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Development and Validation of Stability Indicating HPLC Method for Epigallocatechin Gallate (EGCG)

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Abstract : A new, economical, precise and accurate stability indicating HPLC method wasdeveloped and validated for the EGCG as per ICH guidelines. The study deals with development and validation of HPLC method for estimation of EGCG. Chromatographic separation was performed on C_{18}column fitted with C_{18} guard column using mobile phase Methanol: Acetic acid (0.1%)(75:25v/v). The wavelength used for detection was 276 nm. Regression plots revealed a linearrelationship in the concentration range of 20-120 µg/ml. The retention time for EGCG was found to be 5.3min.TheLOD and LOQ were found to be5.07 and 15.27 µg/ml respectively. The method was validated as per International Conference on Harmonization (ICH) guidelines, demonstrating to be accurate and precise within the corresponding linearity range of titledanalytes. Stability of the drug was studied by exposing drug to acid, alkali, oxidative, photolytic and thermal conditions. Relevant degradation was found to take place under theseconditions. The proposed method has been validated as per ICH Q2 (R1) guidelines. This methodcan be used for routine quality control analysis of EGCG.

Keywords : Validation, Epigallocatechin Gallate (EGCG), HPLC, Stability indicating method.

Introduction

Epigallocatechin Gallate (EGCG) is a herbal extract with anticancer, antioxidant, neuroprotective activity. The chemical name of EGCG is (2R,3R)-5,7-dihydroxy-2-(3,4, trihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-13,4,5trihydroxybenzoate(Fig.1).EGCG is a faint pink powder which is soluble in water, methanol. The molecular formula is $C_{22}H_{18}O_{11}$andthemolecularweight is 458.375 g/mol[1-2].

Literature survey revealed few Spectrophotometric, UV spectrometry[3-4], RP-HPLC[5],HPTLC[6], LC-MS[7], LC-MS/MS[8] methods for the determination of various catechin derivatives. But, to the best of our knowledge, a simple, rapid and economical method for estimation of EGCG by HPLC for routine laboratory analysis is not yet reported. So, the aimof the present work was to develop and validate stability indicating HPLC method fordetermination and quantitative estimation of EGCG.

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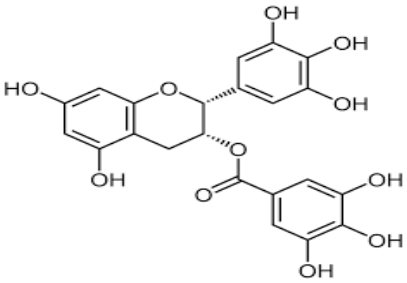

Figure 1: Structure of Epigallocatechin Gallate (EGCG)

Experimental

Instrumentation

HPLC system consisted of Pump (Jasco PU2080 Intelligent HPLC pump)with Auto sampler(JascoAS-4050 HPLCAuto sampler) programmed at 20µl capacity per injection. UV/VIS detector was used (Jasco UV-2075 Intelligent UV/VIS detector).Data was integrated using Chrom NAV software, LC-Net-II/ADC system. The column used was CHROMATOPAK C_{18}column(PEERLESS BASICC_{18}COLUMN, 5µM, 250 MM x 4.6I.D.) fitted with C_{18} guard column (HYPERSIL BDSC_{18}),Syringe filter was used for sample filtration (0.45 µm). Ultrasonic bath and Electronic Balance Shimadzu AX200 were used in the study.

Chemicals and reagents

Gift sample of EGCG was provided by Cipla Ltd., Mumbai. Methanol, acetic acid of HPLC grade was purchasedfrom Merck Specialties Pvt. Ltd., India. Double distilled water was used for mobile phase preparation.

Preparation of stock solution

The stock solution was prepared by weighing 10mg of EGCG accurately then transferred into a 10 ml volumetric flask and dissolved in methanol. Volume was adjusted to 10ml with methanol. Then sonication was done for 20 min. Different concentrations (10 and 100µg/ml) of standard solutions were prepared by dilution of the stock solution using methanol.

Selection of wavelength

The evaluation was performed by using methanol as a blank solution. The solution was scanned using JascoV-630 spectrophotometer between the wavelength range of 400 nm to 200 nm thentheUV spectrum of 10µg/ml solution of EGCG was taken; it showed maximum absorbance at 276 nm. The spectrum of the drug is depicted in (Fig. 2).

