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Development and Validation of HPTLC Method for Simultaneous Estimation of Berberine, Gallic Acid and Ursolic Acid in a Polyherbal Blend

A sensitive high-performance thin-layer chromatography method was developed for simultaneous estimation of berberine, gallic acid and ursolic acid in a polyherbal blend and validated as per ICH guidelines. Polyherbal blend was prepared using widely recommended herbal plants for platelet augmentation activity viz. Carica papaya, Berberis aristata, Ocimum Sanctum, and Tinospora Cordifolia. The optimized separation was obtained with TLC aluminum plates pre-coated with silica gel G60 F254 as a stationary phase and a solvent system containing Toluene : Ethyl acetate : Methanol : Formic acid (3:3:0.2:0.1 v/v/v/v). Berberine and gallic acid were found to demonstrate linearity in the range of 1µg/band – 6µg/band and ursolic acid in the range of 20 µg/band – 100 µg/band with the regression coefficient in acceptable limits. The method was also found to be specific and precise one. The accuracy of the developed method at 80 %, 100 % and 120 % levels was found to be within limits and % RSD was found to be less than 2. LOD and LOQ of all three standards were also determined. Blend was also quantified for the amount of berberine, gallic acid and ursolic acid presence and was found to be 37.8 µg, 46.1 µg and 108 µg per mg, respectively.

Keywords: HPTLC, polyherbal, gallic acid, berberine, ursolic acid, method development, validation.

Introduction

Polyherbal formulations are common in traditional system of medicine. The standardization of these polyherbal formulations with complex chemical composition is a major challenge faced by analysts. High-performance thin-layer chromatography (HPTLC) can be a useful tool for analysis and standardization with the use of markers/biomarkers due to a number of advantages, such as analysis speed, sensitivity, and low operational cost.

In the current study, an analytical method was developed for in-house platelet booster polyherbal formulation comprising of *Carica papaya*, *Berberis aristata*, *Ocimum Sanctum* and *Tinospora Cordifolia*. The selected plants have reported platelet augmentation [1, 2] and anti-dengue activities [3–6].

The markers viz. gallic acid, ursolic acid and berberine (Fig. 1) used for the standardization of the polyherbal formulation are widely found in many herbal drugs. Gallic acid or 3,4,5-trihydroxybenzoic acid with anti-dengue, astringent, cyclooxygenase-2 (COX-2) inhibitory, antioxidant and anti-neoplastic activity [7, 8] is a constituent found in *Carica papaya* [9]. Ursolic acid, a pentacyclic triterpenoid, 3β-hydroxyurs-12-en-28-oic acid present in *Ocimum sanctum* [10] is reported to have activities like anti-inflammatory, antioxidant, antiviral, serum lipid-lowering, and antineoplastic activities [11, 12]. The phytoconstituent of *Berberis aristata and Tinospora cordifolia*, berberine, [13] an isoquinoline alkaloid, with diverse biological activity including antiviral, anti-diabetic, anti-neoplastic, anti-inflammatory and anti-lipidemic activities [14] was also used as a biomarker in the current study.

Several studies employing high-performance thin-layer chromatography (HPTLC) [15–19] and high-performance liquid chromatography (HPLC) [20–23] to estimate these markers alone or in combination have been published. However, a thorough review of the literature found that no validated HPTLC approach for simultaneously estimating all three markers has been published.

Thus, this study aims to establish a simple, reliable, precise, and validated HPTLC approach for estimating these markers simultaneously in a polyherbal preparation.

