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DEVELOPMENT OF VALIDATED HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF FERULIC ACID AND ELLAGIC ACID

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Keywords:

Ellagic acid, Ferulic acid, Simultaneous, HPTLC method, Validation

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ABSTRACT: A simple, precise, accurate and rapid high performance thin layer chromatographic method has been developed and completely validated for the estimation of ellagic acid and ferulic acid in herbal formulation. Quantification was carried out with pre-coated silica gel aluminium plate G60F254 (10x10cm) as stationary phase, mobile phase consisting of Ethyl acetate: toluene: formic acid: methanol (3.5: 4:0.8:0.2 v/ v/ v) and scanned in Absorbance Reflectance mode at 275 nm using Camag TLC scanner 3 with WinCAT software. The R_f value of 0.31 for ellagic acid and 0.54 for ferulic acid was obtained. The proposed method has permitted the quantification of ellagic acid and ferulic acid over the linearity range of 100 to 1000 ng/spot, its percentage recovery was calculated along with intraday and inter day precision. The limit of quantification and the limit of detection were found to be 38.96ng/ spot and 12.85ng/ spot, respectively for ellagic acid and 42.28ng/spot and 27.66ng/spot, respectively for ferulic acid. The suggested method can be successfully used to estimate the drug content of many commercial formulations on a single plate.

INTRODUCTION: Ferulic acid (FA) is a phenolic molecule derived from plants that is a hydroxycinnamic acid (4 – hydroxyl – 3 - methoxycinnamic acid). Ferulic acid is composed of trans-cinnamic acid with methoxy and hydroxy substituents at 3,4 positions, respectively, on the phenyl ring. It is a conjugate acid of a ferulate [1]. It exhibits a variety of pharmacological effects, such as antioxidant, anti-inflammatory, vasodilatory, antithrombotic, antimicrobial, anti-allergic, antiviral, hepatoprotective, and anticancer activities [2].

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Commelinids, grasses, grains, vegetables, flowers, fruits, leaves, beans, coffee, artichoke, peanut, and nut seeds are among the plants that contain FA [3]. Ellagic acid is an organic heterotetracyclic molecule formed *via* oxidative aromatic coupling of gallic acid and intramolecular lactonisation of both carboxylic acid groups of the resultant biaryl. Many fruits and vegetables contain it, mostly berries and pomegranates.

Ellagic acid is a fungal metabolite, a plant metabolite, an antioxidant, a skin lightening agent, an EC 2.7.7.7 (DNA-directed DNA polymerase) inhibitor, and a geroprotector. It is functionally related to a gallic acid [4]. The present study illustrates development and validation of a simple, accurate, precise and specific HPTLC method for the simultaneous estimation of ferulic acid and ellagic acid.

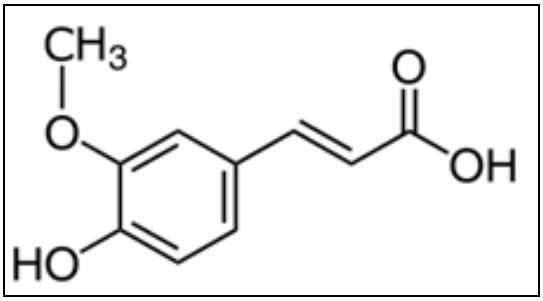

FIG. 1: STRUCTURE OF FERULIC ACID

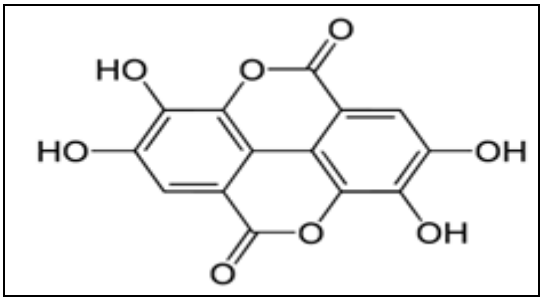

FIG. 2: STRUCTURE OF ELLAGIC ACID

EXPERIMENTAL:

Selection of Marker Compounds: Ferulic acid was selected as the analytical reference marker for *Cynodon dactylon* (durva) extract. It was purchased from P. C. Chem, Mumbai

Ellagic acid selected as the analytical reference marker for *Punica granatum* peel (pomegranate) extract. It was purchased from P. C. Chem, Mumbai

Preparation of Stock Solutions: The stock solution of marker was prepared by dissolving 10 mg of each marker (ellagic acid and ferulic acid) individually transferred in three volumetric flask and volume was made up to 10 ml with methanol to obtain 1000 ppm stock solution. These solutions were filtered through the syringe filter (0.45 µm) and all the stock solutions were stored in refrigerator.