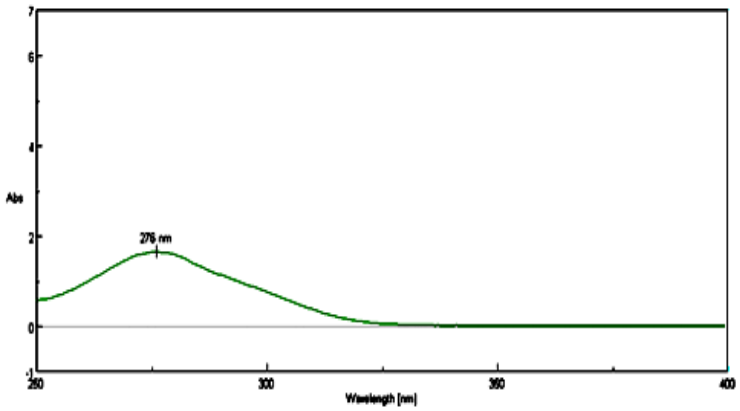

Figure 2: UV Spectrum for selection of wavelength

Selection of mobile phase

Optimization of mobile phase was done based on trial and error method. To increase sharpness various acids were added. The mobile phase was filtered through a 0.45µm membrane and sonicated prior to use.

Method validation

The method was validated for linearity, accuracy and intra-day and inter-day precision, robustness, recovery in accordance with ICH guidelines Q2 (R1)[9-11].

Linearity

EGCG solution (1000µg/ml) was used to prepare various concentrations. From this different volume were accurately transferred to 10 ml volumetric flask and diluted up to the mark to get 20-120 µg/ml concentration range. Six different concentrations of EGCG solutions (20, 40, 60, 80, 100, 120µg/ml) were prepared and injected in triplicate. From this peak area was calculated and the graph was plotted of peak area vs concentrations. The linearity of the method was evaluated by linear regression analysis, using the least square method. The slope and intercept were calculated.

Recovery study

Recovery studies were performed by the standard addition method. An appropriate amount of standard substance was spiked to the predetermined concentrations in triplicates. Then resulting mixtures were analyzed by injecting into the HPLC. Recovered amount of EGCG was estimated by %RSD (relative standard deviation) and the mean recovery was calculated.

Precision

The precision of the proposed analytical method was demonstrated by repeatability (Intra-day) and intermediate (Inter-day) precision studies. The intra and inter-day variations were determined using three different concentration levels 60, 80 and 100 µg/mlof EGCG (n = 3). The precision of the developed method was evaluated by performing the repeatability of the sample application and peak area measurement in six replicates of the same spot. The results are expressed in terms of percent relative standard deviation (% RSD).

Method sensitivity (Limit of detection and limit of quantification)

The LOD and LOQ of the method were calculated using the regression equation. Preparations containing 20-120 µg/ml were prepared and injected. Calibration graph was plotted for the obtained peak area against concentration. The LOD and LOQ were calculated using the equations,

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \qquad \mathrm{LOD} = \frac{10\sigma}{S}$$

respectively where σ is the standard deviation of the y-intercept and S is the slope of the calibration carried out. Thus, the obtained LOD and LOQ values were confirmed by injecting different concentrations of a stocksolution of 10µg/ml of EGCG in triplicates, % RSD values were calculated.

Robustness

In robustness, small changes were made in various parameters like the ratio of the mobile phase, flow rate and detection wavelength. Only one factor was changed at a time and the effect on the t_R and the peak area of the drug were studied and expressed in terms of % RSD.

Forced degradation studies[12,13]

Forced degradation studies were carried out by exposing EGCG solution to stressed conditions. A stock solution of 10 mg of extract was prepared by dissolving it in 10ml of methanol and exposed to a stress condition. Forced degradation was carried out to evaluate the stability of the developed method and for effective separation of drug from degradants.

Acid and alkali hydrolysis

To 5 ml solution of EGCG 5 ml of (0.1 N HCl) and (0.01 N NaOH) were added separately. For acid induced degradation mixture was refluxed separately for up to 6 h at 80°C.For alkali induced degradation mixture was refluxed separately for up to 1.5h at 80°C.100µl of resultant solutions were injected into the column.

Hydrogen peroxide induced degradation

To 5 ml solution of EGCG, 5 ml of 3%H_2O_2 was added. This solution was heated in a boiling water bath for 20 min to remove the excess of hydrogen peroxide completely and refluxed for 2 h at 80°C. 100µl of resultant solutions were injected into the column.

