RESEARCH ARTICLE

Simultaneous Estimation for Diosgenin, Charantin and Hydroxychalcone from Herbal Antidiabetic Formulation using Validated HPTLC Method

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ABSTRACT

Most antidiabetic polyherbal preparations contain *Trigonella foenum-graecum, Momordica charantia* and *Cinnamomum zeylanicum* as active components. Although all ingredients are popular as antidiabetic agents, but the analytical method is not available for simultaneous estimation of marker compounds from herbal formulations due to several challenges. Hence, the HPTLC method was developed for simultaneous estimation of three active phytoconstituents viz. diosgenin, charantin and hydroxychalcone in polyherbal formulation. The stationary phase of the optimized HPTLC method is silica gel 60 GF254 and the mobile phase is chloroform: glacial acetic acid: methanol: water (4:3:2:1v/v). The Rf value of phytoconstituents charantin, diosgenin, and hydroxychalcone was found to be 0.72, 0.61 and 0.30 at detection wavelength 342 nm. According to ICH criteria, the analytical method validation was performed. All three markers showed linear and proportional responses in the 400 to 1400 ng/band range, which confirmed the markers' linearity. Precision measurements were made at the intraday and interday levels, and the results were satisfactory and in line with the specifications. Accuracy was determined by the recovery method and %recovery was found to be in the range of 85.20 to 97.19. The validated HPTLC method was utilized to analyze the marketed Quanto Diab Forte capsule formulation containing charantin, diosgenin, and hydroxychalcone. The proposed validated analytical method was found to be simple, precise and accurate.

Keywords: Planar chromatography, Diosgenin, Charantin, Hydroxychalcone, HPTLC, Analytical Method validation.

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INTRODUCTION

T. foenum-graecum L seeds*, M. charantia* L. fruits and *Cinnamomum verum* J. Presl bark are commonly used in food and are also form common ingredients of polyherbal formulations utilized for the treatment of diabetes.[1-6] Diosgenin from *T. foenum-graecum* and charantin from fruits of *M. charantia* are steroidal saponin glycosides and are active markers of the plants. The bark of cinnamon contains a flavonoid hydroxychalcone, established to have antidiabetic activity.

Herbal remedies are becoming more and more popular since they are widely available, reasonably priced, safe, and people have trust on it. As a result, the World Health Organization (WHO) supports, campaigns for, and promotes the use of ethnomedicine in national healthcare programs.[7] In spite of the popularity of these plants as antidiabetic agents, very little information is available on the simultaneous estimation of these marker compounds from polyherbal formulations.

Standardization of such herbal products is important as a part of production and quality control of herbal medications. Standardization of polyherbal formulation involves quantification of the marker compounds from the extracts or powders of crude drugs to be utilized in the formulation.[8,9]

In the standardization of any herbal formulation, few analytical methods are reported for the quantification of diosgenin and charantin individually.[10-21] No report was found for the estimation of Hydroxychalcone along with the other two marker compounds.[22-24] Hence, there is a need of the development of methods for simultaneous estimation of biomarkers viz. diosgenin, charantin and hydroxychalcone, and its application for assay of formulation.

In this context, the present research work is undertaken to optimize and validate an analytical HPTLC method for the estimation of biomarkers.

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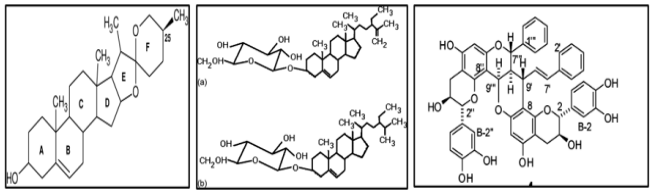

Diosgenin Charantin Hydroxychalcone

Figure 1: Structure of diosgenin (DG), charantin(CT) and Hydroxychalcone (HC)

MATERIALS AND METHODS

Procurement of plant materials and chemicals

The *T. foenum-graecum* seeds and the *C. verum* J. Presl bark procured from the ayurvedic vendor, (batch number; 20448009), Kalamboli, Navi Mumbai, Maharashtra, India. The *M. charantia* L. fruits were procured from the local market Kalamboli, Navi Mumbai, Maharashtra, India.

