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SIMULTANEOUS ESTIMATION OF TRIGONELLINE HYDROCHLORIDE AND BERBERINE DIHYDRATE BY USING HPTLC METHOD FROM HF/R/AD/310 POLYHERBAL ANTIDIABETIC TABLET

Unmesh Keshwar*, Deepmala Matey, Kashish Dhanrajani, Prajakta Pakade, Sharad Dhurde, B. K. Shrikhande

Siddhayu Ayurvedic Research Foundation Pvt. Ltd, Nagpur, Maharashtra, India.

Corresponding Author: Unmesh Keshwar
Siddhayu Ayurvedic Research Foundation Pvt. Ltd, Nagpur, Maharashtra, India.
Email id: hodald@siddhayu.com,

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ABSTRACT

Most of the herbal formulations available in the market widely used for the management of diabetes are not standardized with respect to its active ingredients marker molecule. The present study was designed with an objective of simultaneous estimation of Trigonelline hydrochloride and Berberine dihydrate by HPTLC from HF/R/AD/310 Polyherbal Antidiabetic Tablet. Chromatographic separation was achieved on pre-coated silica gel TLC aluminium plate 60 F_{254} using Ethyl Acetate: Methanol: Water: Glacial Acetic acid, 3:6:2:1 (v/v/v/v) as mobile phase followed by densito-metric measurement at two different wavelength 268 nm and 348 nm by TLC Scanner 4. The retention factors of Trigonelline hydrochloride and Berberine dihydrate was 0.25 and 0.66 respectively. The estimated amount of Trigonelline hydrochloride and Berberine dihydrate was found to be 0.02 % and 0.06 %. Results obtained in validation parameters indicate the accuracy and reliability of the developed simultaneous HPTLC method. The proposed method found to be simple, sensitive, selective and accurate for routinely used in Quality Control Lab.

KEYWORDS: Trigonelline hydrochloride, Berberine dihydrate, Antidiabetic Tablet, Polyherbal Tablet, HPTLC, Diabetes.

INTRODUCTION

According to the key facts pointed out by WHO, there was a 5% increase in premature mortality from diabetes between 2000 and 2016. Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation. In 2019, an estimated 1.5 million deaths were directly caused by diabetes.[1]

In Ayurveda, herbal formulations containing herbs like *Trigonella foenum-graecum and Berberis aristata* have been used for treating diabetes.[2-4] These herbs are considered to be effective and safe alternative option for replacement of synthetic drug medicines such as Metformin, Ondansetron and Pioglitazone which may cause side effects.[5,6]

Methi consists of seeds of *Trigonella foenum-graecum* Linn. (Fam. Fabaceae) an aromatic, 30-60 cm tall, annual herb, cultivated throughout India.[7] It contains the main alkaloid, Trigonelline which has a therapeutic potential for diabetes and central nervous system disease. Its properties include hypoglycemic, hypolipidemic and improving diabetic auditory neuropathy activity.[8,9]

Daruharidra consists of dried stem of *Berberis aristata* DC. (Fam. Berberidaceae); an erect, spinous, deciduous shrub, usually 1.8-3.6 m in height found in the Himalayan ranges at an elevation of 1000-3000 m, and in the Nilgiri hills in South India. [10] It contains an isoqinoline alkaloid, Berberine as the major bio-active constituent which has promising antidiabetic activity. An observational study of the antidiabetic activity of berberine in newly diagnosed type 2 diabetes mellitus patients shows improvement in the glycemic parameters comparable to metformin.[11]

Literature review revealed that various methods have been reported for analysis of Trigonelline hydrochloride and Berberine dihydrate in various herbal formulations by High Performance Liquid Chromatography (HPLC),[12-14] High Performance Thin Layer Chromatography (HPTLC),[15-18] and Ultra violet–visible spectroscopy (UV–Vis).[19] The developed HPTLC method has several advantages over other available methods such as ability to analyze several samples simultaneously in parallel, as well as using small quantities of solvents as a mobile phase which reduces time and cost of analysis.[20]

