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Research Article

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF VICINE, TRIGONELLINE AND WITHAFERIN-A IN A POLYHERBAL ANTIDIABETIC FORMULATION

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ABSTRACT

Purpose: Vicine, trigonelline and withaferin-A are bioactive markers for commonly used herbs *Momordica charantia*, *Trigonella foenum-graceium* and *Withania somnifera*, respectively. No analytical methods reported so far, associated with quality control of polyherbal formulations containing these three drugs. Therefore, there is a need to develop a sensitive, simple, rapid, and reliable method that can simultaneously determine these markers in their combinations. In this regard high performance thin layer chromatography (HPTLC) method has been developed and validated as per ICH guidelines.

Method: The chromatographic analysis was performed on silica gel 60 F_{254} aluminum-backed TLC plates of 0.2 mm layer thickness. The stationary phase was pre-derivetized with the use of 0.02 M sodium acetate. The mobile phase system comprised of n-butanol: acetic acid: water 5:1:5 v/v/v, produced compact spots for vicine, trigonelline and withaferin-A, scanned at wavelength of 235 nm.

Result: The proposed method has shown the linearity in the range of 100-600 ng $band^{-1}$, 50-300 ng $band^{-1}$ and 100-600 ng $band^{-1}$ for vicine, trigonelline and withaferin-A, respectively. The proposed method has been validated as per ICH guidelines for its precision, recovery, robustness and accuracy.

Conclusion: The developed HPTLC method holds potential for detection and quantification of vicine, trigonelline and withaferin-A in polyherbal formulation that contain *Momordica charantia*, *Trigonella foenum-graceium* and *Withania somnifera*.

Keywords: HPTLC, Karela, Aswagandha, Methi, Standardization.

INTRODUCTION

Globally, 80% of the indigenous population in developing countries relies upon traditional medicine and medicinal plants as primary healthcare [1]. The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine [2]. This could be achieved only, if herbal products are evaluated and analyzed using modern sophisticated techniques of standardization.

Vicine, a glyco-alkaloid (Figure 1a) present in all parts of *Momordica charantia* (Karela) possesses the antidiabetic potential, used as a biological marker for karela products [3, 4**]**. Trigonelline is a major alkaloid (Figure 1b) present in *Trigonella foenum-graceium* (Methi) seeds, reported to possess hypoglycemic effect in normal and alloxanized diabetic rabbits [5]. Withaferin-A, steroidal lactones with ergostane skeleton (Figure 1c), is one of the important chemical present in *Withania somnifera* (Aswagandha) roots, possess immune-modulatory activities [6]**.**

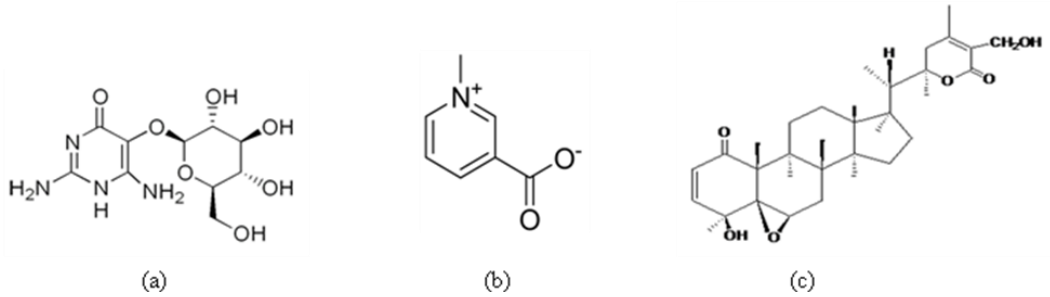

Fig. 1: Chemical structures of vicine (a), trigonelline (b) and withaferin-A (c)

A comprehensive literature survey revealed **that there is no** suitable procedure for the simultaneous determination of these selected markers in any polyherbal formulation. In past two decades, HPTLC has emerged as an efficient tool for the quality control of herbal drugs [7-9]. HPTLC remains the method of choice because of its advantages of reliability in quantitation of analytes at nano-gram levels and cost-effectiveness. Furthermore, it permits the simultaneous assay of several components in a multi-component formulation/herbal extracts [10-12].