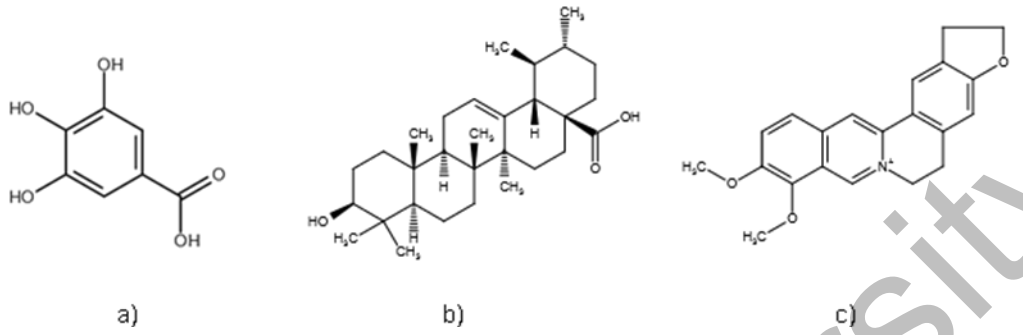

Figure 1: Structure of a) gallic acid, b) ursolic acid, c) berberine

Experimental

Chemicals and reagents: All chemicals and reagents used for the study were AR Grade. The herbal extracts used in the study and the marker ursolic acid was procured from Phyto Life Sciences P. Ltd, Gujarat, India. The markers, namely gallic acid and berberine, were procured from Natural Remedies Bangalore, India and Cayman Chemical Company, USA, respectively.

Preparation of polyherbal blends: The herbal blends were prepared by mixing *Carica papaya* leaf extract *(200 mg)*, *Berberis aristata* root extract (120 mg), *Tinospora cordifolia* stem extract (150 mg) and *Ocimum sanctum* leaf extract (120 mg). Blend was prepared by mixing extracts in increasing concentration.

Standardisation of blend by high-performance thin-layer chromatography

1) HPTLC method development

a. Preparation of standard solutions and a test solution: For a standard solution, 1mg of berberine was dissolved in 1ml of methanol to obtain a solution of 1000 µg/ml concentration, which was used further for spotting on TLC plate. Similarly, stock solutions for gallic acid (1000 µg/ml) and ursolic acid (1000 µg/ml) were prepared in methanol.

For a test solution, 200 mg of polyherbal blend was dissolved in 10 ml methanol by sonicating for 10 minutes followed by filtration through filter paper (Whatman No. 42). Filtrate was further used for spotting on TLC plates.

b. Selection of the mobile phase: A standard stock solution (4 µl) and a test solution (10 µl) were applied on a pre-coated TLC plate as a band (the band size was 6 mm). Different solvents and solvents combinations with varying polarity were used to obtain well-separated sharp bands.

c. Methodology: The Camag Linomat-V sample applicator, semi-automatic equipment was used to apply standard stock solutions of berberine, gallic acid and ursolic acid on pre-coated TLC plates. The development of plate was performed in a twin trough chamber saturated with the mobile phase for 20 minutes. After development, the air-dried material was scanned at 280 nm for densitometric evaluation.

2) The method validation: The developed methodology was validated using Methodological Validation Criteria of the International Conference on Harmonization's (ICH) Q2 (R1) [24–27].

a. Linearity: Linearity for standard berberine and gallic acid was obtained in the range of 1 µg/band – 6 µg/band and that of ursolic acid was found to be 20 µg/band – 100 µg/band by spotting them separately on TLC plates. For each concentration, the peak area was measured, and the calibration curve graph was plotted as concentration versus peak area.

b. Specificity: The determination of the method specificity was carried out by application of methanol as blank, berberine, gallic acid, and ursolic acid as a standard solution, and a polyherbal blend as a test solution into the HPTLC system.

c. Accuracy: Accuracy of the developed method was estimated at levels 80 %, 100 % and 120 % separately for the standards (with 4µl berberine, gallic acid, ursolic acid considered as 100 %, 10 µl blend con-

centration was taken as 100 %). The blend was spiked with standard of known concentration, and the calculation for percent recovery (recovered and expected concentrations) was performed as per ICH guidelines.

d. Precision

System precision: Six replicate bands of standard as 4µl of berberine, gallic acid, ursolic acid separately at 6 tracks were applied to determine system precision. Standard deviation and % relative standard deviation were calculated.

Method precision: The method precision was carried out for blend from six replicate applications (10 µl extract application at 6 tracks) and % relative standard deviation was calculated.

e. Robustness: Robustness was checked by making small deliberate alterations in the method. The standard deviations of peak areas were computed for two parameters as variation in the mobile phase volume (±1 ml) and saturation time of a chamber (± 5 min)

f. Limit of detection (LOD) and limit of quantitation (LOQ): Limit of detection and limit of quantitation were calculated using the standard calibration curve method for berberine, gallic acid, and ursolic acid.

