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A novel high-performance thin layer chromatography method for quantification of long chain aliphatic hydrocarbons from *Cissus quadrangularis*

[Nuevo método de cromatografía en capa fina de alta resolución para cuantificar hidrocarburos alifáticos de cadena larga de *Cissus quadrangularis*]

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Abstract

Context: A high-performance thin layer chromatography (HPTLC) is an analytical technique, which can be used for the determination of constituents or marker components in various parts of the plants. Earlier studies have estimated phytoconstituents from the stem and other aerial plant parts of *Cissus quadrangularis* Linn. Estimation of hydrocarbons can also be successfully done using HPTLC technique using suitable derivatization.

Aims: To develop and validate a simple and rapid method for the estimation of long chain aliphatic hydrocarbons from the leaves of *C. quadrangularis* using HPTLC technique.

Methods: Precoated silica gel 60 F_{254} plates were used as stationary phase. The mobile phase used was hexane (100 %). The detection of spots was carried out using berberine sulphate as detecting reagent.

Results: The method was validated in terms of linearity, sensitivity, accuracy, and precision. Linearity range was found to be 2-10 µg/mL, limit of detection 0.127 µg/mL, and limit of quantification 0.384 µg/mL.

Conclusions: A novel, simple, accurate, precise and sensitive HPTLC method has been developed and validated for the estimation of long chain aliphatic hydrocarbons obtained from the leaves of *C. quadrangularis* Linn.

Keywords: Berberine sulphate; high-performance thin layer chromatography; long chain aliphatic hydrocarbons.

Resumen

Contexto: La cromatografía en capa fina de alto resolución (HPTLC) es una técnica analítica que puede ser utilizada para la determinación de los constituyentes o marcadores en varias partes de las plantas. Estudios anteriores han estimado fitoconstituyentes del tallo y otras partes aéreas de la planta de *Cissus quadrangularis* Linn. La estimación de hidrocarburos también se puede realizar con éxito mediante esta técnica utilizando una derivatización adecuada.

Objetivos: Desarrollar y validar un método sencillo y rápido para la estimación de los hidrocarburos alifáticos de cadena larga de las hojas de *C. quadrangularis* utilizando la técnica de HPTLC.

Métodos: Placas recubiertas de sílica gel 60 F254 fueron utilizadas como fase estacionaria. La fase móvil fue hexano (100%). La detección de las manchas se realizó mediante sulfato de berberina como reactivo de detección.

Resultados: El método fue validado en términos de linealidad, sensibilidad, exactitud y precisión. Se encontró un rango de linealidad de 2-10 µg/mL, límite de detección 0.127 µg/mL y límite de cuantificación 0.384 µg/mL.

Conclusiones: Un nuevo método de HPTLC simple, exacto, preciso y sensible se ha desarrollado y validado para la estimación de hidrocarburos alifáticos de cadena larga obtenidos de las hojas de *C. quadrangularis* Linn.

Palabras Clave: Cromatografía en capa fina de alta resolución; hidrocarburos alifáticos de cadena larga; sulfato de berberina.

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INTRODUCTION

Cissus quadrangularis Linn. (*Hadjod)* is a medicinal plant found in the Indian Subcontinent as well as in Africa. Ancient literature of Ayurveda has prescribed it a tonic and has been used to cure fractures (Nayar, 1959; Justin and Joseph, 2001; Sanyal et al., 2005; Justin and Baby, 2011). Other pharmacological effects reported are anti-oxidant, anti-ulcer, anti-microbial, central nervous depressant, anti-epileptic and anti-cancer activity (Chidambara Murthy et al., 2004; Subhashri et al., 2013; Anusiya et al., 2014).

The phytochemical studies suggest the presence of triterpene derivatives, polyphenols, ketosteroids, carotenoids, stilbenes, flavonoids and iridoids (Sen, 1966; Bhutani et al., 1984; Gupta and Verma, 1990; 1991).