Keeping in mind the need of effective and safe option for treatment of diabetes, Siddhayu Ayurvedic Research Foundation Pvt. Ltd. has developed HF/R/AD/310 polyherbal Antidiabetic Tablet and standardized with respect to its active ingredients marker molecule. The proposed analytical method is also validated as per the International Conference on Harmonization (ICH) guidelines Q2 (R1).[21]

In the present study, an attempt has been made to develop a simple, rapid, accurate, precise and cost effective HPTLC method which can be routinely used in Quality Control Lab for simultaneous estimation of Trigonelline hydrochloride and Berberine dihydrate in HF/R/AD/310 polyherbal Antidiabetic Tablet.

MATERIALS AND METHODS

Ingredients used in HF/R/AD/310 polyherbal Antidiabetic Tablet

Antidiabetic Tablet was formulated using dried aqueous extracts derived from Daruharidra (*Berberis aristata),* Methi (*Trigonella foenum-graecum),* Vijaysar (*Pterocarpus marsupium),* Gudmar (*Gymnema sylvestre),* Manjistha (*Rubia cordifolia),* Giloy (*Tinospora cordifolia),* Saptarangi (*Salacia oblonga),* Vat chhal (*Ficus bengalensis),* Turmeric Extract (*Curcuma longa),* Amla Extract (*Emblica officinalis),* and Giloy Extract (*Tinospora cordifolia).* All these raw materials were screened for identity, purity and strength before formulation of tablet.

Table 1: Instrumentation details.

Instruments	Specification
HPTLC instrument	Camag, Switzerland
Sample applicator	Camag Linomat 5
Detection by	Camag TLC scanner 4
Visualizer	TLC Visualizer
Heating by	TLC Plate Heater III
Spray Cabinet	TLC Spray Cabinet II
Syringe	Hamilton (100 µl)
Software	winCATS (ver.1.4.9)
TLC Plates	Pre-coated Silica Gel 60 F_{254} TLC Plate

Reference standards

Reference standard Trigonelline hydrochloride (99%) and Berberine dihydrate (96.9%), were purchased from Natural Remedies, Bangalore, India.

Chemicals and reagents

All chemicals used throughout this work were of analytical grade or HPLC grade purchased from Merck Chemicals, India. Stationary phase was pre-coated silica gel aluminium plate 60 F_{254} was obtained from Merck, Germany.

Standard solution

The standard solution was prepared by weighing 1.5 mg of the reference standard Trigonelline hydrochloride and then transferred to 10 ml volumetric flask and volume was adjusted with HPLC grade methanol. Also the standard solution of Berberine dihydrate was prepared by dissolving 1.5 mg in 25 ml volumetric flask containing HPLC grade methanol.

Sample preparation

The contents of twenty tablets were grounded to a fine powder. An amount of 2.5 g powder was transferred to 25 ml volumetric flask containing 20 ml HPLC grade methanol and sonicated for 15 min. The solution was diluted up to the mark with HPLC grade methanol and filtered through Whatman filter paper. The resulting solution was used for the study.

Chromatographic condition

The standard and sample solutions were spotted in the form of bands, width 6 mm with a Camag 100 microliter syringe (Hamilton, Bonaduz, Switzerland) in a controlled nitrogen stream on silica gel pre-coated aluminium plate 60 F_{254} plates, using a Camag Linomat V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110˚C for 5 min prior to chromatography. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm/s. The mobile phase was Ethyl acetate: Methanol: Water: Glacial Acetic Acid (3.6:2:1 v/v/v/v). Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 20 min at room temperature (25˚C ± 2) at relative humidity 33 %, saturated with $MgCl_2$. The length of each chromatogram run was 7 cm. Following the development, the TLC plates were dried in a current of air with the help of a TLC plate heater. Densitometric scanning was performed using a Camag TLC scanner 4 in the reflectance /absorbance mode at two different wavelengths 268 nm and 348 nm. Evaluation was done using linear regression analysis through peak areas. The amount of Trigonelline hydrochloride and Berberine dihydrate were computed from peak areas. The chromatograms are shown in Figure 2-4.

