Validation of the Fast Robust and Cost Effective HPTLC Method for the Simultaneous Estimation of Three Phytochemical Standards

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Abstract

Veliparuthi powder sold by Neotea brand is widely used as folklore medicine for treatment of skin deceases, respiratory ailments and relieving body pain. Rheumatigo syrup used for the treatment of rheumatoid pain contains Pergularia Daemia 5 gm/ 100 mL. Amongst major Phytochemicals of Pergularia Daemia, i.e. Kaempferol, Betaine, Lupeol, Hentiracontane and Betasetosterol, Very few analytical methods are published which can provide simultaneous analysis. There is no published method for the simultaneous analysis using HPTLC. Fast, robust and cost-effective method developed for the above phytochemicals, from above Betaine, Hentriacontane opted out, and method finalised for three phytochemicals. Two phytochemicals Betasetosterol and Lupeol required derivatisation and Kaempferol does not required the derivatisation. All three phytochemicals separated with mobile phase composiyion of Toluene: Ethyl acetate: Formic Acid (85:15:1 v/v/v). For method validation, the protocol designed in line with ICH Q (R1) guideline [37]. Method validation performed for the parameters like, Precision, Accuracy, specificity, linearity and robustness. Method validated successfully and data of this validation presented in this paper. The data obtained from this method is comparable with other chromatographic method. The method used to quantify the content of the three phytochemicals in Veliparuthi powder and Rheumatigo syrup.

Keywords: Pergularia Daemia, Phytochemicals, Markers, Validation, HPTLC

INTRODUCTION

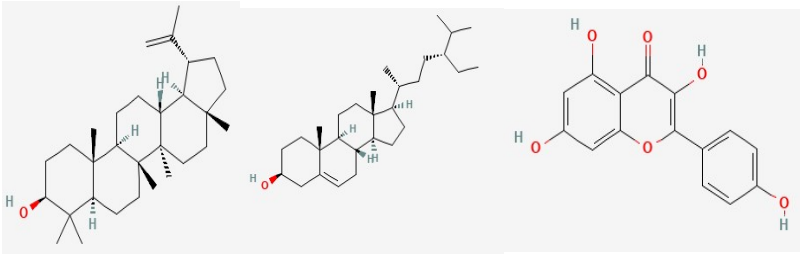
HPTLC is powerful tool for separation of any chemical compounds, but the efforts should be made to develop fast, robust and cost effective method to make that tool more powerful and comparable with other methods. Our quest or interest generated for the phytochemical analysis of few herbal formulations like Veliparuthi tea and Rheumatigo syrup. The formulations containing the Pergularia Daemia as main ingredient. Extensive literature search done for the Pergularia Daemia and related plant for phytochemicals present it. There is no method reported for simultaneous analysis of this phytochemicals (Lupeol, Kaempferol and Betasetosterol).

Pergularia Daemia reported to have many phytoconstituents such as β-Sitosterol, Lupeol, oleanolic acid, calactin, calotropin,

corotoxigenin, daucosterol, sucrose, α-amyrin, β-amyrin , betaine, Hentriacontane and pentacosanoic acid, Calactin, Calotropin, calotropigenin, corotoxigenin, Dihydrocalotropigenin, uscharidin and calotoxin Coroglaucigenin, Corotoxigenin, Uscharidin and Uzarigenin in various parts of the plants. Amongst above the major phytochemicals reported are Betasetosterol, betaine, Hentriacontane, Lupeol and Kaempferol. The method development started using this phytostandards. Betaine dropped as there was no detection for the plant extract and Hentriacontane required different derivatisation reagent hence also dropped as we targeted to develop simultaneous method for the estimation of phytochemicals.

The finalised phytochemicals were Betasetosterol, Lupeol and Kaempferol. The method validation protocol was prepared and executed. The parameters taken from industries best practices. The method validated for the Specificity, Accuracy, Precision, linearity and robustness. Finalised linearity range after optimisation of LLOQ 50-500 PPM.