Therefore, the aim of present work was to develop and validate a simple HPTLC method for the simultaneous determination of vicine, trigonelline and withaferin-A in the polyherbal formulations comprised of karela, methi and aswagandha extracts.

MATERIALS AND METHODS

Instrumentation and chromatographic conditions

A HPTLC system (Camag, Switzerland) equipped with a sample applicator Linomat V fitted with a 100 µL syringe (Hamilton, Switzerland), TLC Scanner III, Wincats 4.02, integration software (Camag, Switzerland) used for the analysis and documentation. The analysis were performed on precoated silica gel 60 F_{254} aluminium backed TLC plates (20 × 10 cm), pre-derivetized with 0.02 M sodium acetate solution. For pre-derivatisation, the 0.02 M sodium acetate solution was prepared by dissolving the weighed amount of sodium acetate in the water. This solution was allowed to over run on the plates, using twin trough glass chamber, which resulted in the development of uniform sodium acetate layer on the plate. The

plates were dried using hair dryer (Philips, India). These dried plates were used for the purpose of separation. The mobile phase system comprised of n-butanol: acetic acid: water 5:1:5 v/v/v. The chromatographic separation was achieved using linear ascending development carried out in a twin-trough glass chamber. The optimized chamber saturation time for the mobile phase was 20 min at room temperature (25 ± 2 °C) at a relative humidity of 50 ± 5 %. TLC plates were allowed to run up to 8 cm. After development, the plates were dried using hair drier and densitometric scanning was performed. The slit dimension was 6 × 0.45 mm, and the scanning speed of 20 mm s^{-1} was employed. Quantitative evaluation of the plate was performed in the absorption mode at 235 nm.

Drug and chemicals

Standards, vicine, trigonelline and withaferin-A were procured from Natural remedies Pvt., Ltd. Bangaluru, India. The lyophilized polyherbal formulation (Form HA) developed at our laboratory, was analysed during the study. **All** the solvent used during the study were of HPLC grade and obtained from (CDH, New Delhi).

Sample extraction

One gram of a laboratory-developed formulation, Form HA (hydro-alcoholic extracts of karela, methi and aswagandha) suspended in 25 mL of methanol and sonicated for 10 min in ultra sonicator. The resultant solution was filtered through a 0.45 µm **syringe** filter. Twenty micro liter of **the** filtrate was applied in triplicates. The plates were developed and scanned as mentioned above. The peak areas were recorded, and the amount of vicine, trigonelline and withaferin-A was calculated using the calibration curve.

Preparation of standard solutions and linearity

The stock solution of the markers (0.5 mg mL–1 for trigonelline and 1 mg mL–1 for vicine and withaferin-A) were prepared by dissolving accurately weighed marker compounds (5 mg, 10 mg and 10 mg of trigonelline, vicine and withaferin-A, respectively) in methanol and volume was made up 10 mL in volumetric flask. One mL of prepared stock solution was mixed in another volumetric flask and volume was made up to 10 mL with methanol. The aliquots (1, 2, 3, 4, 5 and 6 µL) were applied on the pre-derivetized plate. That gave the concentration ranges of 100–600 ng band–1 for vicine and withaferin-A, and 50–300 ng band-1 for trigonelline.

Method validation

The proposed HPTLC method was validated for specificity, accuracy, precision, limit of detection and quantitation and robustness as per the ICH guidelines [13].

Precision

Instrument precision was checked by repeated scanning (n = 9) of same band of all the three markers at the concentration of 200 ng $band^{-1}$. The repeatability of the sample application and measurements of peak area was evaluated without changing the position of the plate. To study the intra-day precision different concentration levels of 100, 200, and 400 ng $band^{-1}$ of vicine and withaferin-A and 50, 100 and 200 ng $band^{-1}$ for trigonelline were spotted six times for intra and inter-day.

Specificity

The specificity of the method was ascertained by analyzing the markers band and the corresponding band in the sample. The presence of vicine, trigonelline and withaferin-A in the Form HA was confirmed by comparing the Rf values and spectra of the samples band with those of the standards. The peak purity was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the band.