Sample assay

Sample and standard solution were spotted in triplicates on a plate, developed under the same conditions. After development and drying of the plates, the analyte was found to be completely separated from other components; hence, the linear and compact zones were scanned at two different wavelengths 268 nm and 348 nm and peak areas Trigonelline hydrochloride and Berberine dihydrate were recorded.

Validation of the method

The proposed analytical method was validated for the parameters like linearity, specificity, accuracy, precision, limit of detection, limit of quantification, and robustness as per the International Conference on Harmonization (ICH) guidelines Q2 (R1).

RESULTS AND DISCUSSION

The mixtures of several mobile phases were tried on silica gel TLC plates for simultaneous estimation of Trigonelline hydrochloride and Berberine dihydrate. A mobile phase consisting Ethyl acetate: Methanol: Water: Glacial Acetic Acid (3.6:2:1v/v/v/v) gave good separation. Standard Trigonelline hydrochloride (*R*f = 0.25) and Berberine dihydrate (*R*f = 0.66) mentioned in Table 2, showed single peak in HPTLC chromatogram in Figures 2, 3 and HPTLC chromatogram of HF/R/AD/310 polyherbal Antidiabetic Tablet in Figures 4.

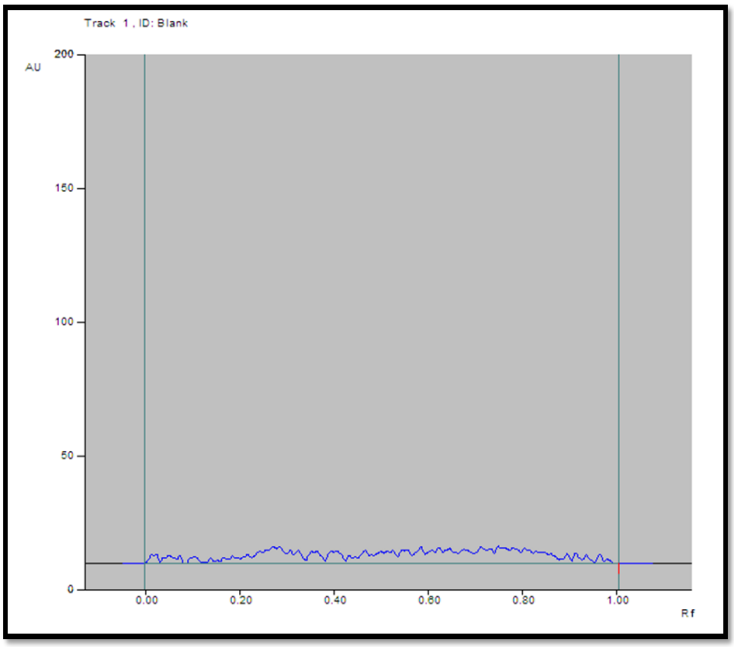

Figure 1: HPTLC chromatogram of blank solution.

