ANALYTICAL METHOD DEVELOPMENT FOR PIPER BETEL LINN.

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ABSTRACT

HPTLC method which is simple, particular and robust has been developed for the hydroxychavicol. The method was validated using parameters such as linearity, specificity, and precision, limit of quantification (LOQ), limit of detection (LOD), accuracy and robustness as per ICH guidelines. The present work deals with development of HPTLC method. Chromatographic separation of the drug was performed on Merck TLC aluminum plates pre-coated with silica gel 60F254 as the stationary phase. Butanol: Ethyl Acetate in the composition of 70:30 (v/v) were optimized for method development. The sample solutions were prepared in ethanol and linear ascending development was carried out in twin trough glass chamber and scanned at 280 nm using Camag TLC scanner. The marker was resolved successfully with Rf values 0.64±0.02 for hydroxychavicol respectively. The regression analysis data indicated good linear relationship for the calibration plots for hydroxychavicol in the range of 200-800 ng/spot and regression coefficient was 0.9997.

Introduction

Materials and Method

Standards and Reagents

All the chemicals of LR grade were procured from S.D. fine chemicals, Mumbai, Maharashtra, India.

Analytical standard hydroxychavicol was isolated in the lab by flash chromatography.

Instrumentation

Chromatographic separation was attained on HPTLC plates using Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with 100µl Hamilton syringe. TLC scanner 3 with win CATS software was used for detection of samples.

Preparation of standard solution

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 spots covering over range of 200-800 ng/spot (200, 300, 400, 500, 600, 700, 800 ng/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 30min. 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.

Optimization of chromatographic conditions

The samples were spotted in the form of bands (6 mm width) with a Hamilton microliter syringe (100 µL) under a controlled nitrogen stream using a Cammag Linomat V sample applicator The slit dimension was kept at 5 mm × 0.45 mm and a scanning speed 10 mm/s. Precoated TLC silica gel aluminium Plates 60 F254 (20 cm × 10 cm, 250 m thickness, Merck, Darmstadt, Germany) were utilized. The chromatographic

ascending development was performed using a mixture of mobile phase Buthanol : Ethyl Acetate(7:3 V/V) to a migration distance of 80 mm. The chamber was previously saturated for 20 min at temperature 25±2°. Densitometric scanning was achieved over Cammag TLC scanner III operated using win CATS software (V 1.44 CAMAG). The source of radiation used was a UV spectrophotometer. The obtained bands were scanned at a wavelength of 280 nm. After completion of scanning the R_f values, peak areas and spectra for the marker were recorded**.** The marker were resolved successfully with R_f values 0.64 ±0.02 hydroxychavicol, respectively.

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

The ability to assess unequivocally the analyte in presence of components which may be expected to be present is known as specificity. Specificity was confirmed by comparing the R_f 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 ability to get test results which may be directly proportional to the concentration (amount) of analyte in the sample is the linearity. Linearity was evaluated by analyzing 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 respectively. Three sets of such solutions were evaluated. Every set was analyzed to obtain a calibration curve. The standard deviation (SD), coefficient of determination (r2), slope and intercept of the calibration curve were estimated to determine the method linearity.

Accuracy (Recovery)

The accuracy is 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. The repeatability 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 smallest amount of analyte which is present in a sample which can be detected but not necessarily quantitated with 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 the y-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 is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and as well as with the help of accuracy. The quantitation limit is one of most suitable the 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.

The LOD and LOQ are expressed as:

$LOD = 3.3 \sigma/S$

$LOQ = 10 \sigma/S$

Where, σ = Standard deviation of response, S = Slope of the calibration curve both of them are obtained from the calibration curve of the individual maker compound.

Robustness

The robustness is a measure of its capacity to remain unaffected by small change, but intentional changes in method parameters and on additionally provides an evidence of its reliability during normal usage. The robustness was studied by changing 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 mobile phase was varied over the range of ± 5% amount. 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.

Results and Discussion

Selection of Analytical wavelength

Analytical methods

- **UV method- Construction of calibration curve**

Lambert-Beer's law is found to be obeying in the concentration range of 0-25 ppm (Figure). The slope and regression coefficient are given in Table.

