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Development And Validation Of HPTLC Method For Simultaneous Estimation Of Rutin And Quercetin In Hydroalcoholic Extract Of Triphala Churna

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Abstract: A new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous determination of Rutin and Quercetin in Ayurvedic formulations *Triphala churna.* The retention factors of rutin and quercetin were 0.01 and 0.76, respectively. linearity was obtained in the range of 200-600 ng for Quercetin and Rutin. Methods are validated according to ICH guidelines and can be adopted for the routine analysis of rutin and gallic acid in hydroalcoholic extract of *Triphala churna*. Satisfactory recoveries of 99.14-99.60% and 98.61-100.56 % were obtained for Rutin and Quercetin. The results obtained in validation assays indicate the accuracy and reliability of the developed simultaneous HPTLC method for the quantification of Rutin and Quercetin in *Triphala churna.*

Key words- HPTLC, Triphala Churna, marker, quercetin, rutin.

INTRODUCTION

Triphala churna is [1:1:1] combination of three compound viz.*Terminalia chebula, Terminalia belerica, Terminalia officinalis* each of which have its own therapeutic value but in combination it enhances overall potential these are used mostly used as antioxidant, antiaging, anti-inflammatory, mental and memory enhancing effect[1]. The extract of *Triphala* was obtained by continuous heat extraction by using Soxhlet extraction process and ethanol water in ratio (70:30). Marker which have been found in *Triphala* by various method are quercetin, rutin ,gallic acid, ellagic acid, ascorbic acid these are found in *Triphala* and known for its effect. Gallic acid is phenyl propanoid, chemically it is 3, 4, 5,-Trihydroxybenzoic acid, and possess astringent activity. Rutin is 5, 7, 3, 4, tetrahydroxy flavonol -3-rhamanoglucoside and widely used in medicine for maintenance of capillary integrity. Both possess antioxidant activity and reduce low density lipoproteins [LDL] oxidation[2-7].

Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemoprofiling and marker compound analysis using modern analytical techniques. The literature revealed that no HPTLC method is not yet reported for the estimation of rutin and gallic acid in *Triphala churna*. The method is developed in solvent Methanol. Method validation is done as per ICH guidelines[8].

Thus, this method is more accurate and cost effective. This paper describes simple, rapid, accurate, precise and economical method for simultaneous determination of rutin and gallic acid in *Triphala extract.*

MATERIALS AND METHODS-

Reagents and Materials:

All chemicals and solvents used were of analytical grade and obtained from E.Merck (Darmstadt, Germany). Flavanoids standards (Rutin and Quercetin) were purchased from Lobo Cherie,

Mumbai, India (purity >97%). TLC aluminum plates pre-coated with silica gel 60 F254 (20 x 2 0 cm, 0.2 mm thick) used were obtained from E. Merck Ltd (Mumbai, India).

Plant material:
The *Triphala churna* is unique combination of three plants viz.*Terminalia chebula, Terminalia belerica, Terminalia officinalis* these all plants are used in equal proportion available in form of powder was purchased from local market from Kolhapur.

Extraction of plant material for HPTLC analysis:
The *Triphala churna* was extracted with ethanol water (70:30) with continuous heat extraction with Soxhlet apparatus and filtered. The extract was concentrated to get dry residue and stored in the dessicator and it was used for subsequent experiments. Preliminary photochemical screening revealed the presence of Polyphenols, flavanoids and glycosides.

Instrumentation:
The method was developed on CAMAG HPTLC system consisting of Linomat V applicator (Camag, Muttenz, Switzerland) CAMAG twin trough chamber,CAMAG TLC scanner, equipped with Win cats software (version 1.4.6) , CAMAG syringe of 100 µL capacity. Separation and identification of quercetin, rutin were performed separately on aluminum backed silica gel 60 F254 (20cm x10cm of plate size, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany).

Preparation of working standard solution of gallic acid, rutin, quercetin:
Standard stock solution of rutin, and quercetin was prepared by dissolving 10 mg of Rutin, Quercetin up to 10 ml of methanol, to get stock solution containing 1000 µg/ml of Rutin and Quercetin.

Preparation of sample solution of *Triphala churna*:
Sample stock solution was prepared by dissolving 100 mg of extract in 5ml methanol sonicated for 10 min any insoluble fraction was removed by filtration and further dilution was made by using 1 ml of stock solution to 10ml with methanol.