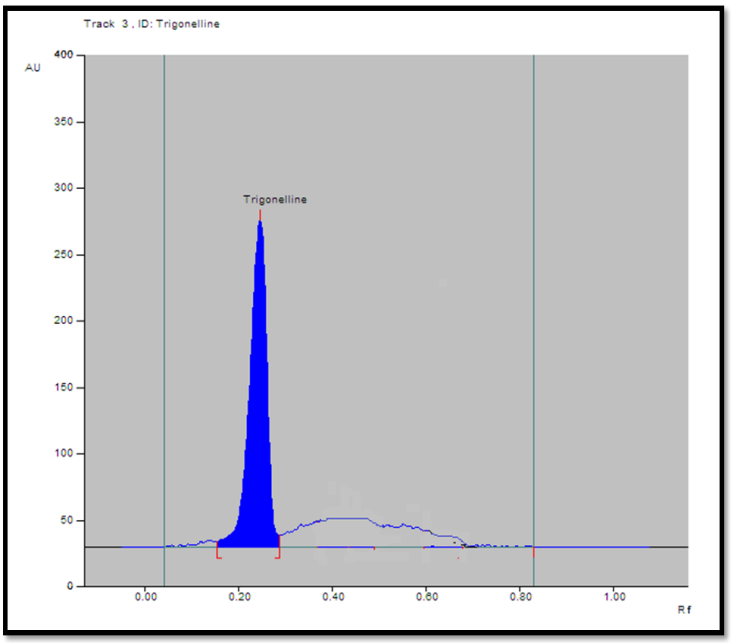

Figure 2: HPTLC chromatogram of standard Trigonelline hydrochloride solution.

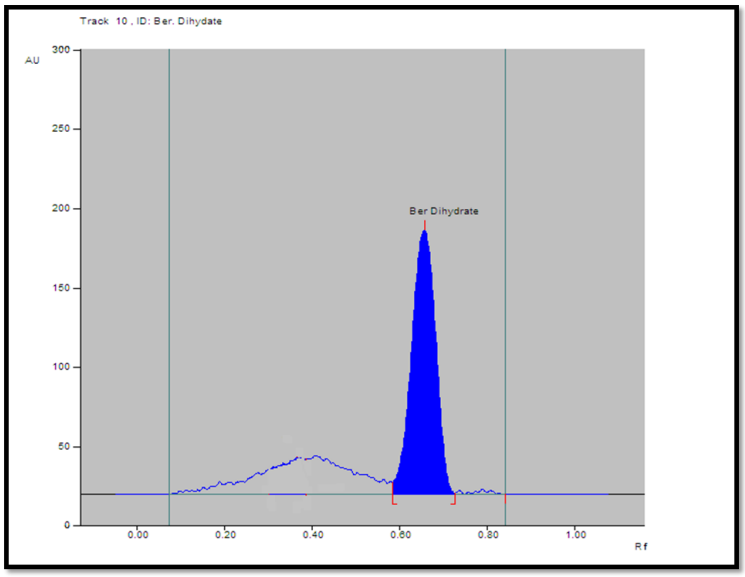

Figure 3: HPTLC chromatogram of standard Berberine dihydrate solution.

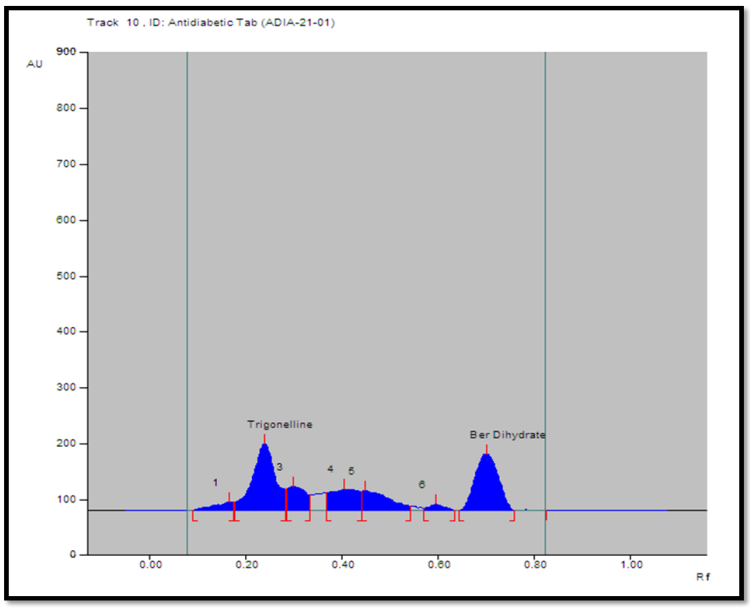

Figure 4: HPTLC chromatogram of HF/R/AD/310 polyherbal Antidiabetic Tablet solution.