The method validation activity performed at Gujarat Technological University, Gandhinagar. The method validation was successfully completed. The method is ready for simultaneous quantification of three phytochemicals i.e. Betasetosterol, Lupeol and Kaempferol (*Image:1*) form the extracts of Veliparuthi powder and Rhematigo syrup along with plant extracts of Pergulaira Daemia. As a scope of method we can use this method for the estimation of this phytochemicals from any plant extracts or its products where they are reported.



a. Lupeol b. Betasetosterol c. Kaempferol

Image 1 Chemical Structures of phytochemicals

MATERIALS AND METHODS

Instruments:

HPTLC system used for the Spiking of the standards and samples is CAMAG® Automatic TLC Sampler 4 (ATS 4). Visual presentation and photo documentation done using CAMAG® TLC Visualizer 3. CAMAG® Derivatizer used for spraying the Anisaldehyde sulphuric acid reagent for derivatisation of the phyto-standards Betasetosterol and Lupeol. CAMAG® TLC

Scanner 4 used to scan the plates before and after the derivatisation using suitable wavelength. 5 µL of the mixed standards were spiked on Pre-coated Silica Gel G60 F254 Aluminum sheets (10 X 10 cm, 20 X 10 cm). Software used to control the system and generate the data is VisionCATS. Weighing done on Mettler Toledo analytical balance.

Phytostandards:

Hentriacontane purchased from Sigma Aldrich, Lupeol, Betaine, Betasetosterol and Kaempferol purchased from Synthocore Biochemie, Navi Mumbai. All the chemicals purchased were of purity >99 %. Other chemicals like, Toluene, Ethyl acetate, Formica acid, Methanol were of high purity and purchased from authorised vendors. The products Veliparuthi, Rheumatigo syrup procured from online sources (amazon). The plant Pergularia Daemia grown inhouse. Authentication performed from Dr. Harshal M. Pandit, Botanist (Professor and HOD of Botany).

Standards preparation:

Preparation of standard solutions

Separate standard stock solutions of Kaempferol, beta-sitosterol and lupeol were prepared by dissolving 4 mg of each marker in 2 mL of methanol to obtain a concentration of 2000 µg/mL.

Combined working standards (50, 100, 200, 300, 400 and 500 ppm) were prepare using the separate standard stock solution with appropriate dilution as follows.

Table no. 01: Preparation of calibration stock levels.

Concentration in ppm	Volume of Mixed stock taken	Volume of methanol added	Total volume
50	25 µL	975 µL	1.0 mL
100	50 µL	950 µL	1.0 mL
200	100 µL	900 µL	1.0 mL
300	150 µL	850 µL	1.0 mL
400	200 µL	800 µL	1.0 mL
500	250 µL	750 µL	1.0 mL

Screening of mobile phase:

Optimisation of mobile phase for the separation of the was challenge as out of three phytochemicals Betasetosterol and Lupeol needed derivatisation and Kaempferol does not require the derivatisation due to presence of chromophore group. During development, several composition of mobile phases tried for aiming to separate three compounds. The mobile phase with composition of Toluene: Ethyl acetate: formic acid (85:15:1 v/v/v) was finalised. Saturation

time of 20 minutes set to avoid necklace effect and consistent separation.

Screening of Derivatisation reagent

In initial stage of the development, Betaine and Hentriacontane separated and derivatised using Dragendorff reagent. Betaine not estimated in products and hence opted out. Other phytocompounds i.e. Betasetosterol and Lupeol were optimised with Anisaldehyde sulphuric acid reagent. Hentriacontane could not be optimised using ANS reagent. Hentriacontane was also opted out from the method of estimation. Another phytocompound i.e. Kaempferol was included in method development, which does not require any derivatisation. Image 5 shows Final developed chromatogram comparison.

RESULTS AND DISCUSSION

The validation of a method, such as High-Performance Thin-Layer Chromatography (HPTLC), is crucial to ensure its reliability and accuracy for quantitative analysis. Here's a structured approach and results reported during the validation of the HPTLC method for the simultaneous estimation of three phytochemical standards.