Table 01: Results of calibration curve of HCV by UV method

Medium	Slope	Coefficient of Regression (R^2)
Ethanol	0.0183	0.9995

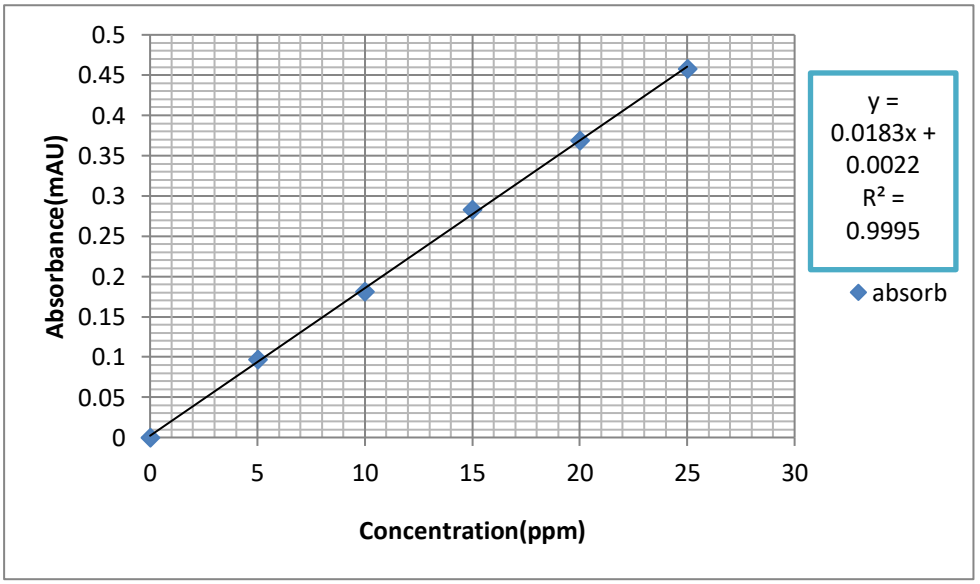

Figure 01: Calibration curve of HCV in Ethanol by UV method

HPTLC ANALYTICAL METHOD

After carrying out several trials by changing mobile phase composition along with injection volume Butanol : Ethyl Acetate in the composition of (70 : 30V/V) were optimized for method development and validation of HCV.

The representative chromatogram as given in figure 64 .The R_f was found to be 0.64

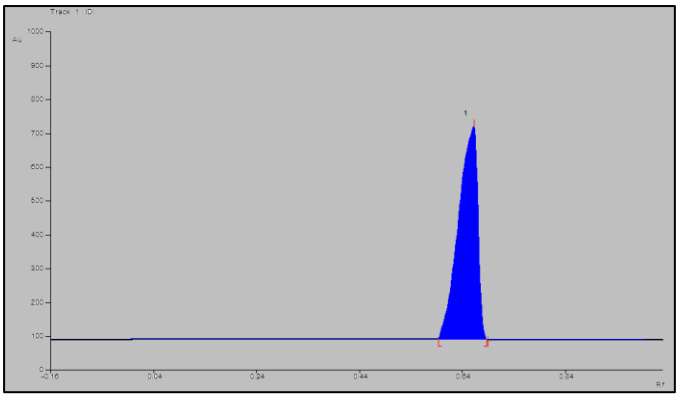

Figure 02. TLC Densitogram showing peak of Hydroxychavicol

Table 02: Optimization Trails of mobile phase for HCV (HPTLC)

Sr.no.	Mobile Pha Tried	Compositi (V/V/V	Application Volume (µL)	Observation	Inference
1	Butanol : Ethyl Acetate : Hcl	40 : 40 : 20	20	R_f was found to be very less	
2	Butanol : Eth Acetate	50 : 50	20	R_f was found to be very less	
3	Butanol : Eth Acetate	60 : 40	20	Broad peak	
4	Butanol : Eth Acetate	70 : 30	20	Sharp peak with de R_f=0.64.	