Studies have reported the presence of three additional components from stems: δ-amyrin acetate, hexadecanoic acid and trans-resveratrol-3-*O*-glucoside (Thakur et al., 2009a). Phytochemical investigations of leaves have revealed the presence of eicosyl eicosanoate, tetratriacontanol, tetratriacontanoic acid, α-amyrin and β-sitoterol (Jain et al., 2009). Isolation and characterization of long chain aliphatic hydrocarbon fraction containing heptacosane ($C_{27}H_{56}$), pentatriacontane ($C_{35}H_{72}$) and hexatriacontane ($C_{36}H_{74}$), using column chromatography followed by IR, Nuclear Magnetic Resonance (NMR) ^{1}H and ^{13}C and GC-MS from leaves is also reported (Jain et al., 2010).

Analytical methods to determine *C. quadrangularis* phytoconstituents (amyrin, amyrone, hexadecanoic acid ethyl ester, quercetin, and kaempferol) are available like GC-MS, HPTLC (Mehta 2001; Sumitra et al., 2013) and HPLC (Thakur et al., 2009b).

Hydrocarbons act as a barrier to the penetration by microorganisms and prevent desiccation. A major problem associated with hydrocarbon analysis is because of their inert nature (Cossio et al., 2000b). They do not give any UV or fluorescent response under the usual analytical conditions. Such compounds provide a fluorescent emission signal when spotted onto a silica gel TLC plate previously impregnated with berberine sulphate and irradiated using 366 nm UV light. Berberine does not affect separation and only allows the detection of saturates (Cebolla et al., 1999; Cossio et al., 2000a).

In our previous studies the isolation of aliphatic hydrocarbon is reported (Jain et al., 2010). In continuation of the earlier report, it was thought worthwhile to develop and validate novel quantification method for hydrocarbons by HPTLC in plant extract.

MATERIAL AND METHODS

Plant material

Leaves of *C. quadrangularis* were procured from in-house medicinal plant garden maintained at Oriental College of Pharmacy, Sanpada, Navi Mumbai and were authenticated by a Prof. H. M. Pandit, Botanist, Khalsa College, Mumbai (Voucher specimen number CQL-1).

Instrumentation and reagents

The stationary phase used was silica gel $60F_{254}$ TLC plates (10 x 10 cm, layer thickness 0.2 mm, E. Merck, Mumbai). All the chemicals and reagents were of analytical grade purchased from S.D. Fine Chemicals. Long chain aliphatic hydrocarbon isolated and characterized in our previous report was used as a standard (Jain et al., 2010). A Camag HPTLC system (Mumbai, India) equipped with Camag Linnomate IV (Mumbai, India) automatic sample applicator, Hamilton syringe (100 µL), Camag TLC Scanner III (Mumbai, India), controlled by Win CATS planar chromatography manager, Camag Twin-trough chamber (10 × 10 cm) were used during study.

Preparation of solutions

Standard solution: Accurately weighed quantity (10 mg) of aliphatic hydrocarbon was dissolved in 10 mL hexane. The above stock solution (1 mg/mL) was then used for spotting to achieve concentrations of 2-10 µg on the plate.

Sample solution: Accurately about 5.0 g of the dried powdered leaves of *C. quadrangularis* were extracted with hexane (50 mL) using Soxhlet extractor for 4 h. Aliquots of the sample were then applied to HPTLC plate for quantitation.

Chromatographic conditions

Chromatography was performed in Camag twin trough chamber, which was pre-saturated with the hexane before use. Precoated silica gel 60 F_{254} plates were used as stationary phase. The plates were pre-washed in methanol for removing any impurities picked up during storage in the laboratory environment. After pre-washing the plates were activated at 110-120°C for 30 min. The plates were developed with hexane (100%) as the mobile phase. Both standard and sample solutions of specific concentrations (as specified above) were applied to the plates as 5 mm bands by means of Camag Linomat IV sample applicator. After development and drying the plates were dipped in berberine sulphate (3 mg/L methanol) used as detecting reagent. Evaluation of standard and extract were performed after derivatization by scanning the plate at 366 nm using Camag TLC scanner III controlled by Win CATS planar chromatography manager.

Statistical analysis

The method was validated as per the ICH guidelines Q2 (R1). Linearity, precision, Limit of Detection (LOD), Limit of Quantitation (LOQ) and accuracy were the parameters performed, to validate the developed method.

Linearity: The linearity was evaluated by analyzing area under the curve as a function of analyte concentration. Test results were evaluated by calculating regression coefficient (r^2).