System suitability: system suitability is somewhat similar to precision experiment, single concentration of the standard spiked six times. The % coefficient of variation is measured for the area counts of each set. The % CV was below 5% for all sets analysed.

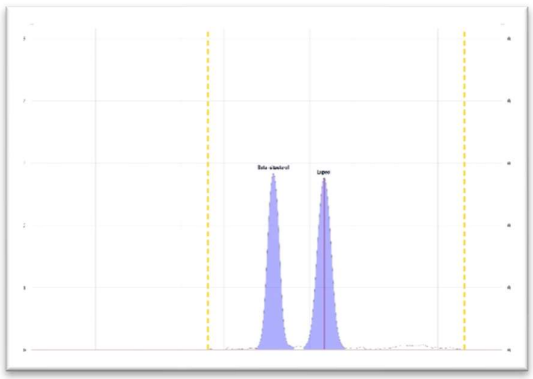


Image 2: Derivatised Beta-Setosterol, Lupeol

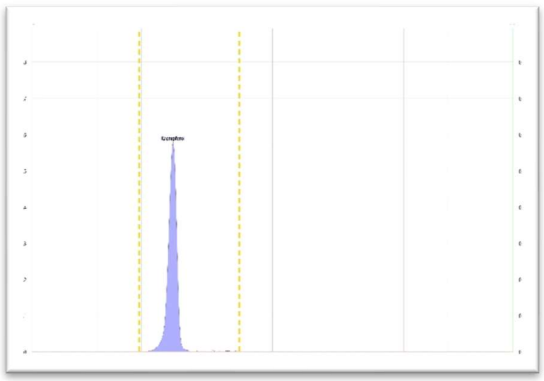


Image 3: Kaempferol

Specificity: The specificity of the method performed by analysing the spot of a blank track, diluent, mobile phase, individual standard solutions, combined standard solution and sample/test solutions. The bands of all three markers were confirmed by comparing Rf values, and comparison of respective UV spectra of marketed preparation with those of standards. Image 2 shows Derivatised Beta-Setosterol, Lupeol image 3 shows Kaempferol underivatised.

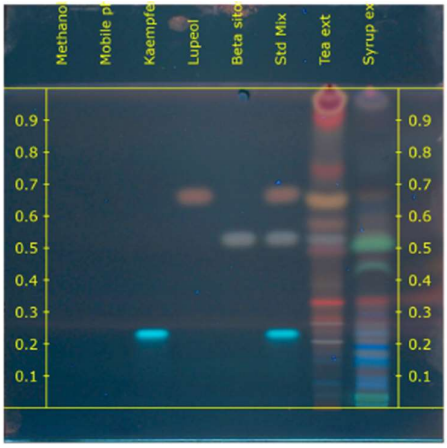


Image 4: Specificity plate

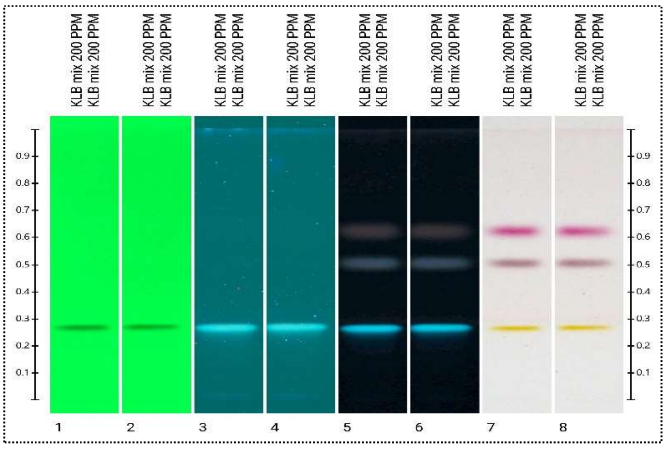


Image 5: Final developed chromatogram comparison

As seen in the image 4: there was no interference from methanol, mobile phase at the Rf value of each individual markers. Also, each individual standards are distinctly separated with different visible colours. Mixed standards also matches with individual Rf values. The products showing Linearity:

lot of bands separated but it can be seen that the bands at Rf values of Kaempferol, Betasetosterol and Lupeol can be distinguished. Hence, the method is specific for the detection of three markers Kaempferol Betasetosterol and Lupeol.