Sensitivity

The sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantitation (LOQ). LOD was determined based on the lowest concentration detected by the instrument from the standard while the LOQ was determined based on the lowest concentration quantified in the sample.

Accuracy

Accuracy was determined by the standard addition method, where pre-analyzed sample were spiked with extra 50, 100 and 150 % of the standards and the mixtures were reanalyzed by the proposed method. The experiment was repeated for six times.

Robustness

It was studied by introducing small changes in the mobile phase composition, mobile phase volume, duration of chamber pre-saturation and analysing the different analyst. Robustness study of the method was done in six replicates at a concentration level of 600 ng $band^{-1}$ and for vicine and withaferin-A and 300 ng $band^{-1}$, for trigonelline. The % RSD of peak areas and Rf were calculated.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The stationary phase pre-derivetized with 0.02 M sodium acetate solution and the mixture of markers and Form HA were then allowed to develop in the mobile phase system comprised of n-butanol: acetic acid: water 5:1:5 v/v/v. These conditions produced the sharp and well defined peaks for all three markers (trigonelline (Rf-0.12), vicine (Rf-0.28) and withaferin-A (Rf-0.76)) from their mixture (Figure 2) as well as in the Form HA (Figure 3).

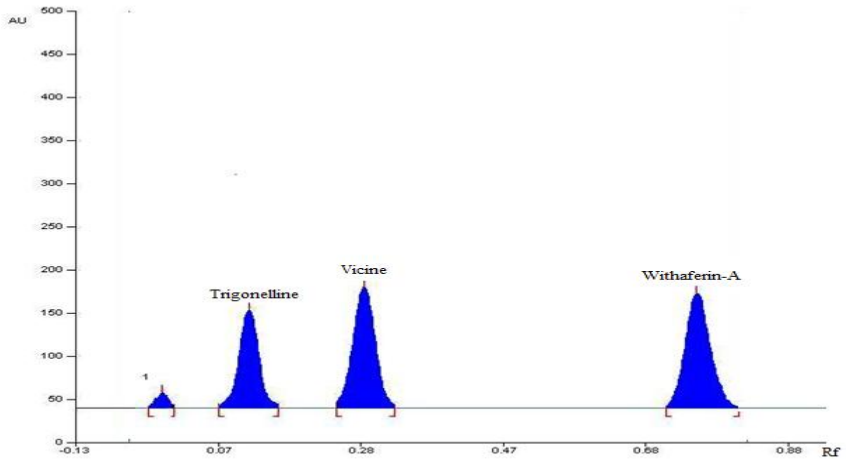

Fig. 2: HPTLC chromatogram of trigonelline (Rf 0.12), vicine (Rf 0.28), and withaferin-A (Rf, 0.76) from their mixtures.

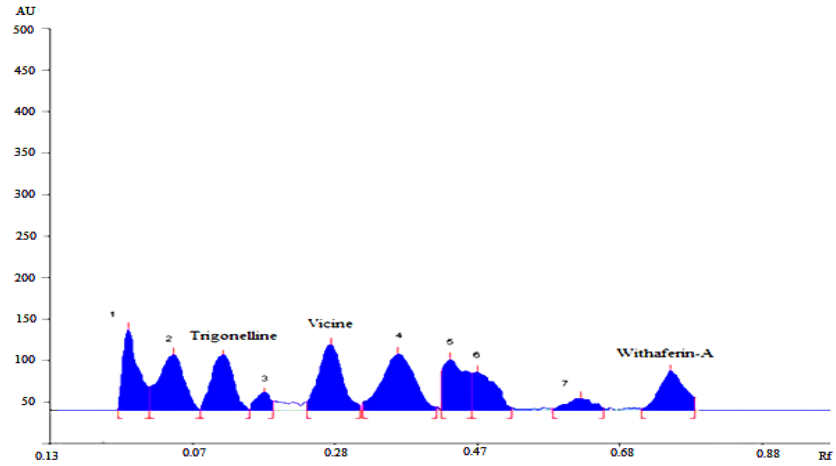

Fig. 3: HPTLC chromatogram of trigonelline (Rf 0.12), vicine (Rf 0.28) and withaferin-A (Rf, 0.76) separated from the Form HA