Table 03: Optimized Chromatographic Conditions for HCV (HPTLC)

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	a. Sample application b. Band width c. Distance between the tracks	20µL 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	90mm
10.	Migration time	20minutes
11.	Densitometry evaluation - Detection wavelength	280nm

TLC PLATE WAS DEVELOPED IN THE MOBILE PHASE

Butanol: Ethyl Acetate in the composition of 70:30 (v/v)

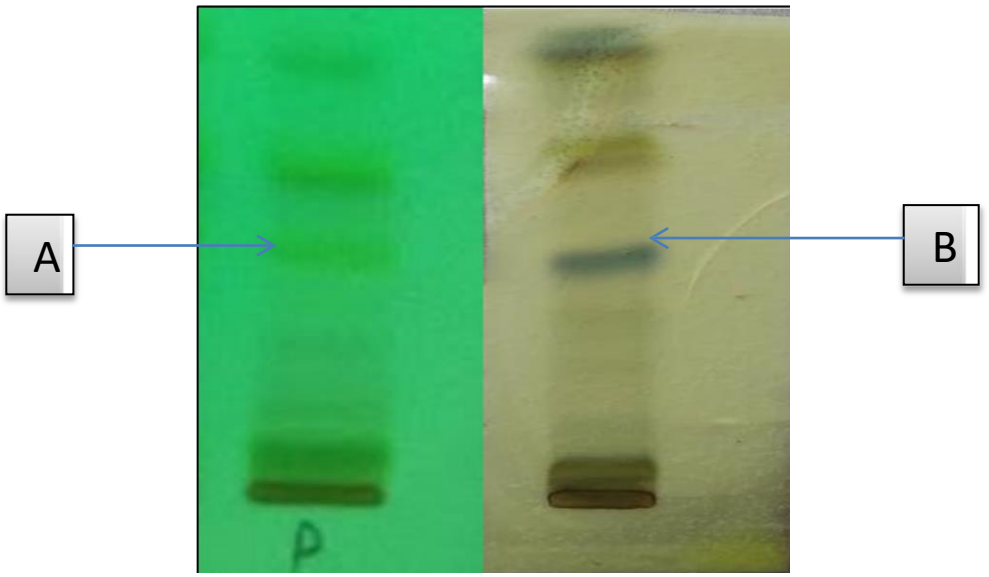

Figure 03. TLC of (A) Piper betel extract (B) P. Blue coloration formed after spraying with FeCl3 reagent determined the presence of Hydroxychavicol.

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The representative chromatogram as given in figure 64 .The Rf was found to be 0.64

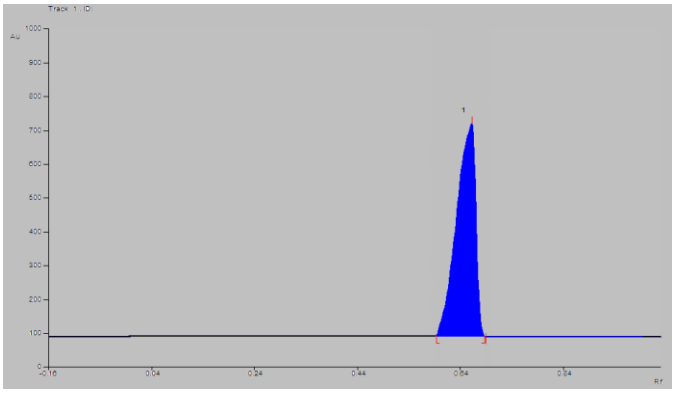


Figure 04. TLC Densitogram showing peak of Hydroxychavicol. (R_f= 0.64)