Dry and Wet heat induced degradation

Dry heat degradation of EGCG was carried out by placing the drug into a hot air oven at 80ºC for 8 h.10 mg of EGCG powder was spread as a thin layer on petridish (50 mmdiameter). The petridish was heated in an oven at 80°C for 6 h.

For wet heat induced degradation, 5 ml of working standard solution of EGCG, 5 ml of HPLC grade methanol was added. Thesolution was refluxed at 60°C for 6 h. The resultant solutions were injected into the column.

Photolytic degradation

A thin layer of 10 mg of EGCG powder was spread on a petri dish (50 mm diameter). Petridish was exposed to direct sunlight for 24 h in the chamber.

Results and Discussion

The mobile phase consisted of methanol: 0.1 %acetic Acid (75:25v/v) showed the symmetrical peak. Retention time (t_R) was found to be 5.3 min at the flowrate of 0.5ml/min(Fig 3). The EGCG showed 276 nm as the detection wavelength.

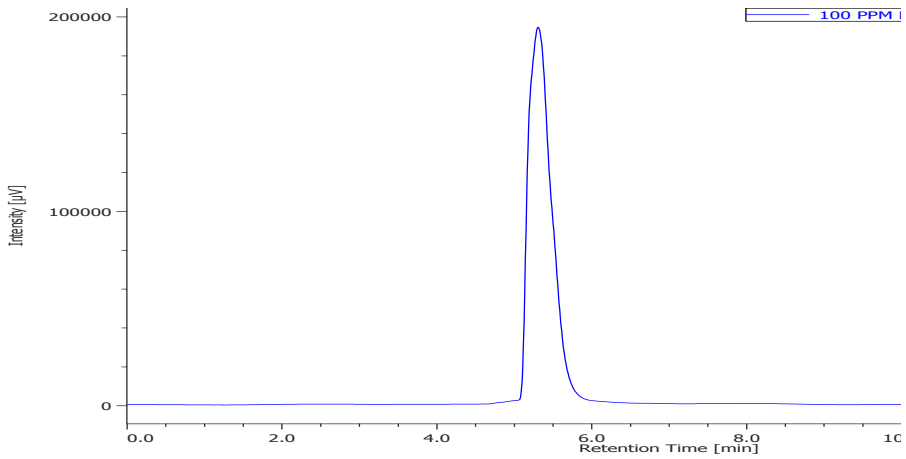

Figure 3: Chromatogram of EGCG

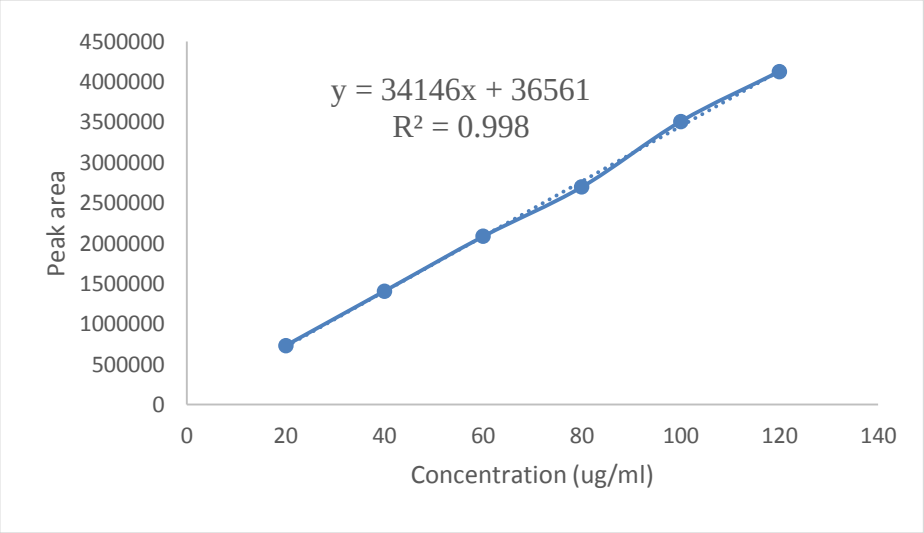

Figure 4: Linearity curve for EGCG

A calibration curve was plotted by mean of peak area versus concentration. For plotting calibration curve concentrations were taken in the range of 20-120 µg/ml. The data of the regression analysis of the calibration curve is shown in (Fig. 4)

The mean recovery and RSD values were calculated. Recoveries of EGCG were in between 97-102 % (Table 1). A result complies with the ICH criteria; hence method was found to be accurate.