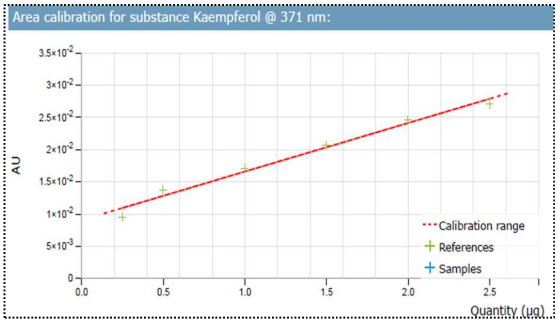


Image 5: Linearity @371 nm Kaempferol

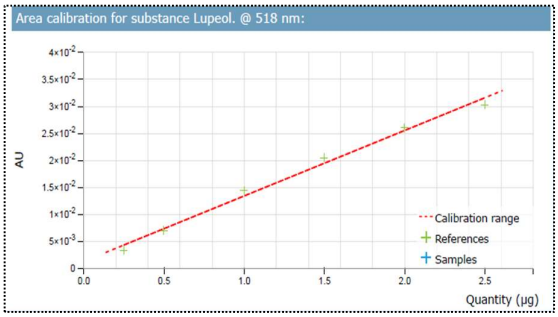


Image 6: Linearity @518 nm Lupeol

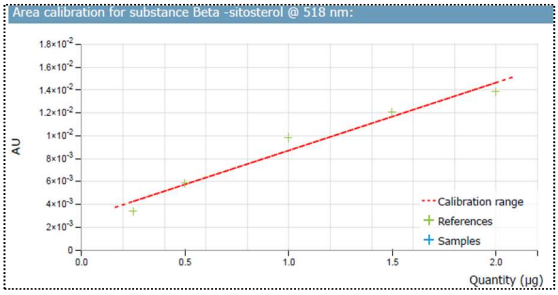


Image 7: Linearity @518 nm Beta-Setosterol

Six concentration levels over a range of 50-500 ppm/ band for all three standards.

For all the standards the regressed line was within acceptance criteria and r2 values were greater than 0.95.

Precision: Repeatability and intermediate precision by using mid concentration of linearity range for mixed standards of Betasetosterol, Lupeol and Kaempferol 200 ppm each, Six replicates of this concentration analysed per day for repeatability, and intermediate precision of the method evaluated by repeating this batch three times within day for intraday and between days for the inter-day precision. In all the batches, the % CV was below 5% for all markers. Repeatability evaluated by using different size chambers 10x10, 20x10 and there was no impact on repeatability of the results.

Accuracy: Determine the accuracy of the method by comparing the measured concentrations of the phytochemical standards with their known concentrations in spiked samples (image 8 and 9). Spike the sample matrix with known amounts of the standards and analyze them using the HPTLC method. 6 point linearity prepared and three point 100 ppm, 200 ppm, 300 ppm mixed with Test samples and resultant concentrations were 50 ppm, 100 ppm and 150 ppm. Accuracy observed was within acceptance criteria.

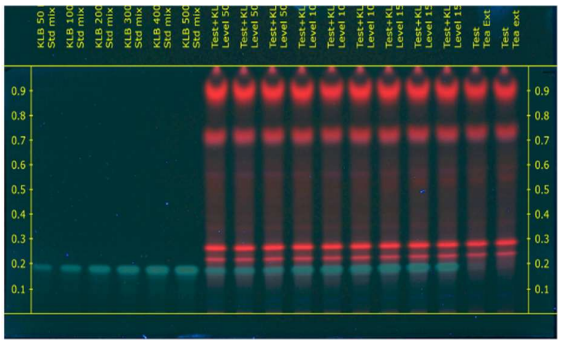


Image 8: Accuracy before derivatisation.