Authentication of all crude drugs was carried out from Guru Nanak Khalsa College Matunga, Maharashtra.

The HPLC grade methanol, ethyl acetate, chloroform and acetonitrile were purchased from Merck, India. Qualigens, India purchased chloroform, distilled water and glacial acetic acid.

Reference standards of diosgenin (DG) and charantin(CT) were purchased from Yucca Enterprises, India, having a purity of 99.30%. Hydroxychalcone was isolated by the researcher of the Department of Pharmacognosy of the institution using the method described by Takshi Tanaka. The isolated hydroxychalcone marker was characterized using spectral studies (HR LC-MS, NMR and elemental analysis) and comparison with the reported data.

Isolation of Biomarker Hydroxychalcone

Hydroxychalcone (HC) was synthesized using the procedure mentioned by Takashi Tanaka.[1,4] In brief, 1 g of catechin and 2 g of cinnamaldehyde were transferred in the round bottom flask and 50 mL of acetone was added in it. The resulting mixture was concentrated in rotary flash evaporator by heating at 100o for 50 minutes. The reaction mixture was allowed to cool and then loaded on Sephadex LH 20 column, followed by elution with ethanol (1.3 mL/min). Two mL of each of the eluents were collected; the first fraction showed the presence of cinnamaldehyde, and hydroxy chalcone was detected in eluents from the third fraction onwards. As the mobile phase, the eluents were analyzed on precoated Silica plates (GF 254) with Chloroform: Methanol: Water (15:5:1 v/v). (Rf of Hydroxy chalcone = 0.5). The fractions indicating the presence of Hydroxy chalcone combined together and evaporated to dryness. The synthesized compound was characterized for its identity and purity using various spectral studies and comparison with the data given by NMR, HR-LCMS and elemental analysis.

Extraction of Crude drugs

The powdered dried seeds of *T. foenum-graecum* L (100 gm) were defatted using Pet. Ether (60–80°C) for 1 hour followed by refluxing marc in 70% v/v hydroalcoholic solution using soxhlet extractor. The extract was dried in a vacuum oven to get extract of about 4 gm.

The fruits of *M. charantia* L and cinnamon bark powder were extracted by refluxing using 70% v/v hydro alcoholic solution for three hours in the soxhlet apparatus separately. The liquid extract (150 mL) was concentrated in a vacuum oven and extracts were then subjected to spray-drying to get extract powder of *M. charantia* (3 gms) and cinnamon (7 gms), respectively.

HPTLC Method Development

The reference standards of DG, CT and HC were individually weighed and dissolved in methanol to get the standard solutions having a concentration 1000 ppm. Stock solutions were diluted suitably to obtain each marker's working standard (100 µg/mL).

Camag linomat V sample applicator (Switzerland), was used to inject various amounts of working standards for the aforementioned marker compounds in the band form (6 mm wide) using a Hamilton microliter syringe (100 µL). The dimensions of slit were 5 mm x 0.45 mm. Appropriate volumes of standard solutions were separately loaded on precoated TLC plates of silica gel $60GF_{254}$ (Merck) to get graded amounts in the 400 to 1400 ng/b range and. The mobile phase of glacial acetic acid: methanol: chloroform: water; 3.0: 2.0:4.0:1.0 v/v showed good resolution of all biomarkers. CAMAG densitometer Scanner was used to scan the HPTLC plate at 342 nm using Wincats Software 1.2.2

Validation of the HPTLC Method[25]

Linearity

Using optimized HPTLC method, a linearity study for the simultaneous estimation of biomarkers was conducted using calibration standards in the 400–1400 ng/band range. The correlation coefficient was computed and the calibration curve was constructed (Table 1). The method was used for quantification of biomarkers DG, CT and HC from the marketed formulation viz. Quanto Diab Forte (75 mg) capsule.