Intra-day and inter-day precision were conductedunderdeveloped chromatographic conditions. %RSD was found less than 2, so the method was found to be precise(Table 2).

LOD and LOQ for EGCG were found to be 5.07and 15.27µg/ml respectively.

The results of the robustness study are summarized in (Table 3)which showed the developed HPLC method was robust. Summary of validation parameters is given in (Table 4).

Table 1: Accuracy data for EGCG

Conc. (µg/ml)	% Amount Added	Total Amount (µg/ml)	Mean	Amount Recovered(µg/ml)	% recovery
60 µg/ml	80	108	5397881.33	108.09	100.09
	100	120	5666023.67	116.44	97.04
	120	132	6110737.33	130.29	98.71
80 µg/ml	80	148	7705913.67	144.16	97.40
	100	160	8221469.67	159.16	99.47
	120	176	8978414.00	181.18	102.94
100 µg/ml	80	180	8563339.33	178.84	99.35
	100	200	9140347.00	197.63	98.81
	120	220	9941184.00	223.71	101.68

Table 2: Precision data for EGCG

Conc. (µg/ml)	Intraday precision (n=3)		Interday Precision (n=3)	
	Mean ±SD	% RSD	Mean ±SD	% RSD
60	2080889 ± 31391.24	1.50	2110355 ± 39408.72	1.86
80	2655378 ± 35534.00	1.33	2734925 ± 46189.35	1.68
100	3271837 ± 42544.81	1.30	3323791 ± 48984.14	1.47

Table 3: Robustness data for EGCG

Parameter	Retention time(min)	Mean ±SD	%RSD
Flow rate (ml/min)			
0.5	5.3	2933153 ± 24622.27	0.83
0.3	8.9	4570080 ±22769.47	0.49
0.7	3.8	2018712 ± 10790.31	0.53
Detection wavelength (nm)			
276	5.3	2933153 ± 24622.27	0.83
274	5.3	2816774 ± 21064.01	0.74
278	5.3	2858964 ± 37039.49	1.29
Mobile phase composition			
75: 25	5.3	2933153 ± 24622.27	0.83
70: 30	5.0	3148183 ± 21812.48	0.69
80: 20	5.5	2771991 ± 21811.99	0.78

Table 4: Summary of the validation parameters

Parameter	Result
Linearity range (µg/ml)	20-120
Correlation co-efficient	0.998
Precision (intraday) % RSD	1.38
Precision (interday) % RSD	1.67
Accuracy (mean % recovery)	99.49
LOD (µg/ml)	5.07
LOQ (µg/ml)	15.27
Robustness	Robust

Forced degradation studies

EGCG was found to be degraded to hydrolytic, oxidative, wet heat condition. EGCG showed maximum degradation under basic condition and was found to be stable in dry heat and photolytic condition.

EGCG was degraded in acid hydrolysis and the chromatogram of acid degraded sample showed one additional peak at t_R4.2 (Fig 5A) and chromatogram of base degraded sample showed two additional peaks at t_R of 7.4 and 9.8 min respectively (Fig5B). In oxidation and wet heat condition the extract showed on additional peak at t_R of 3.1 and3.8 min respectively(Figure 5 C& D).No degradation was found in dry heat and photolytic degradation condition (Figure 5 E& F).