Table 1: Validation parameters, studied for the developed HPTLC method

Parameters	Withaferin-A	Vicine	Trigonelline
Absorption maxima (nm)	226	271	265
Linearity range (ng band^{-1})	100-600	100-600	50-300
Coefficient of determination (r^2)	0.9990	0.9961	0.999
Regression equation (Y^a)	451.7X + 674.5	445.8X + 665	362.1X + 615.7
Slope (m)	451.7	445.8	362.1
Intercept (c)	674.5	665	615.7
Limit of detection LOD, (ng band^{-1})	30	30	10
Limit of quantitation LOQ (ng band^{-1})	90	90	30
Precision, #Repeatability (% RSD)	0.12	0.10	0.18
*Intra-day (% RSD)	0.15	0.19	0.34
*Inter-day (% RSD)	0.12	0.16	0.23

= results of mean (n=9); * = results of mean (n=6); % RSD= relative standard deviation

Linearity

The six-point calibration curves for vicine and withaferin-A were found to be linear in the range of 100–600 ng band^{-1}, for trigonelline the range was 50–300 ng band^{-1}. These values revealed a good correlation coefficient for developed method (Table 1).

Validation

Precision

The instrument was precised based on measurement of peak area of repeatable sample applied and scanned. The percent RSD were 0.21, 0.34 and 0.23 for vicine, trigonelline and withaferin-A, respectively.

The % RSD for intra-day in the range of 1.10-1.68, 1.15-2.14 and 1.4-2.43 and for inter-day 2.07-2.65, 0.92–1.26 and 1.92-2.95 for three different concentrations tried for vicine, trigonelline and withaferin-A, respectively (Table 1).

Specificity

The bands of markers in the formulation were appeared at same Rf as for their respective markers. Furthermore, the compounds was confirmed by overlaying their UV absorption spectra with those of the standards (Figure 4 & 5). The results showed that the method is specific and effective enough to separate the markers from the combination of karela, methi and aswagandha extracts.

Sensitivity

Lower limits of detection obtained were 30 ng band^{-1} for vicine and withaferin-A, for trigonelline the value was 10 ng band^{-1} (Table 1). This indicated that the proposed method exhibits a good sensitivity for the quantification of above compounds.

Accuracy

The method afforded recovery in the range of 99.74 – 99.89 %, 99.80 – 100.06 % and 99.94 – 99.97 % for vicine, withaferin-A and trigonelline, respectively (Table 2). Results of the study showed high extraction efficiency and accuracy of the proposed method for all three compounds. This suggests that the proposed method can be used for determination of all three compounds at different concentration levels in polyherbal formulations.

Robustness

The study showed, when mobile phase composition was altered the % RSD for the peak area and Rf was in the range of 0.20 - 0.40 and 0.17 - 0.36, respectively. Moreover, the changes made in pre-saturation time and analysts, no significant effect had been observed on both peak areas and Rf of all three markers (% RSD<1). The results depicted in table 3.

Table 2: Results of the recovery study (n = 3)

Amount Spike (ng spot^{-1})			Amount found (ng spot^{-1})			% RSD			Recovery (% w/w)		
VC	WTH	TRG	VC	WTH	TRG	VC	WTH	TRG	VC	WTH	TRG
100	100	50	99.89	100.06	49.99	0.72	0.58	0.31	99.89	100.06	99.97
200	200	100	199.4	199.6	99.92	0.53	0.14	0.11	99.74	99.80	99.95
300	300	150	299.4	299.8	148.86	0.38	0.09	0.06	99.82	99.99	99.94

VC= vicine, WTH= withaferin-A and TRG= trigonelline; % RSD= relative standard deviation.

