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Development and Validation of New Analytical methods for Simultaneous estimation of Epigallocatechin gallate, a component of Green Tea extractand Niacin in a Pharmaceutical dosage form

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ABSTRACT

An analytical method developed for simultaneous estimation of Epigallocatechin gallate (a component of Green Tea Extract) and Niacin in a pharmaceutical dosage form in accordance to International Conference on Harmonization guidelines. The method was developed with the help of Ultra Performance Liquid Chromatography and the separation technique which we adopted for the above two components is by gradient method. Two mobile phases used for dilution and to elude the components in chromatography termed as Mobile Phase A and Mobile Phase B. Mobile Phase A comprises of 3.5 mL Orthophosphoric acid, 50 mL Methanol which is diluted to 1000 mL with Water. Mobile Phase B comprises of Acetonitrile and Methanol in ratio of 95:5 (%v/v). Peak areas were recorded for both Epigallocatechin gallate (a component of Green Tea Extract) and Niacin at 278nm and retention time was found to be 2.170 min for Epigallocatechin gallate and 0.255 min for Niacin respectively. The flow rate was maintained at 0.5mL/min during the separation. The developed analytical method has been validated for its linearity, accuracy and precision. The linearity of the analytical method developed was found to be in range of 160 µg $mL^{-1}$to 240 µg mL^{-1} for Niacin and 80 µg $mL^{-1}$to 120 µg mL^{-1} for Epigallocatechin gallate.

Keywords: Niacin;Green Tea Extract; Epigallocatechin gallate; UPLC.

INTRODUCTION

Niacin also known as Vitamin B_3 is chemically termed as pyridine-3-carboxylic acid **(Figure-1)**derivative of pyridine, with a carboxyl group for cardiovascular and atherogenic dyslipidemia [1-4].

Green Tea Extract (GTE) is an herbal extract which contains Green Tea Catching (GTC) which is the prime response for its antioxidant property. The GTC comprise of four major derivatives which includes Epicatechin (EC), Epigallocatechin (EGC), Epicatechin gallate (ECG), and Epigallocatechin Gallate (EGCG) of which, EGCG accounts for more than 40% w/w of the total content [5-6].

EGCG also known as epigallocatechin-3-gallate**,** is chemically termed as [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate **(Fig. 2)** is the ester of Epigallocatechin and Gallic acid.

Literature review reveals that many methods have been reported for the analysis of Niacin by High Performance Liquid Chromatography (HPLC) but there were no analytical method developed for a pharmaceutical dosage form using Ultra Performance Liquid Chromatography (UPLC). UPLC was utilized in a patent for the determination of niacin in blood plasma [7]. Several analytical methods that have been reported for estimation of Epigallocatechin gallate, a component of GTE using HPLC [8] but there were no method developed for a pharmaceutical dosage form using UPLC.

The purpose of this research was to establish and validate, in accordance with ICH guidelines, an accurate, economical and reproducible procedure for quantitative analysis of Epigallocatechin gallate, a component of GTE and Niacin as the bulk drug and in tablet dosage forms. It was thought worthwhile to develop precise, accurate UPLC method for simultaneous determination of Epigallocatechin gallate, a component of GTE and Niacin in a tablet dosage form.

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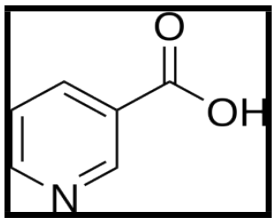

Fig. 1: Chemical structure of Niacin

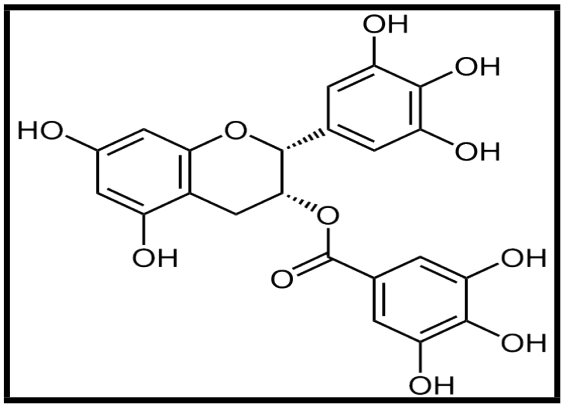

Fig. 2: Chemical structure of Epigallocatechin gallate

EXPERIMENTAL

Chemicals, reagents and samples:

Tablet dosage form containing 500 mg of Niacin and 500 mg of GTE (equivalent to 250mg of Epigallocatechin gallate) was used. UPLC grade Acetonitrile, Methanol and Orthophosphoric acid. Nylon membrane filter (0.22µm), PVDF syringe filter (0.22µm) and Nylon syringe filter (0.22µm) were from J.T. Baker, USA. All the chemical and reagents used were of UPLC grade and purchased from J.T. Baker, USA. Water from MilliQ.