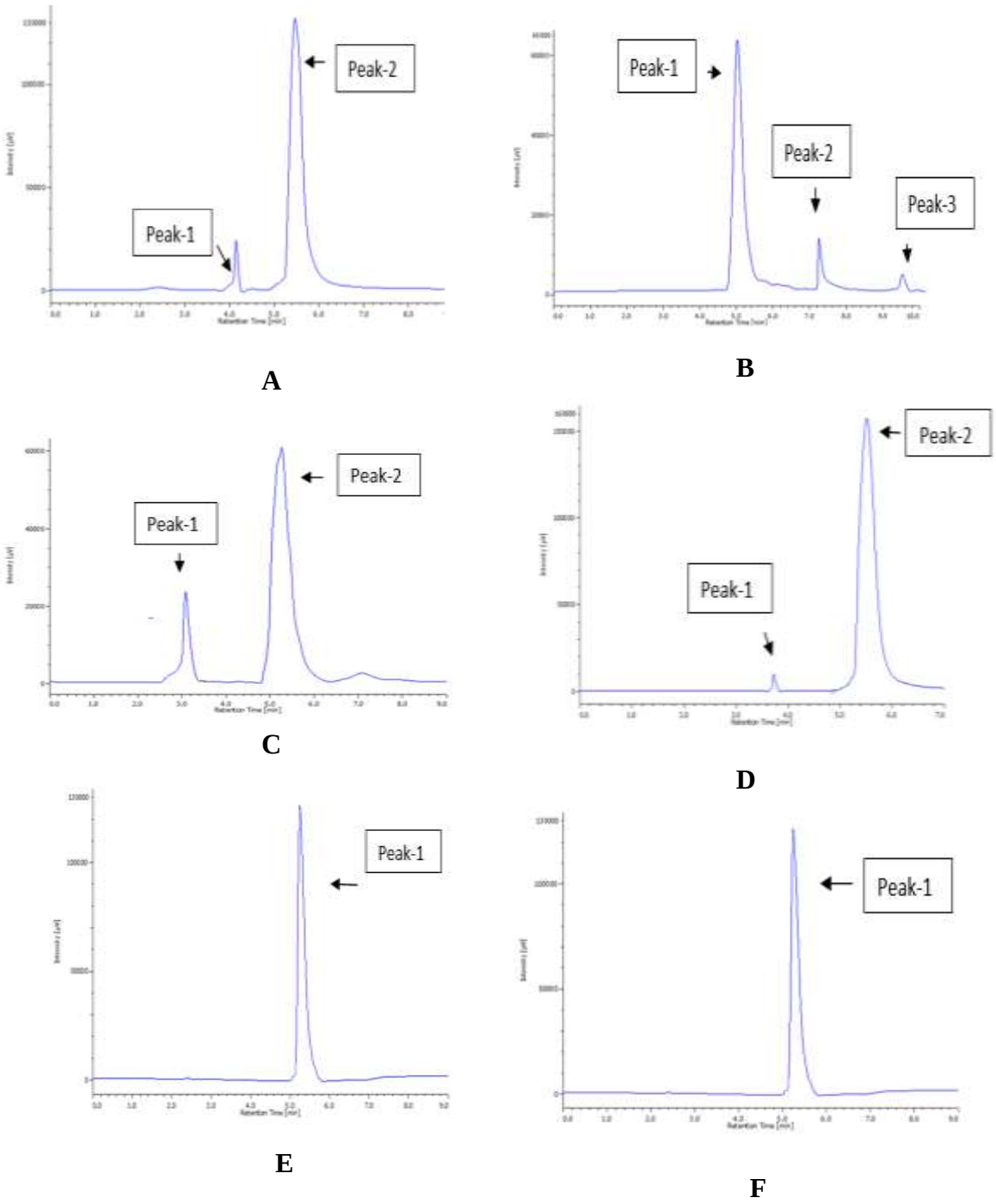

Figure 5: Chromatogram of Degradation study (A - Acid induced degradation condition, B - Alkali induced degradation condition, C - Oxidation Degradation condition, D - wet heat degradation condition, E – Dry heat degradation condition, F – Photochemical degradation condition)

Percent degradation was calculated by comparing the areas of the degraded peaks in each degradation condition with the corresponding areas of the peaks under non-degradation condition. Summary of degradation studies is given in (Table 5).

Table 5: Summary for forced degradation study

Degradation condition	**Time**	**% degradation**	t_R
Acid ,(0.1 N HClrefluxation at 80ºC)	6 h	4%	4.2
Base, (0.01 N NaOH refluxed at 80ºC)	30 min	13%	a.7.4, b. 9.8
Hydrogen peroxide, 3% v/vH2O2 (refluxed at 80ºC)	90 min	7%	3.1
Wet heat (at 80ºC)	6 h	0.2%	3.8
dry heat (at 60 °C)	6 h	-	ND
Photochemical stability (Daylight)	24h	-	ND

In the proposed study, stability indicating HPLC method was developed and validated as per ICH guideline for quantitation and estimation of EGCG. It enables satisfactory resolution of Epigallocatechin Gallate from its degradants and is simple, linear, accurate, precise and reproducible. The proposed method can be regarded as simple, rapid (since it has reduced analysis time), economical and suitable for estimation of Epigallocatechin Gallate for stability study and routine analysis in various pharmaceutical dosage forms in industry.

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Conflict of Interest:

NA

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