3) Quantification of markers by the developed method

For quantification of markers, 10 mg of extract/blend was dissolved in 1ml of methanol (100µg/ml) by sonication for 10 minutes and finally filtered using Whatman filter paper No. 42. A total of 10µl of stock solution was applied, developed and scanned using the optimized chromatographic conditions. Concentration of berberine, gallic acid, ursolic acid in methanol extract/blend was calculated using linearity equation.

Results and Discussion

HPTLC Method Development: The solvent system containing Toluene : Ethyl acetate : Methanol : Formic acid (3:3:0.2:0.1 v/v/v/v) gave good resolution for berberine, gallic acid and ursolic acid. R_f value for standards berberine, ursolic acid and gallic acid were found to be 0.31, 0.53 and 0.65, respectively at 280 nm with optimized chromatographic conditions (Table 1, Fig. 2). At a wavelength of 280 nm, the densitometric evaluation of separated bands was performed. The polyherbal blend was subjected to similar chromatographic conditions to obtain well-separated peaks (Fig. 3).

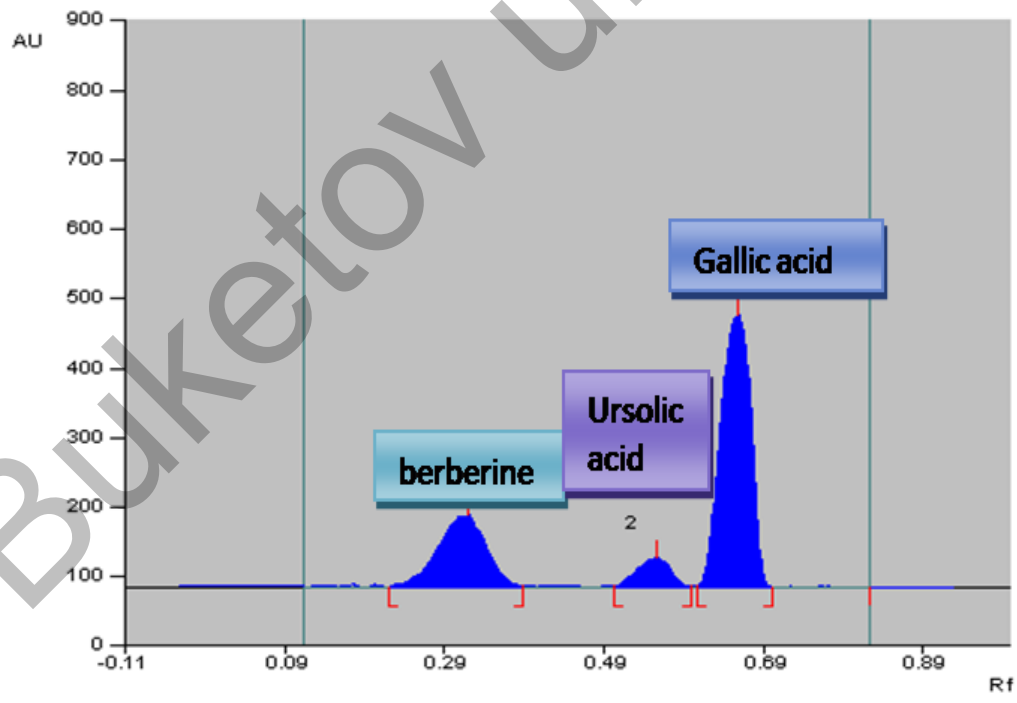

Figure 2. Densitogram of berberine (Rf is 0.31), ursolic acid (Rf is 0.53) and gallic acid (Rf is 0.65)