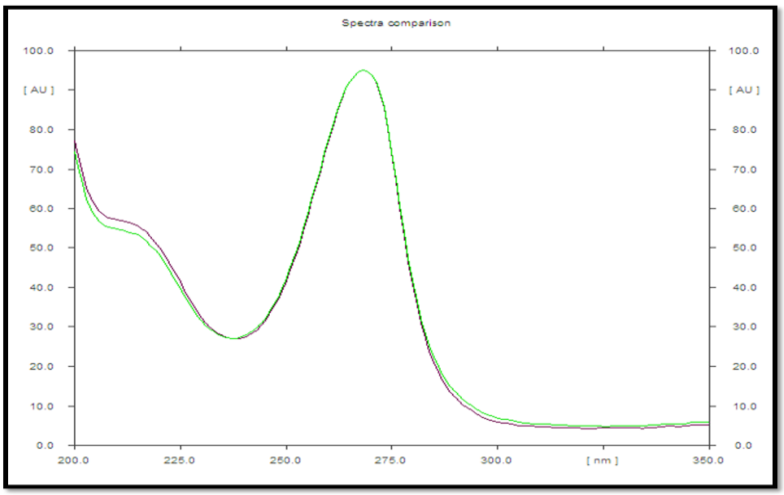

Figure 5: Overlain spectrum of standard Trigonelline hydrochloride solution and HF/R/AD/310 polyherbal Antidiabetic Tablet solution.

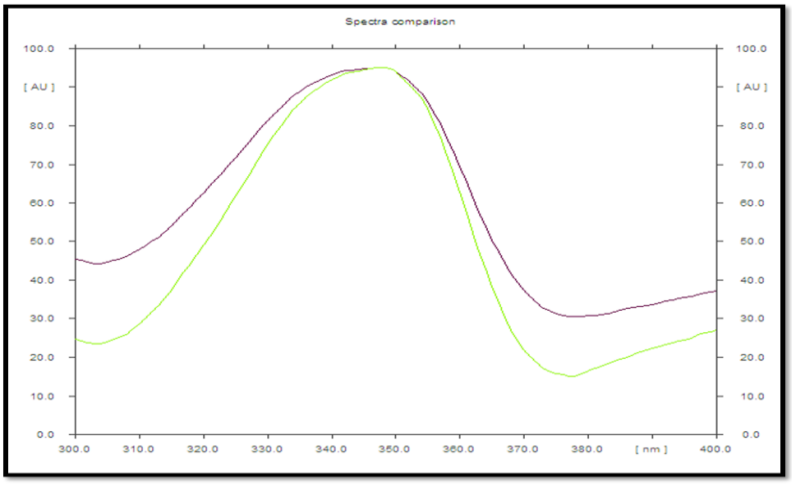

Figure 6: Overlain spectrum of standard Berberine dihydrate solution and HF/R/AD/310 polyherbal Antidiabetic Tablet solution.

Linearity

Linearity was performed by applying standard solution of different concentrations of Trigonelline hydrochloride and Berberine dihydrate. Linearity curve was obtained by plotting a graph of peak area vs. applied concentration. Linear regression data for the calibration curves of standard Trigonelline hydrochloride showed a good linear relationship over the concentration range of 300 - 1050 ng/band. The correlation coefficient (*R*2) was 0.998 and linear regression equation was found to be: Y = 6.936 x + 803.7, and Berberine dihydrate showed a good linear relationship over the concentration range of 240 - 840 ng/band. The correlation coefficient (*R*2) was 0.995 and linear regression equation was found to be: Y = 8.381 x + 899.5. All data were calculated and given in Table 2 and figure 7, 8.