Preparation of Working Solutions: Working solutions were prepared from standard stock solutions of ellagic acid and ferulic acid by withdrawing aliquot of 1 ml from stock solutions of each marker compound and transferred in three separate volumetric flasks of 10 ml. To achieve 100 g/ml solutions, the volume was made up using methanol.

Solutions to be used for calibration curve were made in such a way that application of 15 µl volume gave a series of spots covering over range of 100-1000 ng/spot for ellagic acid and, 100-1000 ng/spot for ferulic acid. These ranges were used for the construction of calibration curve.

Preparation of Sample Solution: Sample solution of formulation was prepared by triturating the tablets. Approximately 2gm of tablet powder was weighed and extracted with 30 ml of methanol by maceration method for 30min. The solution was further cooled and filtered to get methanolic extract. 1 ml of the above extract was diluted to 10 ml with methanol and used for further analysis.

HPTLC Method Development:

Optimization of Mobile Phase: Mobile phase selection was done on trial-and-error basis. 10 µl of each ellagic acid, and ferulic acid were applied individually and were used to study chromatographic behaviour. Different combinations of solvents were tried to obtain a mobile phase in which all the markers show good separation and give quantifiable sharp peaks with no fronting or tailing. Table demonstrates the various compositions tried for optimization of mobile phase [5, 6, 7, 8, 9].

TABLE 1: OPTIMIZATION OF MOBILE PHASE

Sr. no.	Mobile phase composition	Ratio (%v/v/v)	Rf		Comment
			Ellagic acid 0.05	Ferulic acid 0.45	
1.	Hexane: Ethyl acetate: Formic acid: Methanol	7:3:1:0.6	0.45	0.05	No spot for ellagic acid was observed
2.	Toluene: Ethyl acetate: Isopropanol: Methanol	7:4:2:1	0.5	0.20	Rf of ellagic acid is very low
3.	Hexane: Acn: Isopropanol:Glacial acetic acid	5:3:1:0.6	0.36	0.38	No resolution
4.	Toluene: Ethyl acetate: Formic acid: glacial acetic acid	4:3:1:0.8	0.46	0.99	Poor resolution and high Rf values were observed
5.	Toluene: Ethyl acetate: Formic acid: methanol	5:5:1:1	0.48	0.51	Tailing was observed

6.	Toluene: Ethyl acetate: Formic acid: methanol	4:3.5:0.8:0.2	0.31	0.54	Good resolution

Quantification: The developed method was applied for the quantification of ellagic acid in *punica granatum* peel extract and Ferulic acid in *Cynodon dactylon* extract. The sample of ellagic acid and ferulic acid in extract and tablet were analysed successfully by proposed method. The peak area was taken in triplicate and the average of three values was determined.

TABLE 2: QUANTIFICATION OF ELLAGIC ACID FROM *PUNICA GRANATUM* EXTRACT

Cynodon dactylon extract	Average peak area
Extract1	8693
Extract 2	8671
Extract3	8642
Avgare as of three samples	8668
Concentration of Ellagic acid	969.26 ng/spot

The ellagic acid contentin *Cynodon dactylon* extract was found to be 3.23% w/w.

TABLE 3: QUANTIFICATION OF FERULIC ACID FROM *CYNODON DACTYLON* EXTRACT

Punica grantum peel extract	Average peak area
Extract 1	9258
Extract 2	9226
Extract 3	9298
Avgareas of three samples	9260.66
Concentration of ferulic acid	857.47ng/spot

The Ferulic acid content in *Cynodon dactylon* extract was found to be 2.85% w/w.