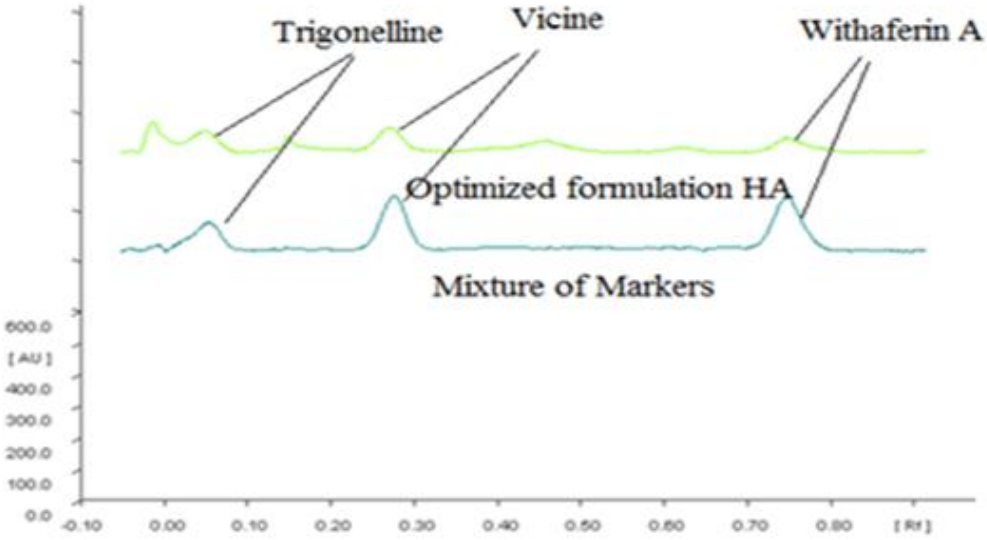

Fig. 4: 2-D Chromatograms of mixtures of markers and of the Form HA

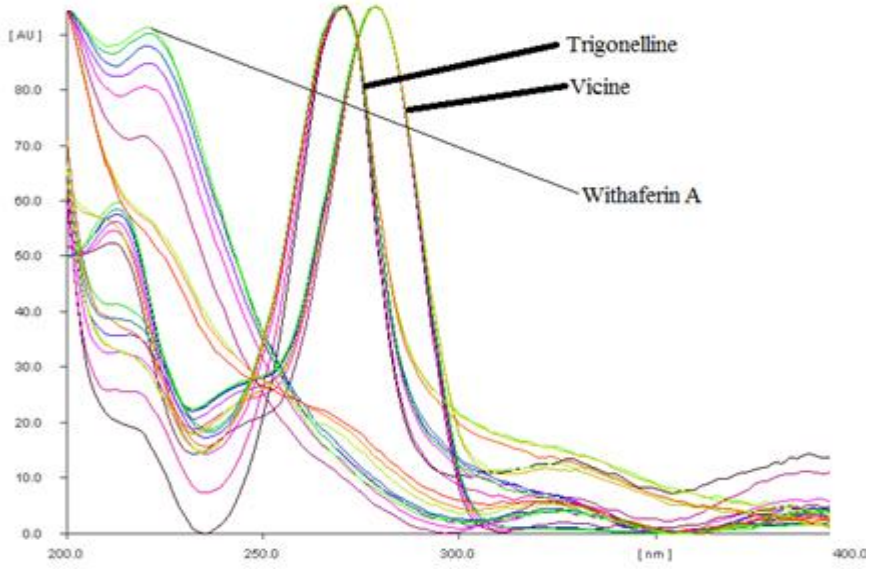

Fig. 5: Overlay UV absorption spectra of markers in the sample track with respective standards

Table 3: Robustness testing (n = 6)

Parameters	Alterations	% RSD for Rf (n=6)			% RSD for Area (n=6)		
		VC	WTH	TRG	VC	WTH	TRG
	-2%	0.31	0.28	0.36	0.30	0.40	0.30
Mobile phase	Optimized	0.24	0.15	0.30	0.21	0.39	0.20
	+2%	0.34	0.17	0.35	0.30	0.39	0.26
	10 min	0.41	0.39	0.34	0.13	0.54	0.40
Saturation Time	20 min	0.38	0.57	0.36	0.11	0.60	0.48
	30 min	0.42	0.61	0.27	0.15	0.39	0.52
	1st	0.31	0.55	0.46	0.32	0.60	0.43
Analyst	2nd	0.32	0.59	0.43	0.24	0.62	0.46
	3rd	0.33	0.58	0.45	0.28	0.62	0.46

VC= vicine, WTH= withaferin-A and TRG= trigonelline; %RSD= relative standard deviation

Determination of all three analytes in polyherbal formulation

The amount of markers (Vicine, trigonelline and withaferin-A) content were estimated in the Form HA by the proposed method, and were found to be 0.89 %, 0.91% and 0.76 % for vicine, trigonelline and withaferin-A.