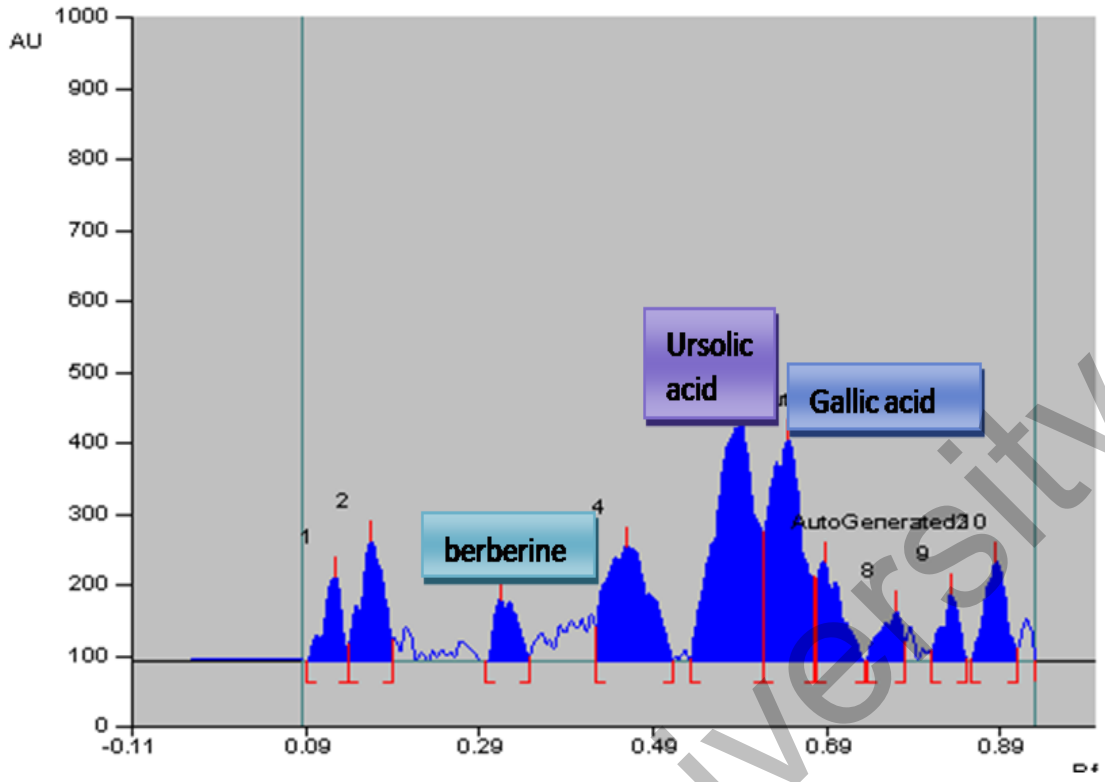

Figure 3. Densitogram of blend with berberine, ursolic acid and gallic acid

Table 1

Optimized chromatographic conditions

Parameters	Specifications
Stationary phase	Aluminium plates pre-coated with silica gel 60 F_{254} (Merck)
Mobile phase	Toluene : Ethyl acetate : Methanol : Formic Acid (3:3:0.2:0.1 v/v/v/v)
Plate size	10 cm × 10 cm
Application mode	Band
Band size	6mm (Distance between two bands: 14mm)
Sample volume	4 µl
Development chamber	Twin-trough glass chamber, 10 cm × 10 cm with stainless steel lid
Saturation time	20 minutes.
Separation technique	Ascending
Migration distance	≈ 80 mm
Scanning mode	Absorbance/Reflectance
Slit dimensions	5 × 0.45 mm
Scanning wavelength	280nm

HPTLC Method Validation:

a. Linearity: Standards berberine and gallic acid were found to be linear in the range of 1µg/band – 6 µg/band. The linearity range for ursolic acid was found to be 20 µg/band – 100 µg/band. Regression coefficients for all the standards were in the acceptable limit according to the ICH guidelines.