Precision

Interday and intraday precision of the analytical method was determined at three concentrations of quality control. The three quality control concentrations at LQC, MQC, and HQC for 400, 800, and 1200 ng/band, respectively. The precision was expressed as %RSD at each concentration of quality control samples. The intra-day precision of the marker compounds was assessed using six replicates of quality control samples on the same day. The inter-day or reproducibility parameter was evaluated by analyzing the QC sample for three consecutive days under the same experimental conditions. The results of precision are expressed in terms of the %RSD (Table 2).

Accuracy

An accuracy study was performed for markers DG, CT and HC by %recovery method. The stock solution was spiked in the formulation with known DG, CT and HC concentrations at different levels viz. 80, 100 and 120% considering all

Table 1: Linearity study for hydroxychalcone, Charantin and Diosgenin.

Concentration (ng/band)	*Mean Area N = 3 (RSD)*		
	Hydroxy chalcone	*Charantin*	*Diosgenin*
400	2058.1 + 3.31	3720.8 + 0.83	4322.6 + 0.07
600	2626.767 + 1.60	5064.5 + 0.21	5218.4 +0.06
800	3137.567 + 1.38	6658.033 + 3.18	5841.7 + 0.03
1000	3501.633 + 2.30	7789.467 + 0.39	6530.1 + 0.27
1200	3904.967 + 3.94	9162.633 + 0.56	7565.7 + 0.04
1400	4408.133 + 3.36	10160.53 + 0.36	8295.6 + 0.03

Table 2: Intraday and interday precision of hydroxychalcone, Charantin and Diosgenin

Quality controls tandard	*CONC (ng/band)*	*Intraday precision %RSD*			*Interday precision %RSD*		
		Hydroxy chalcone	*Char antin*	*Dios genin*	*Hydroxy chalcone*	*Char antin*	*Dios genin*
LQC	400	0.74	1.50	0.34	0.40	0.77	0.62
MQC	800	0.95	0.55	0.55	1.13	1.50	0.64
HQC	1200	1.36	0.21	0.69	1.61	1.15	0.47
Mean RSD		1.01 67	0.75 33	0.52 67	1.04 67	1.14 00	0.57 67

biomarkers. The analysis of all samples by HPTLC method was carried out and the percent recovery of each biomarker was calculated to confirm the accuracy of the analytical method (Table 3).

Specificity

The specificity parameter was evaluated by comparing the chromatograms of the solvent (blank chromatogram) with the marker compound.

System suitability

The system suitability study for the marker compounds was performed using a concentration 400 ng/spot and system suitability was assessed in terms of retardation factor, peak area and peak symmetry (Table 4).

Robustness

The robustness parameter was assessed by slight modification in the chromatographic conditions viz mobile phase composition and wavelength of Analysis. The change in area and Rf value of marker compounds was monitored.

Analysis of Marketed Formulation

The contents of ten Quonto diab forte capsules were weighed containing phytoconstituents viz. seeds of *T. foenum-graecum* 400 mg; Unripe fruit freeze-dried extract of *M. charantia* 200 mg; and bark extract of *C. zeylanicum* 200 mg. Powder corresponding to 75 mg of phytoconstituents was weighed after calculating the average weight of the content of capsules. The weighed quantity was then transferred to 100 mL volumetric flask. Methanol was used as a solvent to make up the volume upto 100 mL (Solution A). Solution A was diluted to 1000 time to get solution B. Solution B (10 µL) was loaded in triplicate on HPTLC plate and analyzed for the content of biomarkers DG and CT was determined using the method described in section 2.3. Standard addition method was used for quantification of HC. Content of DG, CT and HC in formulation was determined from the calibration curve of the respective marker compound.

RESULTS AND DISCUSSION

Authentication of Crude Drugs

The crude drugs *T. foenum-graecum, M. charantia and C. zeylanicum* were authenticated on the basis of microscopic and morphological analysis from G. N. Khalsa College, Mumbai. The voucher specimens (rbs P 014060319, rbs P 013960219,rbs P 013980219)are deposited respectively. Isolated biomarker Hydroxychalcone was characterized by confirming identity, the compound was subjected to spectral analysis (Figure 1S-3S: Authentication certificate of seeds of *T. foenum-graecum* L., *M. charantia* and *C. verum* J. Presl).

