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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR HYDROXYCHAVICOL ISOLATED FROM PIPER BETEL LEAVES

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Keywords:

Piper betel, Hydroxychavicol, HPTLC, validation, flash chromatography

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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 hydroxychavicol isolated from piper betel leaves powder. The isolation of hydroxychavicol was carried out using flash chromatography. Validation was carried out with pre-coated silica gel aluminum plate G 60 F254 (10 x 10 cm) as the stationary phase, mobile phase consisting of Butanol: Ethyl Acetate in the composition of 7:3 (v/v) and scanned in Absorbance Reflectance mode at 280 nm using Camag TLC scanner 3 with WinCAT software. The Rf value of 0.64 was obtained for isolated hydroxychavicol. The proposed method was validated over a linearity range of 200–800 ng/spot, and its percentage recovery was calculated along with intraday and interday precision. The limit of detection and the limit of quantification were found to be 13.890 ng/spot and 40.457 ng/spot, respectively. The suggested method can be successfully used to detect and quantify hydroxychavicol in herbal formulations.

INTRODUCTION: Hydroxychavicol, a phenyl propanoid compound isolated from the chloroform extraction of an aqueous extract of *Piper betel* leaves, showed inhibitory activity against oral cavity pathogens. Hydroxychavicol (HC), is known to possess antiproliferative activity at micromolar doses on various cancer cell lines of different origins while leaving normal cells unharmed [1]. It exhibited an inhibitory effect on all of the oral cavity pathogens tested (MICs of 62.5 to 500 g/ml) with a minimal bactericidal concentration that was two-fold greater than the inhibitory concentration [2]. The HPTLC technique is rapid, comparatively simple, robust, and extremely versatile.

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HPTLC not only confirms but also establishes its identity. It is also an ideal screening tool for adulterations and is highly suitable for the evaluation and monitoring of cultivation, harvesting, extraction processes, and testing of stability.

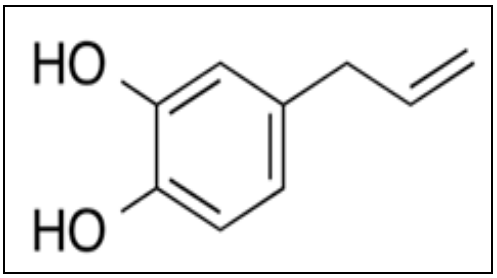

FIG. 1: STRUCTURE OF HYDROXYCHAVICOL

EXPERIMENTAL:

Isolation of Markers: Successful and efficient isolation of Hydroxychavicol from Piper betel was achieved by using Flash Chromatography as a separation technique. Purity was determined by HPLC, the isolated compound was authenticated

using IR-spectroscopy and UV-visible spectroscopy

Preparation of Stock Solutions: Stock solutions of Hydroxychavicol (1000µg/ml) were prepared separately by dissolving 10 mg of accurately weighed standard in 10 ml of ethanol.

Preparation of Working Solution: Working solutions were prepared from standard stock solutions of Hydroxychavicol by withdrawing aliquot of 1 ml from stock solutions of marker compound and transferred in separate volumetric flasks of 10 ml. The volume was made up with ethanol to obtain solutions of 100 ppm. Solutions for calibration curve were prepared such that application of 20 μl volume gave a series a spot covering over range of 200-800 ng/spot (200, 300, 400, 500, 600, 700, 800ng/spot). These ranges were used for the construction of calibration curve.

Preparation of Sample Solution: Tablets were triturated and approximately 2gm of powder weighed and extracted with 30 ml of ethanol by maceration method for 30 min. The solution was further cooled and filtered to get ethanolic extract. 1 ml of the above extract was diluted to 10 ml with ethanol and used for further analysis.

HPTLC Method Development (Optimization of Mobile Phase): Optimization of the mobile phase is one of the most important steps in development of a HPTLC method. Mobile phase selection was done on Trial and error basis. 20 μl of hydroxychavicol was applied and were used to study chromatographic behavior. There are number of factors which should be considered while selecting the mobile phase. For example, solubility of the marker, chemical nature of the marker, melting point of the marker, polarity of marker, mobile phase and stationary phase etc. Different combinations of solvents were tried to obtain a mobile phase in which both the markers show good separation and give quantifiable sharp peaks with no fronting or tailing.