CONCLUSION

In the present work, a validated HPTLC method has been developed and validated for simultaneous quantification of three compounds *viz.* vicine, trigonelline and withaferin-A from the polyherbal formulation, comprised of karela (Fruit), methi (Seed) and

aswagandha (Root) extracts. The proposed method was found to be simple, precise, accurate and robust. Hence, can be used for the quantification as well as quality control of polyherbal formulations those contain *Momordica charantia, Trigonella foenum-graceium* and *Withania somnifera* as their ingredients.

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REFERENCES

1. Mahady GB. Global Harmonization of Herbal Health Claims. J Nutr 2001; 131: 1120S-1123S.
2. Kalaiselvan V, Kalpeshkumar SA, Patel FB, Shah CN, Kalaivani M, Rajasekaran A. Quality assessment of different marketed brands of Dasamoolaristam, an Ayurvedic formulation. Int J of Ayurveda Res 2010; 1(1): 10-13.
3. Raman A, Lau C. Anti-diabetic properties and phytochemistry of *Momordica charantia* L. (Cucurbitaceae). Phytomedicine 1996; 2: 349-362.
4. Dutta PK, Chakravarty AK, Chowdhury US, Pakrashi SC. Vicine, a favism-inducing toxin from *Momordica charantia* Linn. seeds. Ind J Chem 1981; 20B: 669-671.
5. Shah S, Bodhankar SL, Badole SL, Kamble HV, Mohan V. Effect of trigonelline: an active compound from *Trigonella foenum-graecum* Linn. in alloxan induced diabetes in mice. J Cell Tissue Res 2006; 6(1): 585-590.
6. Rahman A, Jamal SA, Choudhary MI, Asif A. "Two. Withanolides from Withania somnifera". Phytochemistry 1991; 30(11): 3824-3826.
7. Poukens-Renwart P, Tits M, Wauters JN, Angenot L. Densitometric evaluation of spiraeoside after derivatization in flowers of *Filipendula ulmaria* (L.) Maxim. J Pharm Biomed Anal 1992; 10: 1085-1088.
8. Corthout J, Naessens T, Apers S, Vlietinck AJ. Quantitative determination of ginsenosides from *Panax ginseng* roots and ginseng preparations by thin layer chromatography-densitometry. J Pharm Biomed Anal 1999; 21: 187–192.
9. Ravishankara M, Srivastava N, Jayathirtha M, Padh H, Rajani M, Sensitive high-performance thin-layer chromatographic method for the estimation of diospyrin, a tumour inhibitory agent from the stem bark of *Diospyros montana* Roxb. J Chromatogr B 2000; 744: 257-262.
10. Ahmad S, Rizwan M, Parveen R, Mujeeb M, Aquil M, A validated stability-indicating TLC method for determination of forskolin in crude drug and pharmaceutical dosage form. Chromatographia 2008; 67: 441-447.
11. Agarawal H, Kaul N, Paradkar AR, Mahadik KR. HPTLC method for guggulsterone. II. Stress degradation studies on guggulsterone. J Pharm Biomed Anal 2004; 36: 33-41.
12. Sharma N, Sharma UK, Gupta AP, Sinha AK, Lal B, Ahuja PS. Simultaneous densitometric determination of shikonin, acetylshikonin, and beta-acetoxyisovaleryl-shikonin in ultrasonic-assisted extracts of four *Arnebia* species using reversed-phase thin layer chromatography. J Sep Sci 2009; 32(18): 3239-3245.
13. International conference on Harmonization (ICH) of technical requirements for the registration of Pharmaceuticals for Human Use, Validation of Analytical Procedures: Methodology, Tripartite Guidelines, Q2B, Geneva 1997.