Characterization of Isolated Marker Hydroxychalcone

The structural elucidation of Hydroxychalcone was performed on Varian (300 MHz) NMR, HR LC-MS and elemental analysis. The results of spectral analysis are given as supplementary material (Figure 4S-13S are supporting files of NMR, HR LC-MS and elemental analysis hydroxychalcone). Spectral studies confirmed the identity and purity of isolated hydroxychalcone (Figure 14 S: TLC plate of Diosgenin,Charantin & Hydroxychalcone).

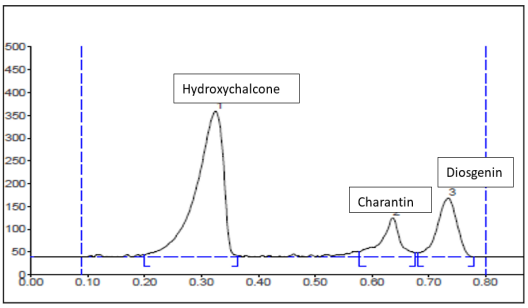

Figure 2: HPTLC chromatogram of hydroxychalcone, charantin and diosgenin

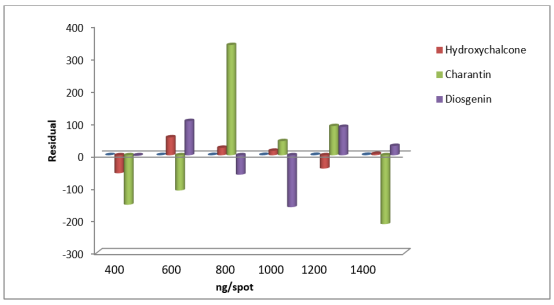

Figure 3: Plot of residuals for linearity study of hydroxychalcone, charantin and diosgenin

Table 3: Accuracy studies on hydroxychalcone, Charantin and Diosgenin by % recovery method

Sample	*Amountofhydroxychalcone present in formulation (mg)*	*Amount of hydroxychalcone added (mg)*	*Amount of total hydroxychalcone recovered (mg)*	*Recovery (%)*	*Average recovery*
hydroxychalcone	10	8	16.72	92.32%	92.22%
	10	10	16.27	89.24%	
	10	12	17.77	95.10%	
Sample	Amount of hydroxychalcone present in formulation (mg)	Amount ofCharantin added (mg)	Amount of Charantin recovered (mg)	Recovery (%)	Average recovery
Charantin	10	8	13.29	85.20%	89.39%
	10	10	12.52	85.78%	
	10	12	13.32	97.19%	
Sample	Amount of Diosgenin present in formulation (mg)	Amount of Diosgenin added (mg)	Amount of Diosgenin recovered (mg)	Recovery (%)	Average recovery
Diosgenin	10	8	15.45	96.93%	95.87%
	10	10	14.84	96.50%	
	10	12	15.56	94.18%	

Table 4: System suitability study for hydroxychalcone, Diosgenin And Charantin

Parameter	*Hydroxyc halcone*	*Charantin*	*Diosgenin*	*Acceptance*
Peak area (mean ± SD) N=3	1961.76 ± 16.29763	1076.37 ± 9.3644	2022.51 ± 9.3035	NA
% CV	0.83	0.87	0.46	NMT 2 %
Peak symmetry	1.3 ± 0.012	1.27 ± 0.099	1.18 ± 0.11	NMT 2