Reverse phase ultra-performance liquid chromatographic method:

A reverse phase ultra-performance liquid chromatographic method was developed for the simultaneous estimation of Niacin and Epigallocatechin gallate, a component of GTE in tablet formulation.

1. Instrument:

The UPLC system is equipped with detector consists of TUV detector. Separation was carried out on AcQuity BEH C18 2.1 X 50 mm; 1.7 µm under reversed phase partition chromatographic conditions. The equipment was controlled by a PC workstation. The work was carried out in an air-conditioned room maintained at temperature 25°C. Chromatograms were recorded using Empower 2 software (Waters, USA).

2. Chromatographic conditions :

The optimal composition of the mobile phase was determined to be 3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted further to 1000 mL with Water (MilliQ) is used as a Mobile Phase A and Acetonitrile : Methanol (95:5, % v/v) as Mobile Phase B. The flow rate was set to 0.5 mL min^{-1} and UV detection was carried out at 278 nm. The mobile phase was filtered through 0.22 µm nylon membrane filter and was degassed before use. Stock solution was prepared by dissolving Niacin (100 mg) and EGCG (50 mg) that were weighed accurately and transferred into 100 mL volumetric flasks. Both drugs were dissolved in Mobile phase B, after the immediate dissolution, the volume was made up to the mark with the same Mobile Phase B. The standard stock solution is observed to contain 1000 µg mL^{-1} of Niacin and 500 µg mL^{-1} Epigallocatechin gallate. From the above stock solutions, dilutions were made in the concentration range of 160 – 240 µg mL^{-1} of Niacin and 80 – 120 µg mL^{-1} of Epigallocatechin gallate, respectively. A volume of 1 µL of sample was injected into column.

Optimization of chromatographic conditions

The primary target of developing the UPLC method is to achieve simultaneous determination of EGCG and Niacin in oral formulation under common chromatographic condition those are applicable to routine quality control of products in pharmaceutical industries.

The optimal composition of the mobile phase was determined to be 3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted further to 1000 mL with Water (MilliQ) is used as a Mobile Phase A and Acetonitrile : Methanol (95:5, % v/v) as Mobile Phase B. The flow rate was set to 0.5 mL min-1 and UV detection was carried out at 278 nm. The mobile phase was filtered through 0.22 µm nylon membrane filter and was degassed before. The Niacin and EGCG chromatogram obtained at the 278 nm.

Evaluation of the Niacin and EGCG by UPLC was indispensable to define certain parameters. By this means, the UV spectra of the Niacin and EGCG peaks were obtained. Comparison of these spectra indicated that these compounds showed two bands that were very similar to the profile found for the Niacin and EGCG standards.

The Waters AcQuity BEH C18 2.1 x 50 mm; 1.7 µm was tested in an attempt to decrease the time required and the volume of solvent used during the analysis. However, under condition G of **Table-1** it was not possible to obtain separation of Niacin and EGCG, and therefore this column was not used for the subsequent analysis.

Table No. 1: Mobile Phases flows tested in separation of Niacin and EGCG.

Condition	Phase A	Phase B	Flow (mL min^{-1})
A	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(95:5)	0.8
B	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(80:20)	0.8
C	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(85:15)	0.5
D	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(95:5)	0.5
E	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(75:25)	0.5
F	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(90:10)	0.5
G	3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted 1000 mL water	Acetonitrile: Methanol(70:30)	0.5

In the proposed method for simultaneous estimation of Epigallocatechin gallate, a component of GTE and Niacin in tablet dosage, an adequate separation of eluted compounds was optimized. Mobile phase and flow rate selection were based on peak parameters (tailing, theoretical plates, capacity factor), run time etc. Several aliquots of standard solutions of Niacin and EGCG were taken in different 10 mL volumetric flasks and diluted up to the mark with a mobile phase, The optimal composition of the mobile phase was determined to be 3.5 mL of Orthophosphoric acid in 50 mL Methanol which is diluted further to 1000 mL with Water (MilliQ) is used as a Mobile Phase A and Acetonitrile : Methanol (95:5, % v/v) as Mobile Phase B (Condition D). The flow rate was set to 0.5 mL min^{-1} and UV detection was carried out at 278 nm which better detector response for drugs was obtained. The average retention times for Niacin and EGCG were found to be 0.255 min and 2.170 min, respectively. The peak shapes of both the drugs were symmetrical and the asymmetry factor was lesser than 2.0.