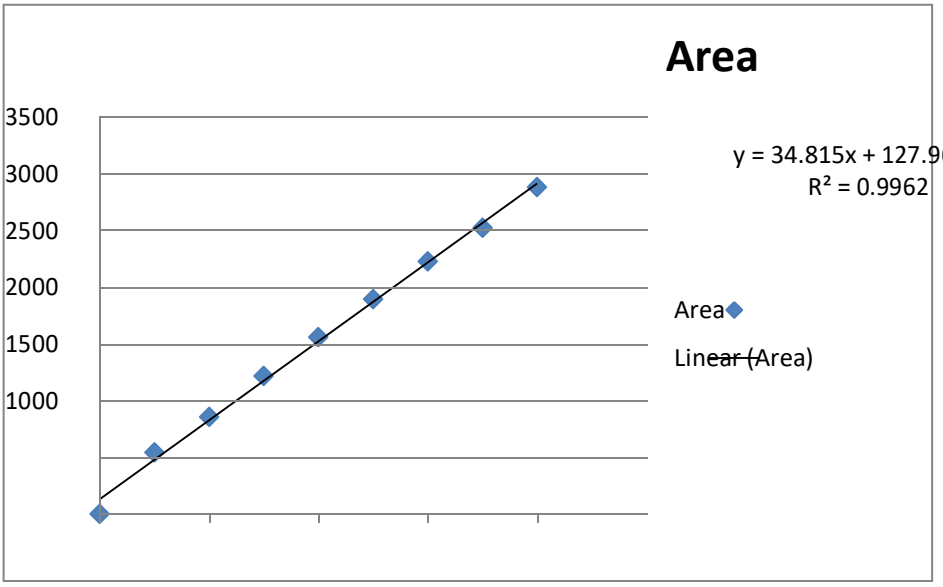
HPTLC ANALYTICAL METHOD VALIDATION

A) 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 is specific.

B) 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 (figure). The linear regression data of calibration plot for HCV is given in the table



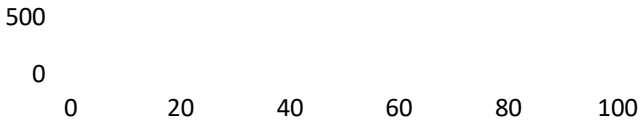


Figure 05: Calibration curve of HCV in Ethanol by UV method

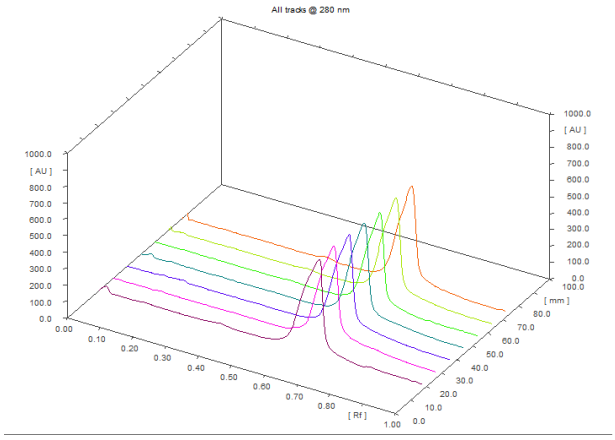


Figure 6: 3D Graph showing linearity

Table 04: 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

C) 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.

Table 05: Accuracy-recovery studies

Drug	Level of percentage recovery (%)	Amount present in extract (ng/spot)	Amount of standard added	Total amount (ng/spot)	% recovery	Average % recovery	% RSD	Inferences
	80	500	400	900	98.79		0.1486	
	100	500	500	1000	101.04		1.0142	
	120	500	600	1100	100.37		1.6030	

D) Precision:

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

Table 06: Intra-day precision studies

				HCV	Inference
Concentration levels		**Low**	**Mid**	**High**	
Concentration (ng/spot)		200	500	800	
	Session 1	1876	3884.1	5881.5	
	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.

Table 07: Inter-day precision studies

		HCV			Inference
Concentration levels		**Low**	**Mid**	**High**	
Concentration (ng/spot)		200	500	800	
	Day 1	1874.1	3895.3	5880.6	
	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.

E) 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 08: LOD and LOQ

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

F) 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 (R_f) 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

Table 09: Robustness results

Robustness parameters	Parameters changed	%RSD of Area
	Buthanol:Ethyl acetate (7.5:2:5 v/v)	0.19
	Buthanol:Ethyl acetate (6.5:3.5 v/v)	0.14
	+ 2	0.14
	- 2	0.17

Conculsion:

The developed and validated method was found to be simple , accurate, precise,reliable for the hydroxychavicol. This can be use as a standard technquie. Hydroxychavicol show showed good resolved spots with selected and optimized mobile phase.

The developed methods were validated as per ICH guidelines in terms of linearity, specificity, precision, accuracy, limit of detection, limit of quantitation and robustness.

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