Table 2: Linear regression data for calibration plots of the analyzed drugs using the proposed HPTLC methods.

Parameters	Results	
	Trigonelline hydrochloride	**Berberine dihydrate**
Wavelength (nm)	268	348
Retention factor (*R*f)	0.25	0.66
Linearity range (ng/band)	300 - 1050	240 - 840
Correlation coefficient (*r*2)	0.998	0.995
Slope	6.58	8.42
Intercept	803.77	899.56
LOD (ng/spot)	40.99	53.52
LOQ (ng/spot)	124.23	162.20

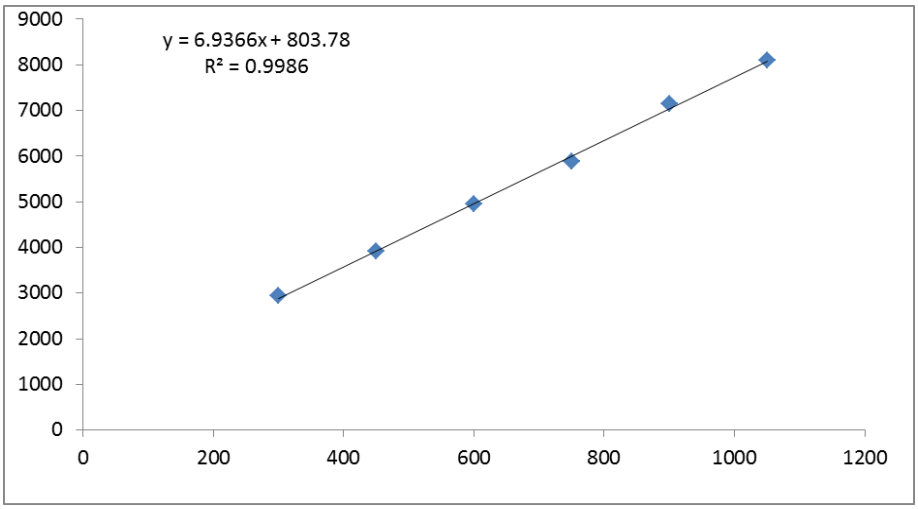

Figure 7: Calibration plot for standard Trigonelline hydrochloride solution.

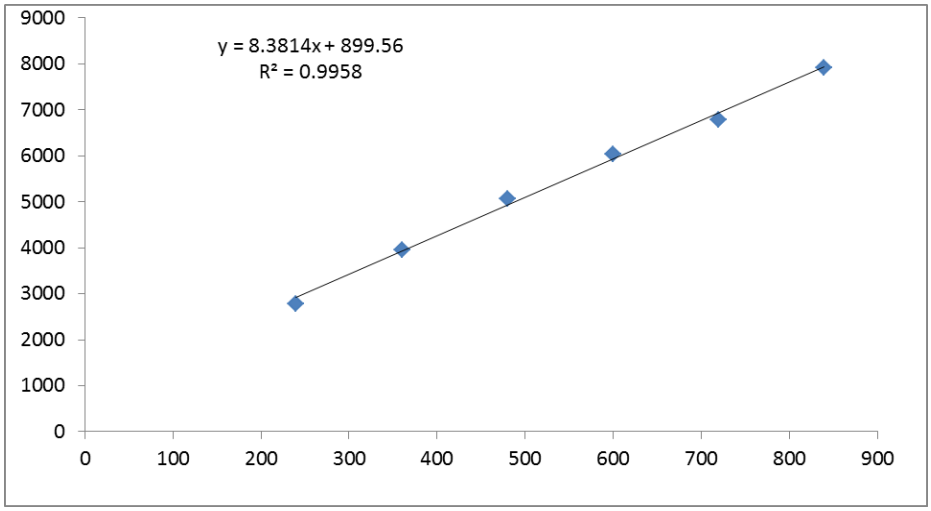

Figure 8: Calibration plot for standard Berberine dihydrate solution solution.