The proposed method was validated as per the standard analytical procedures. Each of the samples was repeated 6 times and the same retention time was observed in all the cases. Precision of proposed UPLC method was found to be 0.39 % (RSD) for Niacin and 0.46 % for EGCG that indicate good precision of the samples analyzed. The correlation coefficient 'r' values (n = 6) for both Niacin and EGCG were > 0.995. Accuracy of the method was calculated by recovery studies (n= 3) at three levels. The method was found to be accurate and precise as indicated by results of recovery studies and % RSD not more than 2%. The mean recoveries obtained for Niacin and EGCG were 100.69% and 100.35 %, respectively. Repeatability, precision and ruggedness of the method seem to be well within the limit.

Analytical Method Validation:

For validation of the analytical method, the guidelines established by the ICH (International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) and by Brazilian regulation RE 899/2003 of the National Health Surveillance Agency (ANVISA) were employed [9-12].

1. Precision:

The precision of the method was determined following ICH guidelines. Precision was evaluated at three levels: repeatability, intermediate precision, and reproducibility. The standard deviation (SD) and relative standard deviation (RSD) of six preparations with two injections each at 100% of the test concentration were evaluated and analyzed intra-day and inter-day, and with different analysts and different instruments and columns. The results were tabulated as given below **(Table-2)**. From the results we came to know that the method is precise. The RSD values are well within the limit.

Table No. 2: Method Precision and Intermediate Precision results

Components	Precision (Day-1)		Intermediate precision (Day-2)	
	Analyst 1		Analyst 2	
	% Assay#	% RSD*	% Assay#	% RSD*
EGCG	100.21	0.46	99.76	0.67
Niacin	99.98	0.39	100.13	0.98

Average of six determinations; * Determined on six values

2. Specificity:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and placebo matrix. Forced degradation studies were performed to demonstrate selectivity and stability indicating capability of the proposed Reverse phase ultra-performance liquid chromatographic method [13].

3. Accuracy:

The accuracy was determined by recovery analysis, adding measured known amounts of Niacin and EGCG working standard to know quantity of placebo. Recovery experiments were carried out by standard addition technique. Three different concentration levels 80%, 90% & 120% of the test concentration in the method of analysis. The recovery experiments were performed in triplicate for the both preparation. The mean of percentage recoveries (n=3) and RSD was calculated.The recovery data were determined by dividing the value obtained for the sample prepared with the added standard, by the amount added, and then multiplying by 100%. The result of recoveries for assay is shown in **Table-3**.

Table No. 3: Accuracy results

Components	Parameters	At 80 %	At 100 %	At 120 %
Niacin	% Recovery#	101.23	100.26	100.58
	% RSD*	0.60	0.95	0.13
EGCG	% Recovery#	99.29	100.51	101.25
	% RSD*	0.79	0.60	1.20

* Determined on three value; # Mean of the three determinations.

4. Linearity:

Linearity was determined by the calibration curves obtained from the UPLC analysis of the standard solutions of Niacin and EGCG. The range (interval between the upper and lower concentrations of analyte in the standard) of the appropriate amount of standard was determined. The slope and other statistics of the calibration curves were calculated by linear regression and analysis of variance (ANOVA). The Niacin and EGCG standards were dissolved in Mobile Phase B to get concentrations of 80%, 90%, 100%, 110% and 120% of Niacin and EGCG, a component of GTE. The solutions were filtered through 0.22 µm membrane filter. Evaluation of each point was conducted in replicates, and the calibration curve was fitted by linear regression.

Then linearity of an analytical method is its ability to elicit test results that are directly or by a well mathematical transformation, proportional to the concentration of analyte. The Response was found linear for niacin and EGCG of standard concentration and correlation coefficient was also found greater than 0.999.The result of correlation coefficient, Y-intercept of the calibration curve niacin and EGCG are presented in the **Table-4**.

5. Limit of detection and limit of quantification:

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curve based on equations (1) and (2) and the results were presented in the **Table-4.**

$$LOD = (3.3 \times SD) / S \quad \ldots\ldots (1)$$
$$LOQ = (10 \times SD) / S \quad \ldots\ldots (2)$$

Table No. 4: Evaluation of LOD, LOQ and linearity data

Parameter	Niacin	EGCG
LOD (µg/mL)	0. 074	0.038
LOQ (µg/mL)	0.26	0.12
Linearity Range (µg/mL)	160-240	80-120
Correlation coefficient	0.9998	0.9999
Intercept (a)	112.558	99.364
Slope (b)	1245.563	18962.3

6. Robustness:

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was studied by testing influence of small changes in flow rate (0.49 mL min^{-1} and 0.51 mL min^{-1}), change in column oven temperature (25 ± 5°C). In system suitability parameters such as theoretical plates, tailing factor and %RSD of Niacin and EGCG standards were studied.