Chromatographic conditions:
The experiment was performed on a silica gel 60 F254 (0.2 mm thickness) HPTLC plates (20×10cm) and (10×10) without prewashing. Samples were applied to the plates as 8 mm bands, 8 mm, with a Camag Linomat. The plates were developed by the ascending technique, to a distance of 80 mm, at 25 ± 5°C, relative humidity 50–60%, in a Camag twintrough glass chamber with a stainless steel lid, using a mobile phase, composed of ethyl acetate: formic acid: acetic acid: water (10: 1.1: 1.1: 0.6), for, Rutin and Quercetin. The chamber saturation time was kept as 20 min. After development, plates were dried with a hot-hair dryer, viewed in a Camag UV cabinet at 254 nm & 366 nm, and then scanned with a Camag TLC Scanner, using win CATS software (version 1.4.6) , in absorbance mode, with slit dimensions 6.00 x 0.45 mm, Micro. The detection wavelength 254 nm was selected. The *R*f values were 0.01, 0.76 for rutin and quercetin respectively.

Calibration curve for standard quercetin and rutin
The standard solutions 200-600 ng was applied on TLC plate and further it was developed and scanned as per the chromatographic conditions mentioned above. The peak areas were recorded. Calibration curve of Quercetin and Rutin were prepared by plotting peak area against concentration of quercetin and rutin applied.

VALIDATION OF THE PROPOSED METHOD:
ICH guidelines were followed for the validation of the analytical methods developed (precision, accuracy, Ruggedness, Robustness, linearity, LOD, LOQ, specificity).

Linearity:
Standard stock of 200-600 ng rutin and quercetin were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the analyte solutions were analyzed in triplicate, and then the calibration curves were constructed by plotting the mean peak areas *versus* the concentration of each analyte.

Inter-day and intra-day precision:
The inter-day precision (RSD) was determined by analyzing standard solution of quercetin and rutin over the entire calibration range for six different days. The intra-day precision (RSD) was determined by analyzing standard solution of quercetin and rutin over the entire calibration range for six times on the same day.

Limit of detection:
The limit of detection (LOD) was determined using following formulae. LOD = 3.3(SD)/S Where, SD = Standard Deviation of response**,** S = avg. of the slope of the calibration curve. The

minimum detectable limit was found to be ng/spot for quercetin and ng/spot for rutin.

Limit of quantification:
Limit of quantitation (LOQ) were determined using following formulae. LOQ = 10 (SD)/S, Where, SD = Standard Deviation of response**,** S = avg. of the slope of the calibration curve. The minimum quantified limit was found to be ng/spot for quercetin and rutin.

Specificity:
It was observed that other constituent's presents in the formulation did not interfere either with the peak of quercetin and rutin. Therefore the method was specific. The peak purity of the gallic acid was assessed by comparing the spectra at three different levels, viz. peak start, and peak apex and peak end positions of the spot.

Robustness of the method:
By introducing small changes in the mobile phase development distance, mobile phase volume and duration of mobile phase saturation, the effects on the results were examined. Robustness of the method was done six times at a concentration level of 5µg/spot and the % R.S.D. of peak area was calculated.

Ruggedness of the method:
It expresses the precision within laboratories variations like different analyst. Ruggedness of the method was assessed by spiking the standard 6 times with different analyst by using same equipment.

Accuracy:
The accuracy was determined by standard addition method. Accuracy of the method was tested by carrying out recovery studies at different spiked level by standard addition method. Standard quercetin and rutin solution was added at three different levels (80,100 and 120%). At each level three determinations were performed and results were calculated by the difference between the spiked and un-spiked sample analyzed under the same conditions. To a fixed amount of preanalyzed. The Percentage recovery of quercetin and rutin were calculated at each level.