Method Validation:

Optimization of Chromatographic Conditions [10]: With a Hamilton microliter syringe (100µL), the samples were spotted in the form of bands (6 mm wide) under a controlled nitrogen stream using a Camag Linomat V sample applicator. The slit dimension was 5 mm × 0.45 mm and a scanning speed was set 10 mm/s. Precoated TLC silica gel aluminium Plates 60 F 254 (20 cm × 10 cm, 250 m thickness, Merck, Darmstadt, Germany) were utilized. The chromatographic ascending development was performed using a mixture of mobile phase Toluene: Ethyl acetate: Formic acid: methanol (4: 3.5: 0.8: 0.2 v/v/v/v) to a migration distance of 80 mm. The chamber was previously saturated for 15 min at temperature 25±2 °C. Densitometric scanning was achieved over Camag TLC scanner III operated using win CATS software (V 1.44 CAMAG). A UV spectrophotometer was utilised as the radiation source. The obtained bands were scanned at a wavelength of 275 nm. After completion of scanning the Rf values, peak areas and spectra of the markers were recorded.

HPTLC Method Validation [6, 11, 12]: The developed method of HPTLC was validated as per ICH guidelines Q2 (R1) for different parameters. Following are the various parameters for which the method was validated.

Specificity: To study specificity, the test compound was applied with standards on the same plate.

Linearity: The linearity for both ellagic acid and ferulic acid was assessed in the range of 100-1000 ng/spot of 100ppm solution and 100-1000 ng/spot of 100 ppm solution. Different concentration of the standard solutions were applied three times to study linearity. The area under the peak vs drug concentration data was analysed using linear least square regression.

Accuracy: The accuracy study was carried out by addition of known amounts of standards to the product. Standards added were 80%, 100% and 120% of the quantitated amount. Three determinations were done to study the recovery. The % recovery of acyclovir and ferulic acid were compared with the actual amounts.

Precision: To study the precision of method, both intraday and interday precision were done.

Intraday Precision: Intraday precision was assessed by using 9 determinations of three different concentrations. Three concentrations for ellagic acid (10, 50 and 70 µg/ml) and ferulic acid (100,500 and 1000 µg/ml) and three replicates for each were carried out to see variation in their peak areas within a same day.

Interday Precision: The interday precision was assessed by using 9 determinations of three different concentrations. Three concentrations for Ellagic acid (10, 50 and 7 0 µg/ml) and ferulic acid (10, 50 and 70µg/ml) and three replicates for each was carried out to see variation in their peak areas for three different days.

Limit of Detection (LOD): Detection limit was calculated from the calibration equations obtained from the experiment. The determination of LOD was based on the standard deviation of the response and the slope. The slope was estimated from the calibration curve of the analyte and the estimate of the standard deviation was carried out from the standard deviation of the y-intercept. The Detection limit may be expressed as:

$$LOD = 3.3\ \sigma / S$$

Where σ = standard deviation of the response, S = slope of the calibration curve.

Limit of Quantification (LOQ): Quantification limit was calculated from the calibration equations obtained from the experiment. The determination of the Quantification limit was based on the standard deviation of the response and the slope. The slope was estimated from the calibration curve of the analyte and the estimate of the standard deviation was carried out from the standard deviation of the y-intercept.

The Quantification limit may be expressed as:

$$LOQ = 10\ \sigma / S$$

Where, σ = standard deviation of the response, S = slope of the calibration curve.

Robustness: In order to study the robustness of the method, slight but deliberate changes were made in some parameters. Parameters used for the robustness study were the mobile phase composition and chamber saturation period.

RESULT AND DISCUSSION:

Analytical Method Development and Validation [6, 11]: A new, simple and rapid method was developed for the formulation. The developed method was applied for the detection and quantification of ellagic acid and ferulic acid in the herbal extract tablet.

Optimized Conditions for Validation: For method validation, the following conditions were used:

Stationary Phase: pre-coated silica gel aluminium plate G 60 F254 (10 x 10 cm).

Mobile phase: Toluene: Ethyl acetate: Formic acid: methanol (4:3.5: 0.8: 0.2).

Application Rate of Micro Syringe: 10µl/spot

Chamber Saturation Time: 15 minutes.

Length of Chromatogram Run: 9 cm.

Detection Wavelength: 275 nm.

The peaks for ellagic acid and ferulic acid were observed at Rf 0.31 and 0.54 respectively in the densitogram of the herbal formulation extract.