TABLE 1: DEMONSTRATES THE VARIOUS COMPOSITIONS TRIED FOR OPTIMIZATION OF MOBILE PHASE COMPOSITION

Sr. no.	Mobile Tried	Phase	Composition	Application (µl)	Observation	Inference
1.	Butanol: Ethyl Acetate: HCL		4: 4: 2	20	R_f was found to be very less	Butanol: Ethyl Acetate in the composition of 7:3 (v/v) were optimized for method development
2.	Butanol	: Ethyl Acetate	5: 5	20	R_f was found to be very less	
3.	Butanol	: Ethyl Acetate	6: 4	20	Broad peak	
4.	Butanol	: Ethyl Acetate	7: 3	20	Sharp peak with desired $R_{f=}$0.64.	

HPTLC Method Validation: 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: Specificity is the ability to assess unequivocally the analyte in presence of components which may be expected to be present. Specificity was confirmed by comparing the Rf value and UV spectra of the standard marker compounds with chromatogram of the components obtained from the extract of ODTs Tablet. UV spectrum of marker compound was overlaid on the UV spectra of extract of formulated tablet.

Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity was evaluated by analysing the plot of area and plotting a graph of area v/s concentration. The test results obtained were assessed by calculating regression coefficient against concentration of analyte and the results of test are evaluated by calculation of regression coefficient (R2). Standard stock solution was diluted to obtain 200–800 ng/spot solution of hydroxychavicol espectively. Three sets of such solutions were evaluated. Every set was analyzed to obtain a calibration curve. The standard deviation (SD), coefficient of determination (r^2), slope and intercept of the calibration curve were estimated to determine the method linearity.

Accuracy (Recovery): The accuracy of an analytical procedure expresses the closeness of

agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Recovery of hydroxychavicol from formulation was checked by spiking a known quantity of standards at three concentration level (*i.e.,* 80%, 100%, and 120% of the quantified amount) to the test samples in triplicate using HPTLC. This way recovery was calculated for nine determinations over a specified range and mean recovery was calculated.

Precision: The present method was validated for intraday and interday precision. Intraday precision was determined in triplicate with the same method on the same day for three different concentrations of (200, 500 and 800 ng/spot) respectively. The interday precision of the method was verified by per-forming a similar method on different days under the same set of experimental situations. There peatability of the sample application and calculation of the peak area for the analyte were articulated in terms of the %RSD.

Limit of Detection (LOD): The detection limit of an individual analytical procedure is the lowest amount of analyte in sample which can be detected but not necessarily quantitated as an exact value. 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 they-intercept. The Limit of Detection was expressed as:

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

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

Limit of Quantitation (LOQ): The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

$$LOQ = 10\sigma / S$$

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

Robustness: The robustness of an individual analytical procedure is a measure of its capacity to remain unaffected by small, but intentional changes in method parameters and provides evidence of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. Following the introduction of small changes in the mobile phase composition (±0.2 ml for major component), the effect on the results was examined.

The amount of mobile phase was varied over the range of ± 5%. The saturation time of development chamber was varied by ± 5 min. The robustness of the method was determined at concentration level (200-800 ng/spot) for hydroxychavicol.

RESULT AND DISCUSSION:

Analytical Method Development: A new, simple and rapid method was developed for the formulation. The developed method was applied for the hydroxychavicol in the herbal extract tablet.

TABLE 2: OPTIMIZED CONDITIONS FOR VALIDATION

Sr. no.	Parameters	Optimized Chromatographic Conditions
1.	Stationary phase	Silica Gel 60 F_{254} precoated HPTLC plates,
2.	Mobile phase composition	Buthanol: Ethyl Acetate (7:3 V/V)
3.	Sample application. Distance between the tracks	20µ, 6mm
4.	Chamber saturation time	20 minutes
5.	Temperature (^{0}C)	25±2
6.	Relative humidity (%)	55±5
7.	Technique of separation	Ascending
8.	Total amount of mobile phase used	9.3Ml
9.	Solvent front	90 mm
10.	Migration time	20 minutes
11.	Densitometric evaluation - Detection wavelength	280 nm

TLC Plate was Developed in the Mobile Phase: Butanol: Ethyl acetate in the composition of 7:3 (v/v).