Chromatogram of *Triphala churna,* standard rutin and quercetin

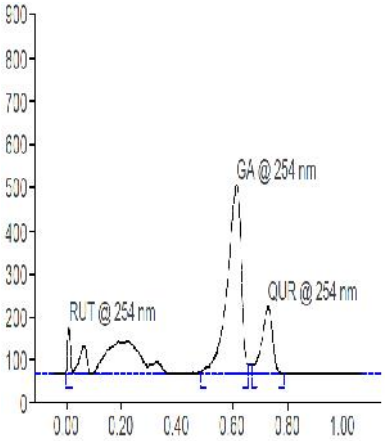


Fig.1

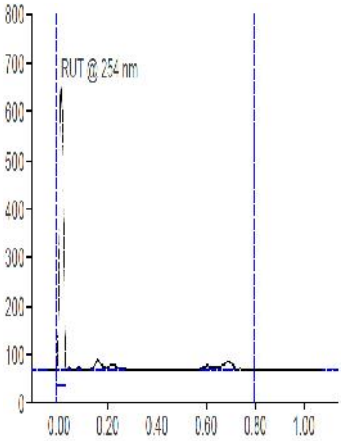


Fig.2

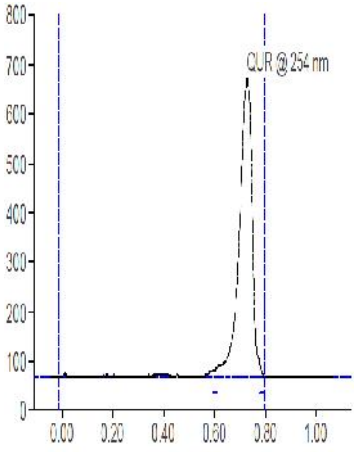


Fig.3

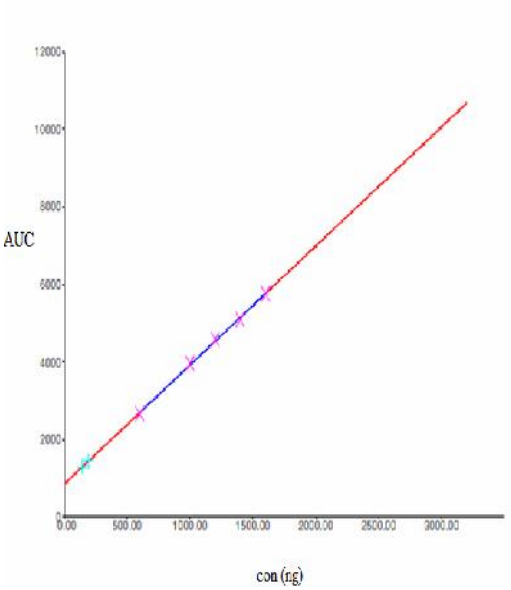


Fig.4

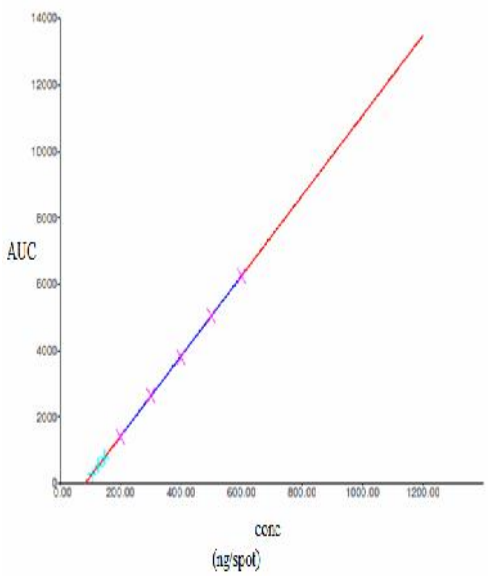
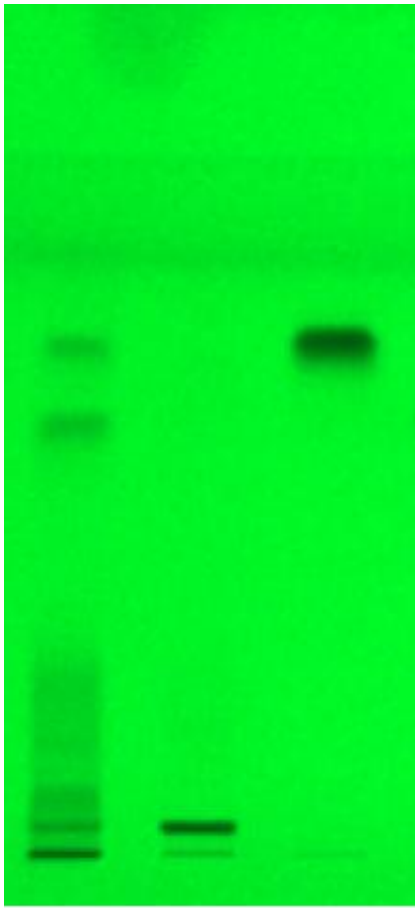


Fig 5



A B C

Fig 6: A=Hydroalcoholic extract of Triphala churna, B=standard = Rutin, C= standard Quercetin

RESULT AND DISCUSSION:

HPTLC procedure was optimized with view to quantify the herbal extract. Ethyl acetate formic acid acetic acid water in ratio of (10:1.1:1.1:0.6 v/v) gives good resolution with Rf value for rutin 0.03 and for quercetin 0.76. Well defined spot were obtained after chamber was saturated for 20 min at room temperature. TLC plate was visualized under UV light at 254 nm without derivatization. The identity of rutin and quercetin were confirmed by comparing chromatogram of standard rutin and quercetin with that of extract and by comparing retention factor of reference with standard.

