



resorcinol

Structure 4. DP-III



pyrocatechol

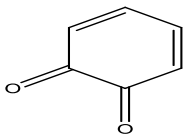
Structure 5. DP-IV

Oxidative Degradation Sample

One degradation product was observed in the chromatogram of Gallic acid subjected to oxidative stress conditions. Mass spectrum of DP-V contains parent peak at m/z 105. Major fragments were observed at m/z 59 and 45.

Mass spectrum of degradation product V (Fig 16) in H_2O_2 revealed enough information about the structure. From m/z value of parent peak and fragment peak, degradation products were characterised as cyclohexa-3,5-diene-1,2-dione

Predicted structures of degradation products were given as below



cyclohexa-3,5-diene-1,2-dione

Structure 6. DP-V

TABLE 6 SUMMARISED RESULTS OF FORCED DEGRADATION STUDY

Stress Conditions	Strength	Temp. ^0C	Time Hrs	Degradation % w/w
Acidic degradation	1 NHCL	Room Temp.	24	69.53
Alkaline degradation	0.1NNaOH	Room Temp.	24	37.61
Oxidative degradation	0.3 % H_2O_2	80	8	19.04
Thermal degradation	----	110	12	No degradation
Photolytic degradation	ʎ=254 nm	----	12	No degradation

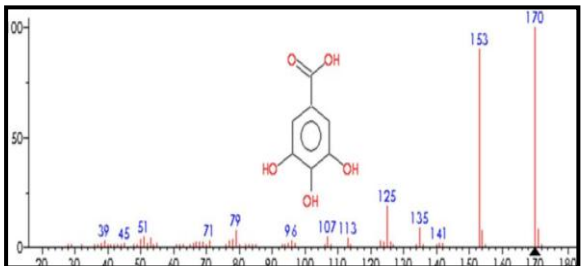


Figure 11 Mass Spectrum of Std. GA

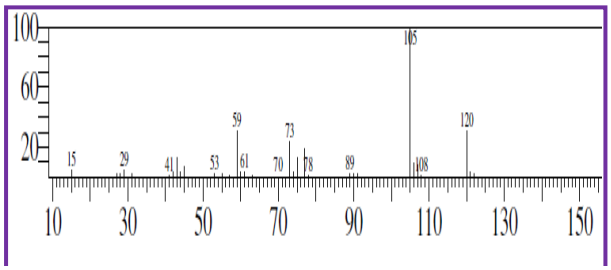


Figure 12 Mass Spectrum of DP-I in 1 M HCl

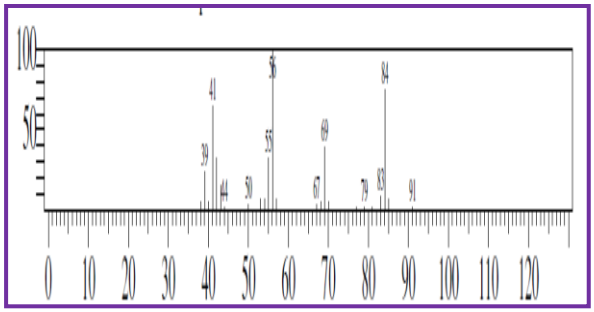


Figure 13 Mass Spectrum of DP-II in 1 M HC

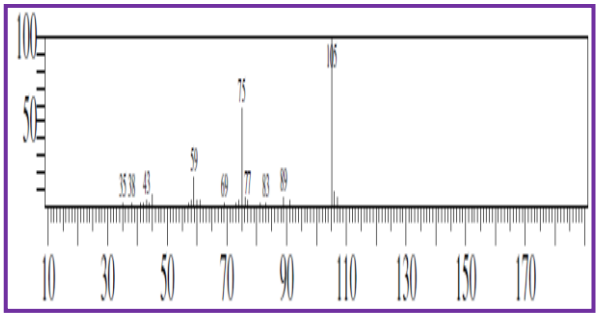


Figure 14 Mass Spectrum of DP-III in 0.1 N NaOH

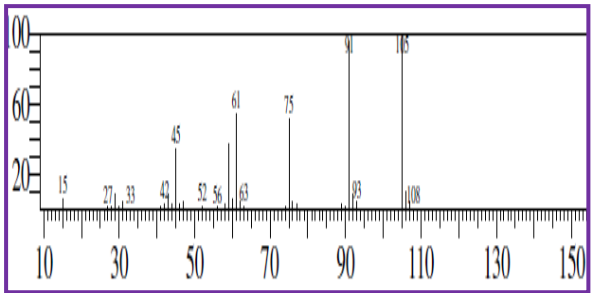


Figure 15 Mass Spectrum of DP-IV in 0.1 N NaOH

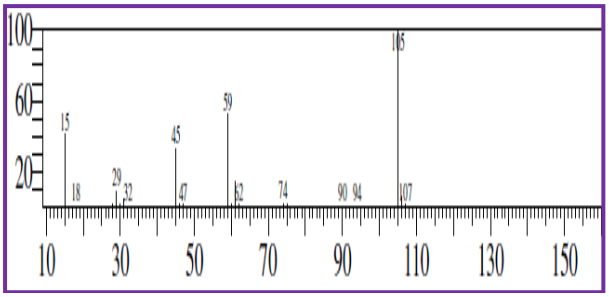


Figure 16 Mass Spectrum of DP-V in 0.3 % H_2O_2

CONCLUSION

Simple, sensitive, rapid, robust, precise and accurate rp-hplc stability indicating method has been developed and validated as per ICH Q1B. The results of stress testing are analysed in accordance with ICH principles, revealing that the procedure is both specific and stable. Application of this method for the analysis of Gallic acid exposed to different stress conditions shows that degradation products did not interfere with the analytical determination. The proposed method was effectively utilised, and recommended for estimation of GA in pharmaceutical formulations for quality control when economy and speed are critical, and therapeutic efficacy is assured.

CONFLICT OF INTEREST: Authors declare no conflict of interest.

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