Specificity

The specificity of method was ascertained by applying methanol as blank, standard solutions and test sample on TLC plate. The solutions were spotted on TLC plate in triplicate and run. The band for of Trigonelline hydrochloride and Berberine dihydrate in the samples were confirmed by comparing the R_f values and spectrum with that of standards. The chromatogram of the polyherbal Antidiabetic tablet obtained using the developed method showed peaks at R_f of 0.25 and 0.66 for Trigonelline hydrochloride and Berberine dihydrate, respectively, and was found to be at the same R_f for both standard drugs chromatogram shown in figure 1-4.

Precision

The present method was validated for intraday and interday precision. The intra-day precision was assessed based on three replicates of three different concentration (600, 750 and 900 ng/band for Trigonelline hydrochloride; 480, 600 and 720 ng/band for Berberine dihydrate) on same day, and the measured peak area is expressed in terms of the per cent relative standard deviation (% RSD). The inter-day precision study was performed on three different days using the aforementioned concentrations of both drugs in triplicate. The repeatability of the sample application and calculation of the peak area for the analyte were articulated in terms of the % relative standard deviation (RSD) which was found to be less than 2.0 in all cases indicate no significant variations. The results were illustrated in Table 3.

Table 3: Intra-day and inter-day precision of Trigonelline hydrochloride and Berberine dehydrate.

Analyte	Conc. (ng/band)	Avg. Peak Area	SD	% RSD
Intra-day Precision				
Trigonelline hydrochloride	600	4950.40	26.37	0.53
	750	5884.97	27.83	0.47
	900	7142.47	42.51	0.60
Berberine dihydrate	480	5052.93	33.22	0.66
	600	6147.97	31.58	0.51
	720	6792.40	46.30	0.68
Inter-day Precision				
Trigonelline hydrochloride	600	4976.17	30.21	0.61
	750	5845.23	36.81	0.63
	900	7137.17	36.17	0.51
Berberine dihydrate	480	5096.90	43.16	0.85
	600	6145.20	36.64	0.60
	720	6762.03	29.97	0.44

Accuracy

The accuracy of the methods was determined by calculating recoveries of Trigonelline hydrochloride and Berberine dihydrate by the standard addition method. Known amounts of standard solution of Trigonelline hydrochloride (600, 750, 900 ng) and Berberine dihydrate (480, 600, and 720 ng) were added to pre-quantified sample solutions. Analysis was performed in triplicates and the average percentage recovery at each concentration level was evaluated. The results are illustrated in Table 4. The amount of drug recovered in accuracy study indicates that the method is accurate.

Table 4: Recovery Study of Trigonelline hydrochloride and Berberine dehydrate.

Compound	Amount Found (ng)	Amount of Std. Added (ng)	Theoretical amount of standard found (ng)	Amount of standard Recovered (ng)	Recovery (%)	Average Recovery (%)
Trigonelline hydrochloride	208.85	600	808.85	802.13	99.17	98.74
	208.85	600	808.85	798.80	98.76	
	208.85	600	808.85	795.11	98.30	
	208.85	750	958.85	933.95	97.40	97.72
	208.85	750	958.85	943.19	98.37	
	208.85	750	958.85	933.84	97.39	
	208.85	900	1108.85	1104.54	99.61	98.80
	208.85	900	1108.85	1084.70	97.82	
	208.85	900	1108.85	1097.29	98.96	
Berberine dihydrate	632.14	480	1112.14	1107.00	99.54	99.32
	632.14	480	1112.14	1098.28	98.75	
	632.14	480	1112.14	1108.35	99.66	
	632.14	600	1232.14	1214.90	98.60	99.45
	632.14	600	1232.14	1234.48	100.19	
	632.14	600	1232.14	1226.74	99.56	
	632.14	720	1352.14	1332.22	98.53	98.86
	632.14	720	1352.14	1339.66	99.08	
	632.14	720	1352.14	1338.15	98.97	

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were calculated by using the values of slopes and intercepts of the calibration curve. The limit of detection (LOD) values for Trigonelline hydrochloride and Berberine dihydrate were 40.99 and 53.52 ng, respectively, and the limit of quantification (LOQ) values were 124.23 and 162.20 ng, respectively, which shows the adequate sensitivity of the method all results given in Table 2.