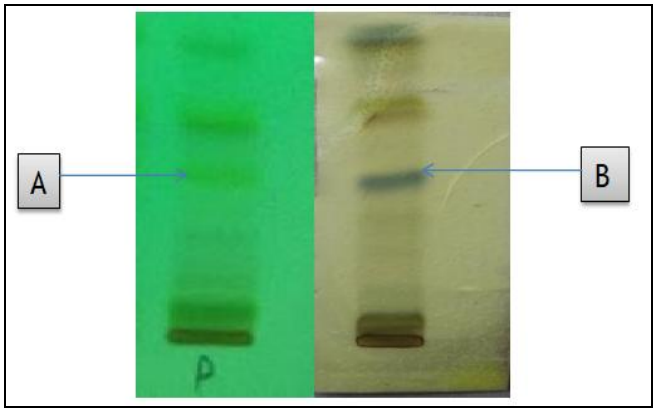

FIG. 2: TLC OF (A) PIPER BETEL EXTRACT (B) P. BLUE COLORATION FORMED AFTER SPRAYING WITH $FeCl_3$ REAGENT DETERMINED THE PRESENCE OF HYDROXYCHAVICOL.

After carrying out several trials by changing mobile phase composition along with injection volume Butanol: Ethyl Acetate in the composition of (7: 3V/V) were optimized for method development and validation of HCV. The representative chromatogram as given in **Fig. 2**. The Rf was found to be 0.64.

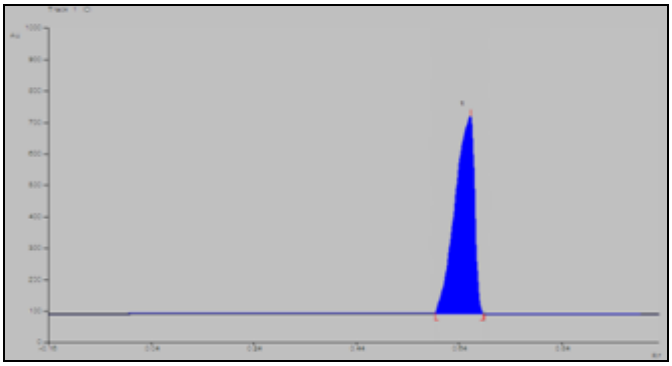

FIG. 3: TLC DENSITOGRAM SHOWING PEAK OF HYDROXYCHAVICOL. (RF= 0.64)

HPTLC Analytical Method Validation:

Specificity: When the densitogram of standard was overlaid with the densitogram of sample (tablet extract) it was observed that the densitogram of standard was exactly matching with the densitogram of tablet extract. Therefore, the method isspecific.

Linearity: Linear relationship was observed by plotting peak area against sample concentration. The calibration graph indicated that HCV produced a linear response across the range of 200-800ng/spot **Fig. 3.**