Linearity: Linear responses for both the markers ferulic acid and Ellagic acid were obtained at 100-1000 ng/spot. Linearity was validated by interpreting regression line and by determining regression coefficient (R^2).

TABLE 4: LINEAR REGRESSION DATA FOR ELLAGIC AND FERULIC ACID

Parameters	Ellagic acid	Ferulic acid
Linearity range (ng)	100-1000 ng/spot	100-1000 ng/spot
Equation of regression Line	7.5024x+1396.2	Y=6.9192x+3327.6
Coefficient of regression (r^2)	0.9983	0.9922
Slope	7.5024	6.9192
Intercept	1396.2	3327.6

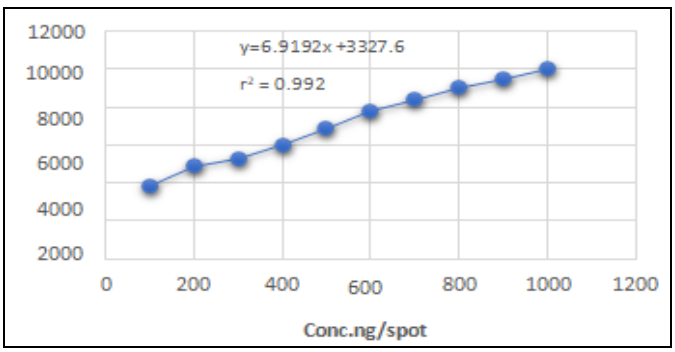

FIG. 3: GRAPH SHOWING LINEARITY OF ELLAGIC ACID

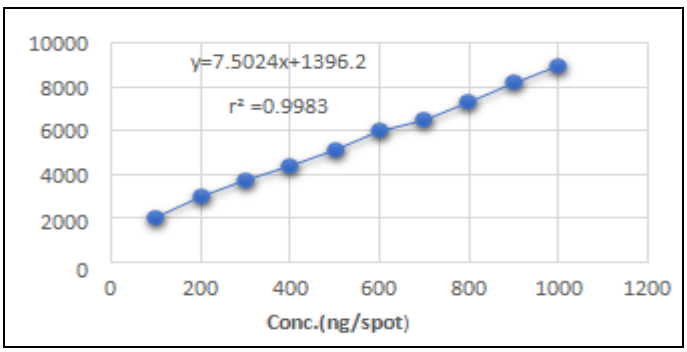

FIG. 4: GRAPH SHOWING LINEARITY OF FERULIC ACID

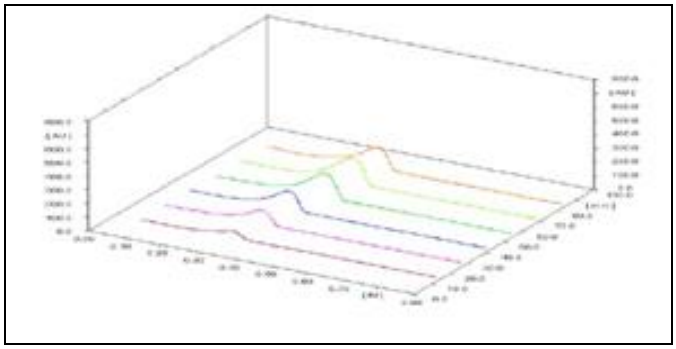

FIG. 5: GRAPH SHOWING LINEARITY OF ELLAGIC ACID

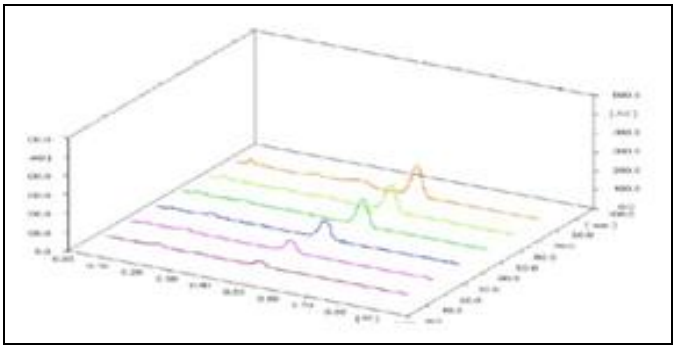

FIG. 6: GRAPH SHOWING LINEARITY OF FERULIC ACID

Accuracy: Accuracy of the method is reported as percent recovery of known added amount of analyte in the sample. The accuracy of the method was established by performing recovery studies in triplicates of three concentration levels *viz*. 80%, 100%, 120% by adding known amount of ellagic acid and ferulic acid. Results obtained are given in table.