7. Stability of analytical solutions:

The stability of the working solutions was determined over a period of 2 days at ambient temperature. Powdered tablet was re-analyzed every 24 hours time intervals and assay was determined against the standard solution which was initially injected. The variability in the assay of all two substances was within ± 1% during solution stability. The results from solution stability experiments confirmed that sample solution was stable for up to 48 hr during assay determination which are presented in **Table-5**.

Table No. 5: Solution stability results

% Assay	Initial	After 24 hrs.	After 48 hrs.
Niacin	100.35	100.22	100.50
EGCG	100.12	99.96	100.26

Analysis of final formulation:

The retention time of Niacin and EGCG a component of GTE was found to be 0.255 min and 2.170 min, respectively. According to the United States Pharmacopeia, system suitability tests are integral part of liquid chromatographic methods. Retention time, capacity factor, number of theoretical plates, and asymmetry factor were calculated for standard solutions. The values for resolution, number of theoretical plates, retention time, resolution and peak asymmetry were calculated for the working standard solutions and it is represented in **Table-6** and **Table-7**. The values obtained demonstrated the suitability of the system for the analysis of these drugs in combination. The typical chromatogram of standard solution is as shown in **Fig. 3.**

Table No. 6: Analysis of data of tablet formulation

S. No.	Parameters	UPLC	
		Niacin	EGCG$
1	Label claim	500 mg	250 mg
2	Drug Content*	99.52%	98.86%
4	%RSD	0.58	0.38

SD is Standard Deviation; RSD is Relative Standard Deviation; * Value for Drug content (%) is the mean of five estimations; $ A component of GTE.

Table No. 7: System suitability parameters of UPLC method

S. No.	Parameters	Niacin	EGCG
1	Tailing factor	1.35	1.45
2	Theoretical plates	2261	10648
3	Resolution factor	-	10.38

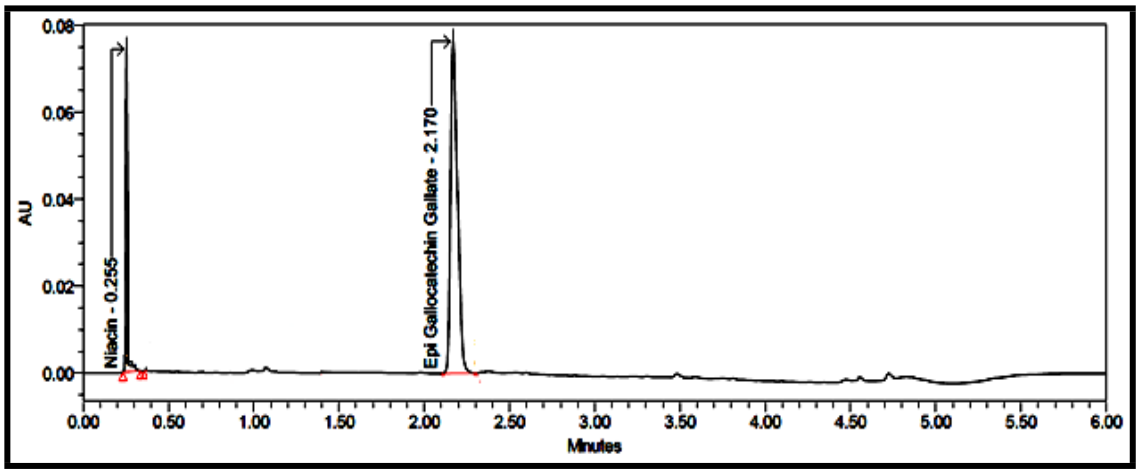

Fig. 3: Representative UPLC chromatogram of Niacin and EGCG

CONCLUSION

A simple and rapid stability-indicating AcQuity UPLC H-CLASS -TUV method was developed and validated successfully for the analysis of Niacin and EGCG in bulk and in-house tablet formulation. The method validation results have proved that the method is selective, precise, accurate, linear, robust, filter compatible stability indicating. Moreover, it may be applied for individual and simultaneous determination of Niacin and EGCG in-vitro release test profiling pharmaceutical dosage forms, where sample load is higher and high throughput is essential for faster delivery of results. The results of the recovery studies performed show the high degree of accuracy of the proposed methods. The method was validated according to the ICH guidelines

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