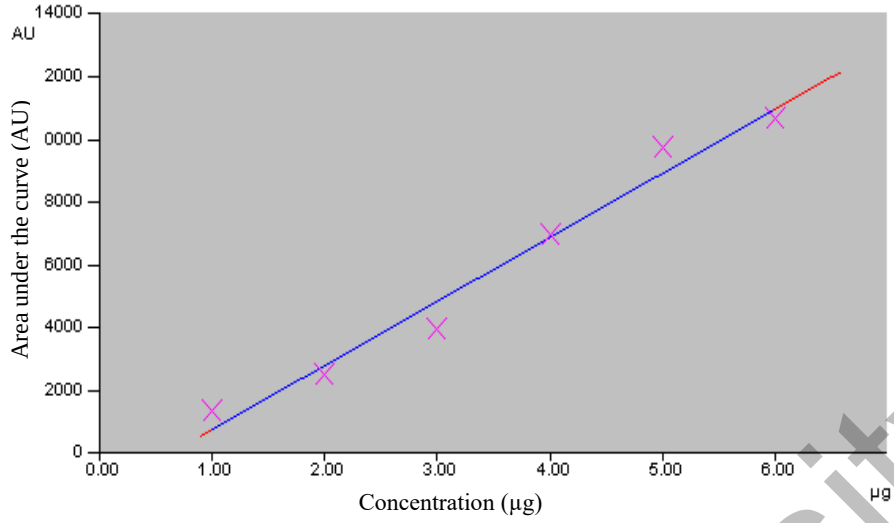

Figure 4. Linearity of berberine standard (1 µg/band – 6 µg/band)

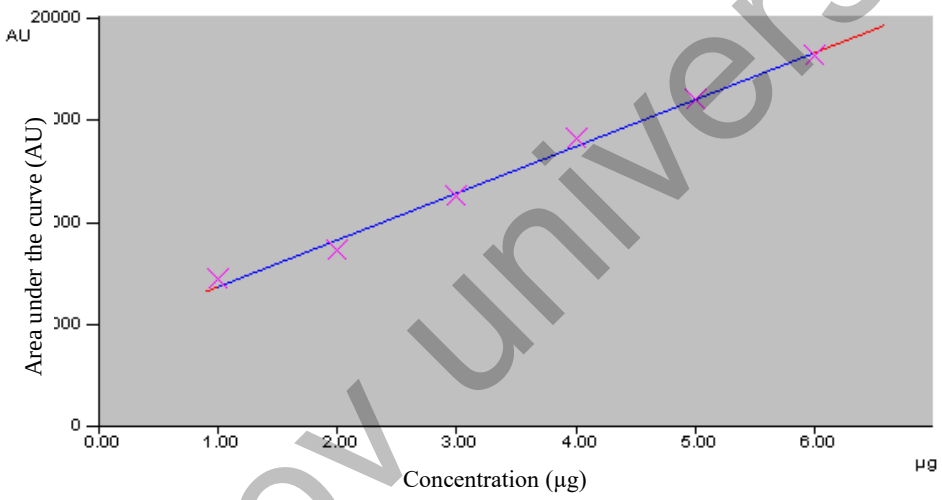

Figure 5. Linearity of gallic acid standard (1 µg/band – 6 µg/band)

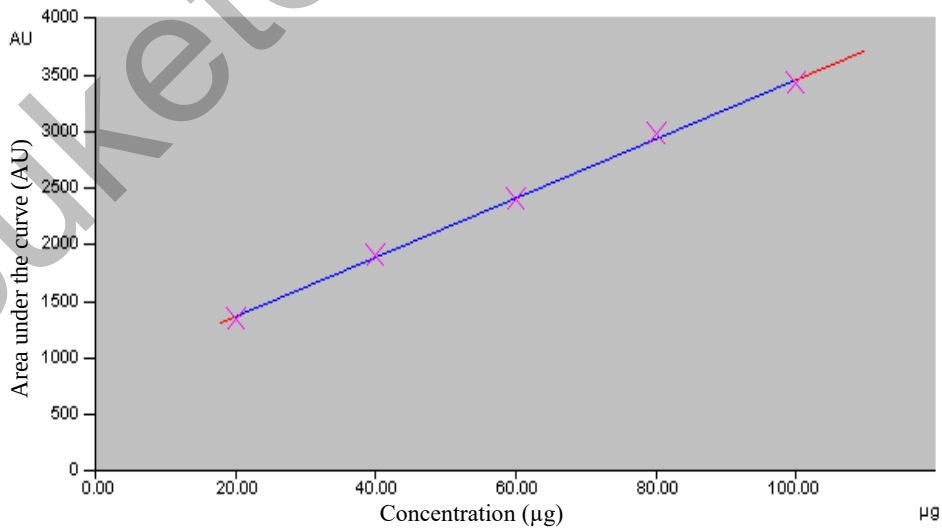

Figure 6. Linearity of ursolic acid standard (20 µg/band – 100 µg/band)

Table 2

Linearity result of standards

Standard	Equation	Regression Co-efficient
Berberine	Y = –1298 + 2039*x*	0.987
Gallic Acid	Y = 4529 + 2283*x*	0.997
Ursolic Acid	Y = 837 + 26.14*x*	0.999

Specificity: Good correlation values and satisfactory peak purity were obtained with no interference in the quantification of ursolic acid, gallic acid, berberine, which proves that the method is specific.