Precision: Precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed condition. Precision was determined in terms of percent relative standard deviation. Three replicates of standard at three different concentration levels (2, 6, 10 µg/spot) were analyzed on three separate days for inter-day precision (reproducibility) and at three different times on the same day for intra-day precision (repeatability).

LOD and LOQ: The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were established using the calibration curve parameters viz. slope and standard deviation. They are expressed as:

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ = standard deviation and S = slope of the curve.

Accuracy (recovery): Accuracy study was performed by spiking 80, 100, and 120% amount of standard drug externally added to the pre-analyzed sample. The experiment was conducted in triplicate. It was conducted to check the recovery of the drug at different levels.

RESULTS AND DISCUSSION

Method development

Alkanes in the presence of berberine sulfate provide an enhancement of fluorescent signal, depending on the alkane concentration, when the system is irradiated with UV light. Computational analysis indicates an ion-induced dipole between berberine sulfate and alkanes is responsible for this phenomenon. This interaction can properly model the experimentally obtained fluorescent response (Fig. 1) (Cossio et al., 2000b).

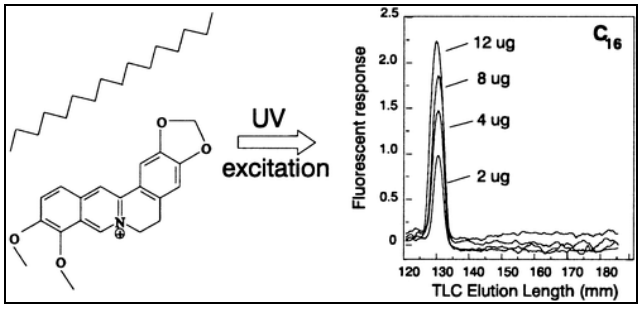

Figure 1. Fluorescent response after berberine sulfate and alkane interaction and its UV activation.

[Adapted with permission from Cossio et al., 2000b Copyright (2000) American Chemical Society].

The underlying principle for fluorescence enhancement of alkane-berberine interaction is due to an ion-dipole induced interaction between berberine cation and the corresponding saturated hydrocarbon. There occurs a weak electrostatic interac-

tion between the alkane molecule and the deficient electron π-system of berberine and that there are intermolecular distances close to van der Waals contacts in several positions. The role of alkanes is to provide a non-polar environment to the excited berberine cation, which hinders alternative relaxation mechanisms and favors the fluorescence emission. Thus, at 366 nm the fluorescence can be observed for hydrocarbons using berberine sulphate.

A solvent system, which would provide dense, compact, accurate spots with significant R_f values was selected for quantification of the hydrocarbons. The mobile phase hexane (100%) provided R_f value of 0.69 for the standard long chain aliphatic hydrocarbon (Fig. 2). The purity of the compound was 95% as determined by HPTLC.

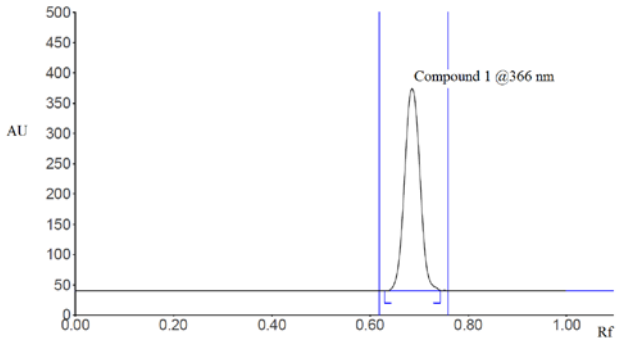

Figure 2. HPTLC chromatogram of standard long chain aliphatic hydrocarbon.

Calibration curve for the concentration range 2-10 µg was plotted. The linear regression equation (n=5) was found to be y = 50x – 80 (r^2 = 0.999), which shows that the method is linear over the range of 2-10 µg. Low values of LOD and LOQ indicate the method is sensitive.

Repeatability studies were carried out by sample application five times and also on the basis of peak area measurement for five times and the plated were developed and analyzed. The Percent Relative Standard Deviation (%RSD) was found to be 0.98 and 0.55% for repeatability of application and repeatability of measurement respectively (Table 1). Lower %RSD values indicate that the method can be successful applied for application and measurement of the sample at different concentrations.