Robustness

The estimations were performed by introducing variations in the mobile phase distance development, saturation time in development chamber and change in mobile phase composition; the effects on the results were examined. Mobile phase development distance was changed by ±1 cm. The saturation time of mobile phase in the chamber was varied by ±1 min. The composition of Methanol in mobile phase was changed by ± 0.2 ml. The % RSD was found to be less than 1.0 in all cases indicates no significant variations in the analysis of Trigonelline hydrochloride at the concentration 750 ng and for Berberine dihydrate at the concentration of 600 ng. The summary of the robustness study is given in Table 5 and 6.

Table 5: Robustness evaluation of the method for Trigonelline hydrochloride.

Std. Conc. (ng/band)	Robustness Parameter		Average Peak Area	SD	RSD (%)	Average RSD (%)
750	Development Distance (cm)	6	5334.77	14.26	0.27	0.44
		7	5352.60	31.37	0.59	
		8	5344.03	24.28	0.45	
	Saturation Time (minute)	19	5318.17	18.32	0.34	0.43
		20	5352.60	31.37	0.59	
		21	5373.03	19.68	0.37	
	Mobile Phase Composition (Change in Methanol composition)	5.8	5374.93	21.26	0.40	0.50
		6.0	5352.60	31.37	0.59	
		6.2	5357.57	28.47	0.53	

Table 6: Robustness evaluation of the method for Berberine dehydrate.

Std. Conc. (ng/band)	Robustness Parameter		Average Peak Area	SD	RSD (%)	Average RSD (%)
600	Development Distance (cm)	6	6034.77	14.26	0.24	0.36
		7	6046.87	26.16	0.43	
		8	6044.03	24.28	0.40	
	Saturation Time (minute)	19	6018.17	18.32	0.30	0.35
		20	6046.87	26.16	0.43	
		21	6073.03	19.68	0.32	
	Mobile Phase Composition (Change in methanol composition)	5.8	6074.93	21.26	0.35	0.42
		6.0	6046.87	26.16	0.43	
		6.2	6057.57	28.47	0.47	

Estimation of Trigonelline hydrochloride and Berberine dihydrate from HF/R/AD/310 Polyherbal Antidiabetic Tablet

Estimation of Trigonelline hydrochloride and Berberine dihydrate was performed by the developed HPTLC method. The analysis was repeated in triplicate. Area was recorded. Percentage assay was determined from linearity equation for Polyherbal Antidiabetic Tablet. The assay % content for Trigonelline hydrochloride and Berberine dihydrate were found to be 0.02 % and 0.06 %, respectively results are illustrated in Table 7.

Table 7: Results for simultaneous estimation of Trigonelline hydrochloride and Berberine dihydrate in HF/R/AD/310 Polyherbal Antidiabetic Tablet.

Analyte in Antidiabetic Tablet	Sample conc. (µg/band)	Average Peak Area	Average Amount Found (ng)	Average Amount found (%)	% RSD
Trigonelline hydrochloride	1001.72	2252.7	208.85	0.02 ±0.0002	0.88
Berberine dihydrate	1001.72	6197.43	632.14	0.06 ±0.0007	1.04

CONCLUSION

The developed HPTLC method for simultaneous estimation of Trigonelline hydrochloride and Berberine dihydrate in HF/R/AD/310 Polyherbal Antidiabetic Tablet was found to be simple, sensitive, selective and accurate. The method can be used for routine analysis in Quality Control Lab.

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