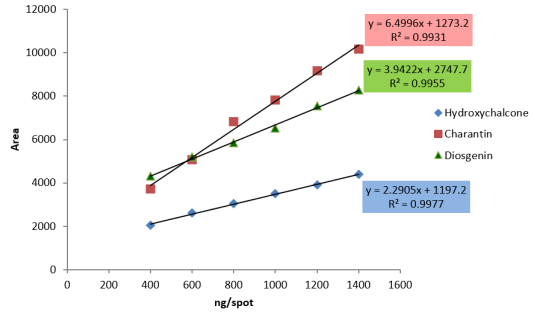

Figure 4: Calibration curve of hydroxychalcone, charantin and diosgenin

HPTLC Method Development

Several chromatographic settings were investigated to achieve effective separation of DG, CT and HC for quantitative analysis. Various mobile phases were tried for the resolution of DG, CT, HC and any potential impurity (Table 1S-3S are supporting files of ^{1}H, ^{13}C NMR values of isolated Hydroxychalcone and elemental analysis of hydroxychalcone. Table 4S: Optimization of chromatographic condition for resolution of hydroxychalcone, charantin and diosgenin by HPTLC method). Good resolution was observed for HC(Rf value = 0.33), CT (Rf value = 0.64) and DG (Rf value = 0.74) with mobile phase having composition glacial acetic acid: methanol: chloroform: water; 3.0: 2.0:4.0:1.0 v/v on precoated silica gel GF_{254} TLC plates. The Detection was carried out using densitometer III at 344 nm (Figure 2).

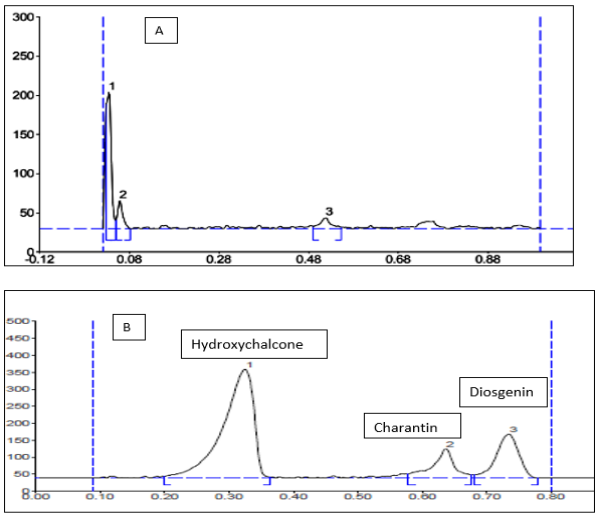

Figure 5: Chromatogram of a. chromatogram of blank sample and b. standard hydroxychalcone (Rf = 0.33), Charantin (Rf = 0.64) and Diosgenin (Rf = 0.74)

HPTLC Method Validation

ICH guideline was used to validate the developed HPTLC technique.[3]

Linearity

Analytical method exhibited linearity in the 400 to 1400 ng/spot range for all three marker compounds. Results of linearity and representative chromatogram are presented in Table 1 and Figure 2. Residual analysis of the calibration curve indicated a random distribution of residuals, indicating the linearity of the working range (Figure 3). A good correlation was observed between concentration and mean peak areas within the 400 to 1400 ng/spot concentration range. Linear regression equations with the coefficient of correlation (R^2) values were observed to be Y = 1222.2 + 2.2758x, (0.995) for HC, Y = 1227.16 + 6.5173x, (0.9961) for CT and Y = 2747.633 + 3.9422x, (0.9955) for DG respectively. Results are presented in Table 2 and Figures 4 and 5.

Table 5: Robustness Study Of Hydroxychalcone, Charantin And Diosgenin

Parameters	*level*	*N=3, % RSD*			*Retardation factor (Rf)*		
		HCN	*CHA*	*DIO*	*HCN*	*CHA*	*DIO*
Mobile phase compositionChloroform: Glacial acetic acid: Methanol: water (4.0:3.0:2.0:1.0) V/V	Volume-9.5mL (3.5:3.0.0:2.0:1.0) V/V	0.80	0.76	0.176	0.31	0.61	0.69
	Voume-10.5mL (4.0:3.0:2.5:1.0) V/V	0.81	0.77	0.165	0.31	0.61	0.69
Change in wavelength of analysis(nm)	340 nm	0.82	0.771	0.17	0.27	0.60	0.69
	344 nm	0.76	0.17	0.27	0.31	0.69	0.70

Table 6: Assay of Marketed Formulation

markers	*Mean Content of Markers (mg) in Quanto Diab Forte per 75 mg capsule (n=3)*
Diosgenin	1.028±0.02
Charantin	0.524±0.01
Hydroxychalcone	0.082±0.005