Precision was determined as intra-day precision and inter-day precision. This was done by measuring three different levels of the standard solution with three replicates of each sample. The low %RSD values indicate the method is precise (Table 2). For recovery studies, to the pre-analyzed sample, three concentrations were added (80, 100, and 120 %) and analyzed using standard addition method. Percent recovery for extracted sample was found to be in the range of 100.86 to 101.54%. The recovery studies indicated that the sample consisted of no interference of other components (Table 3).

Table 1. Method validation parameters.

Parameters	Values obtained
Linearity range (µg/spot)	2 - 10
Correlation coefficient ©	0.999
Regression Equation (y = mx + c)	y = 50x – 80
Slope (m)	50
Intercept ©	-8
Limit of detection (LOD)	0.127
Limit of quantification (LOQ)	0.384
Repeatability of application* (% RSD)	0.98
Repeatability of measurement* (% RSD)	0.55
n	5

% RSD: Percent Relative Standard Deviation.

Table 2. Method precision data.

Component	Concentration (Levels)	Intra-day precision (n=3) (%RSD)	Inter-day precision (n=3) (%RSD)
Standard hydrocarbon	2	0.70	0.98
	6	0.55	0.89
	10	0.63	0.94

Method precision was done as per ICH guidelines according as described in Materials and Methods. % RSD: Percent Relative Standard Deviation.

Table 3. Recovery studies performed on standard long chain aliphatic hydrocarbon.

Component	Amount present (µg/spot)	Amount added (µg/spot)	Amount found (µg/spot)	Amount recovered (µg/spot)	% Recovery	Avg.	SD	%RSD
Standard hydrocarbon	2.4	1.94	4.37	1.97	101.54 ± 0.21	101.253	0.3523	0.35
	2.4	2.32	4.74	2.34	100.86 ± 0.33			
	2.4	2.92	5.36	2.96	101.36 ± 0.47			

Avg.: Average; SD: Standard deviation; % RSD: Percent Relative Standard Deviation.

Sample analysis

As per the developed chromatographic method, *C. quadrangularis* leaves extract was analyzed. The retention factor was found same as that of the standard value. The leaves extract had a R_f value near to 0.69 (Fig. 3). The spots on the plates of TLC could be visible under fluorescence using berberine sulfate reagent. The total aliphatic hydrocarbon content in the *C. quadrangularis* leaves was found to be 0.24% w/w as determined by the linear regression equation. It proves that the method can be successfully applied to determine the hydrocarbon content present in the leaves of *C. quadrangularis*.

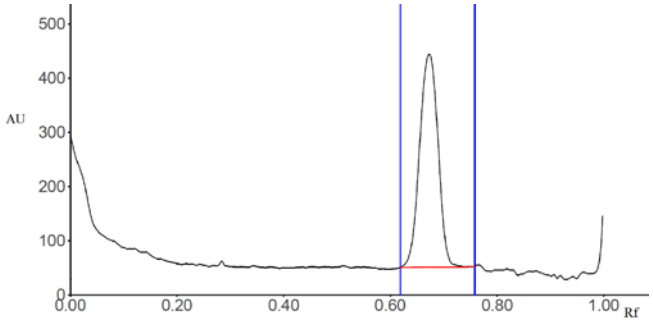

Figure 3. HPTLC chromatogram of *C. quadrangularis* leaves extract.

CONCLUSIONS

The developed HPTLC technique is a novel technique to determine the long chain aliphatic hydrocarbons from *C. quadrangularis* leaves. The technique uses berberine sulphate as a detecting reagent, which helps to determine hydrocarbons easily at 366 nm. The technique can be applied for the determination of other hydrocarbons and the results imply that technique is reproducible and selective for determination of hydrocarbons using HPTLC technique. Also, the method focuses on the determination of hydrocarbon from the leaves of *C. quadrangularis*, which has not been quantified earlier. The developed technique was found to be simple, sensitive, precise, and accurate and can be successfully applied for the determination of long-chain aliphatic hydrocarbons obtained from other plants or other species.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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