Accuracy: Results from accuracy studies were reported as percentage recoveries calculated against respective levels (Table 3). The average % recovery of berberine, gallic acid, ursolic acid was found to be within the acceptance limit (98–102 %). The accuracy of the developed method was good as indicated by the low % RSD values.

Table 3

Result of accuracy study

Analyte	Recovery Level	Average % Recovery (%)	S.D	% R.S.D
Berberine	80 %	101.06	0.013	0.012
	100 %	100.89	0.011	0.010
	120 %	100.71	0.017	0.016
Gallic acid	80 %	100.87	0.003	0.0029
	100 %	101.25	0.005	0.0030
	120 %	101.28	0.005	0.0032
Ursolic acid	80 %	101.7	0.011	0.010
	100 %	100.94	0.010	0.09
	120 %	102.11	0.013	0.012

d. Precision: Method and system precision was carried out using berberine, gallic acid and ursolic acid; % relative standard deviation was calculated. The % RSD is in the acceptable limit, that is, less than 2.0, which indicates that the method has an acceptable level of precision.

Table 4

Results of precision studies

Analyte	Method Precision		System Precision	
	SD	% RSD	SD	% RSD
Berberine	0.32	0.02	0.82	0.011
Gallic cid	0.65	0.03	0.34	0.002
Ursolic acid	0.78	0.09	0.26	0.08

e. Robustness: The optimized parameters were intentionally varied depending on the chamber saturation time (±5 min) and the mobile phase volume (±1 ml). The unaffected R_f values and low values of the % RSD (less than 2) of peak areas indicate the method's robustness (Table 5).

Table 5

Results of the Robustness study

Factor	Level	Ursolic acid (Rf)	Berberine (Rf)	Gallic acid (Rf)
1	2	3	4	5
Saturation time				
15 min	–5	0.53	0.32	0.66
20min	0	0.53	0.31	0.65
25 min	+5	0.52	0.31	0.67
SD ± RSD		0.006±1.09	0.006±1.84	0.01±1.5

Continuation of Table 5

1	2	3	4	5
Mobile phase volume				
6 ml	–1	0.51	0.31	0.63
7 ml	0	0.53	0.31	0.65
8 ml	+1	0.52	0.32	0.64
SD ± RSD		0.01±1.9	0.006±1.84	0.01±1.56

f. Limit of detection and limit of quantitation: The LOD and LOQ values for berberine, gallic acid, ursolic acid were calculated, which showed the adequate sensitivity of the developed method (Table 6).

Table 6

Limit of detection and limit of quantitation

Compound	LOD (µg/band)	LOQ (µg/band)
Berberine	0.031	0.10
Gallic acid	0.020	0.07
Ursolic acid	0.080	0.010

3.4 Quantification of marker compounds in the polyherbal formulation by the developed method

The biomarkers were quantified in the in-house blend using the developed method (Table 7). The calculations were done using linearity curve of markers. The blend was found to contain 37.8 µg, 46.1 µg and 108 µg per mg of berberine, gallic acid and ursolic acid, respectively.

Table 7

Quantification of markers

Sr. No.	Marker	Area (AU)	Percentage in blend (%, Calculated from linearity curve)
1	Berberine	6427.49	3.78 ± 0.02
2		6450.56	
3		6370.4	
4	Gallic acid	14717.39	4.61 ± 0.137
5		15091.32	
6		15337.19	
7	Ursolic acid	1119.44	10.80 ± 1.197
8		1087.98	
9		1150.56	

Conclusions

The HPTLC method was developed for the determination of berberine, gallic acid, and ursolic acid in the polyherbal blend and validated as per ICH guidelines. The study illustrates that the HPTLC method is simple, accurate and precise for the simultaneous estimation. The method is found to be specific and robust for the analysis. The method resulted in well-resolved sharp peaks. The recovery for all the biomarkers was close to 100 %, which confirmed no interference of other phytoconstituents of the extracts. The present method can be used for quantification of these biomarkers in the blend. Also, it was found that ursolic acid was present in the highest concentration in the formulated blend.