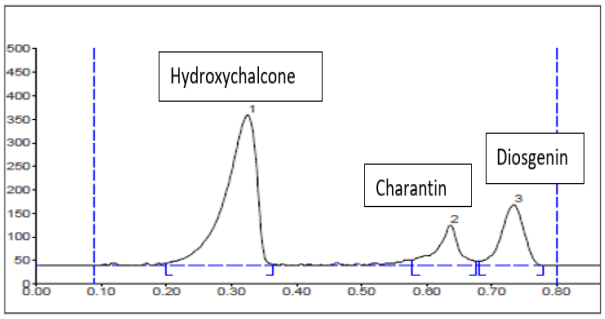

Figure 6: Chromatogram of biomarkers present in marketed formulation (Quanto Diab Forte capsule)

Hence, the method is considered linear as the correlation coefficient is within the acceptance criteria for DG, CT and HC.

LoD and LoQ values for HC (99 and 302 ng/band), CT(29.79 and 89.34 ng/band) and DG (32.2 and 97.26 ng/band). Although the Calculated LoD and LoQ were low for DG and CT, the working range (400–1400 ng/band) of the calibration curve for these marker compounds was utilized as the plant extracts contain much less marker compounds.

Precision

To assess reproducibility, the precision was calculated at three distinct concentration levels for LQC (400 ng/spot), MQC (800 ng/spot), and HQC (1200 ng/spot) samples. The results presented in Table 2 indicate the %RSD, the average %RSD for all levels of QC standards is in the range of 0.52 to 1.14, demonstrating that the method is acceptable for the precision parameter. The intraday and inter-day precision study results pass as per the specification range.

Accuracy

Accuracy parameter was determined using percent recovery of spiked reference standards viz. DG, CT and HC in the respective samples. For this study, spiking was done on the drug substance and drug product. The calculated % recoveries against respective levels are presented in Table 3. According to ICH guidelines, the mean percent recovery for DG, CT, and HC was discovered to be between 85 and 115%. This confirms that the analytical method qualifies the accuracy parameter by the recovery method.

Specificity

The chromatograms presented in Figure 4A and B indicate that there are no interfering chromatograms at the R_f of the biomarkers i.e. HC (Rf= 0.33), CT (Rf= 0.64) and DG (Rf=0.74). It was observed that no peak was co-eluted with analyte. Hence, the method is considered to be specific for analysis of biomarkers.

System suitability

The system suitability study for DG, CT and HC (400 ng/spot) was performed by evaluating mean peak area, retardation factor and symmetry. The results were within the boundary of acceptance criteria and presented in Table 4. As per guidelines, the % RSD of peak area, and retardation factor for the peaks should not be more than 2%. The results presented in Table 4 confirmed that the % RSD for the peaks of all the reference standards are between the acceptable range for the stated parameter. Hence, the system complies system suitability test. All the parameters are in the range of the acceptance limit so the method passes system suitability test.

Robustness

Robustness parameter was measured by making thoughtful modifications in the mobile phase composition and analysis wavelength. The effect of deliberate change on the marker compounds' peak area and retardation factor (DG, CT and HC) is presented in %RSD for triplicate analysis. Table 5 indicate the %RSD is in the range of 0.17 to 0.82. Hence it can be concluded that the method is robust.

Applications of Validated Analytical Method

Analysis of marketed formulation

The assay of the marketed formulation was determined using the validated HPTLC method and the Biomarkers DG, CH and HC content reported in Figure 6 and Table 6.

The validated Analytical method was further modified for its utility for the estimation of biomarkers in biological matrices. The bio-analytical method was developed and validated, then successfully applied for the pharmacokinetic study of biomarkers.[26]

CONCLUSION

A simple, specific, accurate and reproducible HPTLC analytical method for simultaneous estimation of biomarker compounds viz. DG, CT and HC from an antidiabetic formulation is developed and validated. The analytical method was successfully used for analysis of herbal preparations comprising these marker compounds.

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