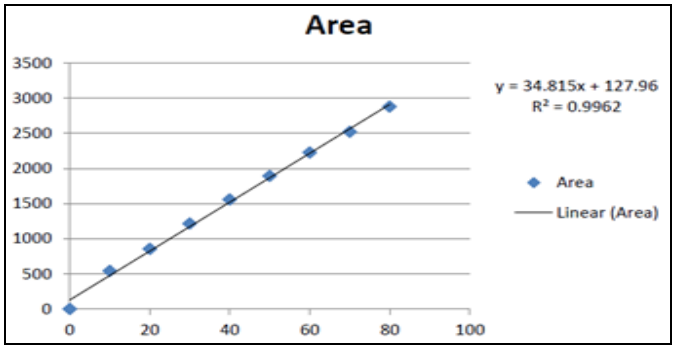

FIG. 4: LINEARITY OF HYDROXYCHAVICOL

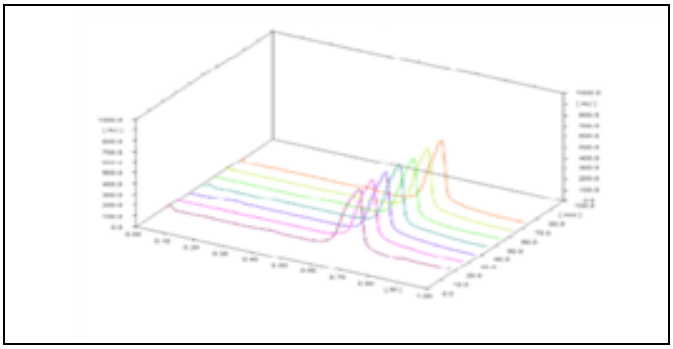

FIG. 5: GRAPH SHOWING LINEARITY OF HYDROXYCHAVICOL

TABLE 3: LINEAR REGRESSION DATA OF CALIBRATION PLOT FOR HCV

Sr. no.	Parameters	Results
1.	Range	200-800ng/spot
2.	R^2	0.9997
3.	y- intercept	205.64
4.	Slope	3.3444

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 HCV. Results obtained are given in **Table 4.**

TABLE 4: ACCURACY-RECOVERY STUDIES

Drug	Level of percentage recovery (%)	Amount present inextract (ng/spot)	Amount of standard added	Total amount (ng/spot)	% recovery	Average % recovery	% RSD	Inferences

HCV	80	500	400	900	98.79		0.1486	Acceptable
	100	500	500	1000	101.04	100.06	1.0142	recovery
	120	500	600	1100	100.37		1.6030	was found

Precision:

Intra-day Precision: It was performed at three different concentration levels low (200ng/spot), mid (500ng/spot) and high (800ng/s pot) respectively within the same day at three different times (session 1, 2, 3) [3, 9].

TABLE 5: INTRA-DAY PRECISION STUDIES

		HCV			Inference
Concentration levels		**Low**	**Mid**	**High**	
Concentration (ng/spot)		200	500	800	Acceptable % RSD,
Peak Area	Session 1	1876	3884.1	5881.5	hence precise
	Session 2	1874	3883.5	5883.1	
	Session 3	1875	3889.3	5885.2	
Average peak area		1876.0	3888.6	5882.4	
Standard deviation		2.27	5.72	7.15	
% RSD		0.12	0.14	0.12	

Inter-day Precision: It was carried out at same concentration levels on three consecutive days, using same homogeneous sample [9].

TABLE 6: INTER-DAY PRECISION STUDIES

		HCV			Inference
Concentration levels		**Low**	**Mid**	**High**	
Concentration (ng/spot)		200	500	800	Acceptable % RSD,
Peak area	Day 1	1874.1	3895.3	5880.6	hence precise
	Day 2	1876.4	3894.2	5878.4	
	Day 3	1879.1	3892.2	5884.8	
Average peak area		1876.0	3893.2	5881.6	
Standard deviation		2.15	2.00	2.95	
% RSD		0.11	0.05	0.06	

The % RSD values for both intra-day and inter-day precision were found within acceptable limit as shown in tables and respectively.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Values of LOD and LOQ calculated using slope of calibration curve are tabulated in table.

TABLE 7: LOD AND LOQ

Parameters	Calculated values
LOD	13.890ng/spot
LOQ	40.457ng/spot

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 standard deviations (%RSD). From the results obtained it was concluded that the developed method was found to be robust [9].

TABLE 8: ROBUSTNESS RESULTS

Robustness parameters	Parameters changed	%RSD of Area
Mobile phase composition (v/v)	Buthanol: Ethyl acetate (7.5:2:5 v/v)	0.19
	Buthanol: Ethyl acetate (6.5:3.5 v/v)	0.14
Chamber saturation time (20minutes)	+ 2	0.14
	-2	0.17

CONCLUSION: HPTLC simultaneous estimation method for detection of hydroxychavicol 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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