TABLE 5: RECOVERY RESULTS OF ELLAGIC ACID AND FERULIC ACID

Drug	% Level	Initial amount (ng/spot)	Spiked amount (ng/spot)	Total Expected amount (ng/spot)	Amount recovered (ng/spot)	% Recovery ± SD*	%RSD
Ellagic acid	80	500	400	900	892	99.11±2	0.22
	100	500	500	1000	996	99.6 ± 1	0.10
	120	500	600	1100	1089.6	99.05±1.25	0.12
Ferulic acid	80	1000	800	1800	1795.6	99.73±1.10	0.06
	100	1000	1000	2000	1997.96	99.89±0.153	0.01
	120	1000	1200	2200	2196.5	99.84±1.5	0.07

Precision:

Intra-day Precision and Inter-day Precision: Intra-day precision was carried out under same laboratory conditions on same day, over specific time intervals whereas inter-day precision was carried out by maintaining the same laboratory conditions on three different days at different time points. Precision was determined in triplicates by analysing three quality Control (QC) samples of Ferulic acid and Ellagic acid at three distinct concentration levels (200 ng/spot, 500 ng/spot, and 700 ng/spot) each i.e. low quality control (LQC), mid quality control (MQC) and high quality control (HQC). The results obtained were further statistically analysed for standard deviation and percent relative standard deviation (coefficient of variance). % RSD of the peak areas obtained was less than 2%, which indicates that the developed method was precise.

TABLE 6: RESULTS OBTAINED FOR LOD AND LOQ OF BOTH THE MARKER COMPOUNDS

Compound	Concentration (ng/spot)	Intraday precision			Interday precision		
		Average Area (AU)	Standard Deviation (S.D.)	%RSD	Average area (AU)	Standard Deviation (S.D.)	%RSD
Ellagic acid	200	2627.3	29.22	1.11	3641.3	65.99	1.68
	500	4268.2	11.54	0.27	3268.2	29.54	0.43
	700	6231.2	51.57	0.52	6131.2	14.81	0.16
Ferulic acid	200	6482.4	58.00	1.97	5946.2	37.06	1.26
	500	7783.1	36.19	0.55	7583.3	68.24	0.88
	700	9231.4	66.81	0.67	9321.8	66.81	0.67

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

TABLE 7: RESULTS OBTAINED FOR LOD AND LOQ OF BOTH THE MARKER COMPOUNDS

Parameter	Ellagic acid	Ferulic acid
Limit of detection (LOD) in ng / spot	12.85	27.66
Limit of quantitation (LOQ) in ng / spot	38.96	42.28

Specificity: It was observed that other constituents present in the extract did not interfere with the peak of Ellagic acid and ferulic acid. Therefore, the method is specific. The spectrum of standard ellagi acid and ferulic acid corresponds with tablet extract.

Robustness: Robustness of method was studied by making slight but deliberate changes in chromatographic conditions such as changes in mobile phase composition and chamber saturation time. Effects of these changes on both the retention factor (Rf) and peak area were evaluated by calculating the relative stand ard deviations (%RSD). From the results obtained it was concluded that the developed method was found to be robust.

TABLE 8: ROBUSTNESS RESULTS FOR ELLAGIC ACID AND FERULIC ACID

Mobile phase composition	% RSD	
	Ellagic acid	**Ferulic acid**
Toluene: ethyl acetate: formic acid: Methanol (3:3.5:0.5:0.8:0.2) v/v/v/v)	0.24	0.19
Toluene: ethyl acetate: formic acid: Methanol (4: 4: 0.8 :0.2 v/v/v/v)	0.49	0.36
Saturation Time (±)	Ellagic acid	Ferulic acid
8 min	0.16	0.10
12 min	0.13	0.08

CONCLUSION: HPTLC simultaneous estimation method for detection of ferulic acid and ellagic acid in the extract and formulation was developed and found to be rapid, simple, precise, specific, and accurate and repeatable.

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CONFLICTS OF INTEREST: The authors have no conflict of interest.

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