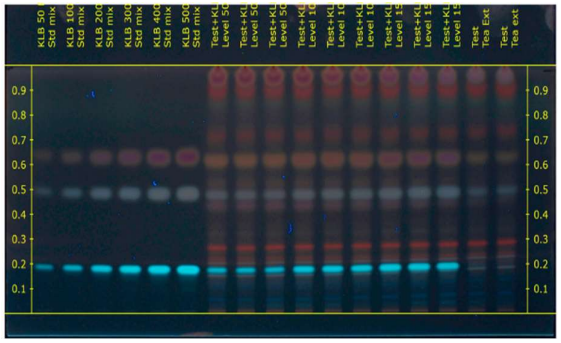


Image 9: Accuracy before derivatisation.

Limit of Quantitation (LOQ): LLOQ set for 50 ppm for all standard concentrations.

LOD was calculated from calibration curve as

Robustness: Evaluate the method's robustness by intentionally varying parameters, Robustness for saturation time performed for 18 min and 22 min, wavelength was varied ± 2 nm. Mobile phase composition varied ± 5%, injection volume was set to 5 µL, the robustness was performed with ± 1µL. In all above variations the results, i.e. precision experiment performed and results were within acceptance criteria.

Conclusion: the method of simultaneous analysis of three phytochemicals was developed and successfully validated for specificity, accuracy, linearity, precision and robustness. The regression for three anaytes were greater than 0.95, Interday and intraday variation was within limit. Robustness for all the parameters were within limit, % CV for mobile phase variation was above 5%, so care should be taken to prepare the mobile phase accurately.

The method validated can be used for analysis three phytochemicals i.e. Betasetosterol, Lupeol and Kaempferol form the plant extracts of Pergularia Daemia and products. It can be also used for any herbal product or plant containing the phytochemicals.

Table no.01: Summary of the HPTLC Method validation results:

Parameters	Results
Linearity range	50-500 ppm
Regression equation (n=6)	$Y=mx+c$
Slope	K=$6.94x10^{-9}$ B=$8.359x10^{-9}$ L=$1.536x10^{-8}$
Intercept	K=$1.972x10^{-3}$ B=$1.506x10^{-3}$ L=-6.66610^{-3}
r^2	K=0.997267 B=0.976988 L=0.997246
Precision (%RSD)	<5.0%
Repeatability @ 6.0µL	K=2.64% B=2.11% L=4.78%
Intraday (Precision)	K=2.10% to 4.50% B=3.88% to 4.47% L=3.93% to 4.79%
Interday Precision	K=3.94% to 4.44% B=2.10% to 4.22% L=4.15% to 4.79%
Accuracy	Between 90-110%
LOD	~20 ppm
LOQ	50 ppm
Robustness (n=6) (%RSD)	
Mobile phase ratio (range)	K=2.64 to 5.41%; B=3.57% to 9.11% L=4.55% to 7.26%.
Wavelength change ± 3 nm	K=4.77 to 4.97%; B=3.13% to 4.30% L=2.02% to 2.64%.
Saturation time ± 2 min	K=2.80 to 4.66%; B=4.32% to 4.66% L=5.28% to 5.47%.

Note: some robustness parameters are above 5% e.g. mobile phase composition. Care should be taken to prepare it accurately.

Table no.02: Summary of the HPTLC Method validation parameters:

Stationary phase	**Pre-coated Silica Gel G60 F254 Aluminum**		
Solvent	Methanol		
Mobile phase	Toluene: MeOH: FA (8.5:1.5:0.1) (V/V/V)		
Temperature	Room temperature		
Chamber saturation time	20 min		
Band	8 mm		
Migration distance	80 mm		
Development time	17 min		
Derivatisation	Anisaldehyde Sulphuric acid followed by heating at 100 °C for		
Visualization	R 254, R 366, White Light		
Detection	371 nm (absorbance mode) Before derivatization 518 nm (absorbance mode) after derivatization		
Slit width	6*0.3mm		
Scanning speed	20 mm/s		
Rf value	Kaempferol	Beta-sitosterol	Lupeol
	0.27±0.01	0.51±0.01	0.63±0.01

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