CALIBRATION CURVE:

Calibration plot shown in fig 4, 5 indicates the response is linear function of concentration in the range 200-600 ng for quercetin and 200-600 ng for rutin. The correlation coefficient, intercept and slope for quercetin 0.9999, -1082, 12.09 and rutin 0.9999, 804.3, 3.07.

METHOD VALIDATION:

Precision:

The measurement of peak area six times inter-day and intar-day showed %R.S.D (< 2%) which suggest precision of the method (**Table.1).**

Robustness:

The low value of S.D AND % R.S.D obtained after introducing small deliberate change in developed HPTLC method indicates the robustness of method (**Table.2).**

The limit of detection (LOD) and limit of quantitation (LOQ):

The limit of detection (LOD) and limit of quantitation (LOQ) of Quercetin and Rutin were which indicate the adequate sensitivity, which indicate the adequate sensitivity of method (**Table.1).**

Recoveries:

The result from recoveries studies, listed in table were within acceptable limit indicating accuracy of method was good (**Table 2, 3).**

Ruggedness:

Low % R.S.D values of between the peak area values proved the ruggedness of method indicating that quercetin and rutin were gives reproducible result for proposed method (**Table.5).**

Specificity:

The peak purity of rutin and quercetin was accessed by comparing the spectra at peak start, peak apex and peak end position of the spot. Good correlation (0.999) and (0.994) was obtained between the standard and sample of rutin and quercetin.

Table 1: Method validation data for HPTLC quantification of Quercetin and Rutin present in *Triphala Churna.*

Method property	value
Linearity for Quercetin(ng/spot)	200-600ng
Linearity of Rutin (ng/spot)	200-600ng
Correlation coefficient for Rutin (r)	0.999
Correlation coefficient for Quercetin (r)	0.999
Intraday precision (RSD % n=6) on different days for Rutin	1.05
Intraday precision (RSD % n=6) on different days Quercetin	1.13
Interday precision (RSD % n=6) on same day Rutin	1.03
Interday precision (RSD % n=6) on same day Quercetin	1.11
Limit of quantitation of Rutin (ng/µl)	0.077
Limit of detection Quercetin (ng/µl)	00.018
Limit of quantitation Quercetin (ng/µl)	0.055
Limit of detection Rutin (ng/µl)	0.025
Specificity	Specific

Table 2: Recovery of Rutin

Level	**0**	**80**	**100**	**120**
Recovery (%)	0.00	99.14	99.41	99.60
Overall average recovery (%)		99.38		

Table 3: Recovery of Quercetin

Level	**0**	**80**	**100**	**120**
Recovery (%)	0.00	98.61	99.96	100.56
Overall average recovery (%)		99.71		

Table 4: Robustness of HPTLC method (n=6)

Parameter	**S.D of peak area**	**% RSD**
Development distance of Rutin (7.6, 8, 8.4 mm.)	57.22	1.42
Mobile phase volume of Rutin (9.5, 10, 10.5 ml.)	54.49	1.34
Duration of mobile phase saturation Rutin (19, 20, 21 min.)	57.17	1.41
Duration of mobile phase saturation Quercetin (19, 20, 21 min.)	61.97	1.4
Development distance Quercetin (7.6, 8, 8.4 mm.)	66.88	1.5
Mobile phase volume Quercetin (9.5, 10, 10.5 ml.)	57.39	1.37

Table no.5. Rugedness of the HPTLC method

Parameter	**S. D. of peak area**	**% RSD**
Analyst 1 rutin and quercetin	58.11 and 63.87	0.949 and 1.54
Analyst 2 rutin and quercetin	59.10 and 52.75	1.43 and 1.23

CONCLUSION:

A rapid, simple, accurate and specific HPTLC method for quantitative estimation of Rutin and Quercetin present in *Triphala churna* has been developed and validated. The data could be used as a QC standard the method used in this work resulted in good peak shape enable good resolution of Quercetin and Rutin from other constituents of the plant material. Because recovery there were no interference with Rutin and Quercetin peaks from other constituents present in plant.

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