In summary, the developed method can be used for the routine standardization and quality control of herbal formulations containing the above mentioned markers, which are common ingredients of many polyherbal formulations. The method can also be used for quantification of the selected markers.

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С.С. Читланж, С.Р. Чандани, С.П. Ганди, П.А. Торат, Х.Б. Лэд

Полишөпті қоспадағы берберинді, галл қышқылын және урсол қышқылын бір мезгілде анықтауға арналған ЖТЖҚХ әдісін жасау және валидациялау

Полишөпті қоспадағы берберинді, галл қышқылын және урзол қышқылын бір мезгілде анықтауға арналған сезімталдығы жоғары, тиімді жұқа қабатты хроматография әдісі ICH ұсыныстарына сәйкес әзірленді және валидацияланды. Шөп қоспасы тромбоциттердің белсенділігін арттыру үшін ұсынылатын белгілі шөптерді, атап айтқанда *Carica papaya, Berberis aristata, Ocimum Sanctum* және *Tinospora Cordifolia* өсімдіктерін пайдалана отырып дайындалды. Онтайландырылған бөлуге стационарлық фаза ретінде G60 F254 силикагелімен алдын ала қапталған алюминий TLC пластиналарын және құрамында толуол : этилацетаты : метанол : құмырсқа қышқылы (3:3:0,2:0,1 көлемі) бар еріткіш жүйесі арқылы қол жеткізілді. Берберин мен галл қышқылы 1 мкг/жолақ – 6 мкг/жолақ, урзол қышқылы — 20 мкг/жолақ – 100 мкг/жолақ диапазонында регрессия коэффициентімен рұқсат етілген шектерде сызықтылықты көрсететіні анықталды. Сондай-ақ әдіс нақты және дәл болып шықты. Жасалған әдістің дәлдігі 80 %, 100 % және 120 % деңгейінде рұқсат етілген шектерде, ал % RSD екіден аз екені анықталды. LOD және LOQ барлық үш стандарт үшін де анықталды. Қоспа сонымен бірге құрамында берберин, галл қышқылы және урзол қышқылының бар екендігіне тексерілген, олар бір мг үшін сәйкесінше 37,8 мкг, 46,1 мкг және 108 мкг құрады.

Кілт сөздер: ЖТЖҚХ, полиграфикалық қоспа, галл қышқылы, берберин, урсол қышқылы, әдісті жасау, валидация.

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Разработка и валидация метода высокоэффективнойтонкослойной хроматографии для одновременного определения берберина, галловой кислоты и урсоловой кислоты в политравной смеси

Был разработан чувствительный метод высокоэффективной тонкослойной хроматографии (ВЭТСХ) для одновременного определения содержания берберина, галловой кислоты и урсоловой кислоты в политравной смеси и утвержден в соответствии с рекомендациями ICH. Политравная смесь была приготовлена с использованием широко известных травяных растений, рекомендуемых для увеличения активности тромбоцитов, а именно *Carica papaya, Berberis aristata, Ocimum Sanctum* и *Tinospora Cordifolia*. Оптимизированное разделение было достигнуто с помощью алюминиевых пластин для ТСХ, предварительно покрытых силикагелем G60 F254 в качестве стационарной фазы, и системой растворителей, содержащей толуол : этилацетат : метанол : муравьиная кислота (3:3:0,2:0,1 об./об./об./об.). Установлено, что берберин и галловая кислота демонстрируют линейность в диапазоне 1 мкг/полоса — 6 мкг/полоса, урсоловая кислота — в диапазоне 20 мкг/полоса — 100 мкг/полоса с коэффициентом регрессии в допустимых пределах. Метод также оказался специфичным и точным. Было обнаружено, что точность разработанного метода на уровнях 80 %, 100 и 120 % находится в допустимых пределах, а % RSD меньше двух. Также были определены LOD и LOQ для всех трех стандартов. Смесь также была исследована на содержание берберина, галловой кислоты и урсоловой кислоты, которое составило 37,8 мкг, 46,1 мкг и 108 мкг на мг соответственно.

Ключевые слова: ВЭТСХ, политравная смесь, галловая кислота, берберин, урсоловая кислота, разработка метода, валидация.

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