Validation of HPTLC Method for the Analysis of Taraxerol in Clitoria ternatea

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Abstract: A new, simple, sensitive, selective and precise HPTLC method has been developed for the determination of taraxerol in Clitoria ternatea L. Determination of taraxerol was performed on TLC aluminium plates. Linear ascending development was carried out in twin trough glass chamber saturated with hexane and ethyl acetate (80:20 v/v). The plate was then dried and sprayed with anisaldehyde reagent. A Camag TLC scanner III was used for spectrodensitometric scanning and analysis at 420 nm. The system was found to give compact spots for taraxerol ($R_f$ 0.53). The calibration plot was linear in the range of 100–1200 ng of taraxerol. The correlation coefficient of 0.9961 was indicative of good linear dependence of peak area on concentration. The concentration of taraxerol was found to be 12.4 mg/g w/w in the hydroalcoholic extract of Clitoria ternatea root. To study the accuracy and precision of the method, recovery studies were performed. Recovery values from 99.65 to 99.74% showed excellent reliability and reproducibility of the method. The limits of detection and quantification were determined to be 31 and 105 ng/spot, respectively. The proposed HPTLC method for quantitative monitoring of taraxerol in Clitoria ternatea can be used for routine quality testing of C. ternatea extract used in Ayurvedic formulations. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Clitoria ternatea L; taraxerol; HPTLC; Ayurveda; marker.

INTRODUCTION

Unlike the single chemical entity that forms the basis of modern pharmacology and drug development, the paradigm of Ayurvedic medicine views multicomponent, multi-ingredient preparations as representing the activity of the herbal drug. Selection of individual analytical compounds for determining either efficacy or quality is contrary to Ayurvedic medicine principles. The common clinical use of Ayurvedic medicine requires the combination of two or more herbals based on recipes and formulae derived from traditional references and empirical evidence of Ayurvedic medicine practitioners. This creates a challenge in establishing quality control standards for raw materials and the standardisation of finished herbal drugs. Currently, there is a common practice of selecting one or more compounds as markers for purposes of identification and quality assessment (Mukherjee, 2002; Mukherjee and Wahile, 2006).

Clitoria ternatea L., commonly called Shankapushpi, is used traditionally for various ailments (Sivarajan and Balachandran, 1994). Roots, seeds and leaves of C. ternatea are commonly used in the Ayurvedic system of medicine. The roots are bitter, refrigerant, laxative, intellect-promoting, diuretic, anthelmintic, tonic and are useful in dementia, hemicrania, burning sensations, leprosy, inflammation, leucoderma, bronchitis, asthma, pulmonary tuberculosis, ascites, fever, otalgia, hepatopathy and as a cathartic (Nadkarni, 1976). Extracts of this plant have been used as an ingredient in ‘Medhya Rasayana’ as a rejuvenating recipe used for treatment of neurological disorders and are considered to enhance the intellect (Sharma and Bhagwan, 1988). C. ternatea has been shown to have number of pharmacological activities such as possessing nootropic, anxiolytic, antidepressant, anti-convulsant, antistress (Jain et al., 2003), sedative (Kulkarni et al., 1988), antipyretic, anti-inflammatory and analgesic activities (Parimaladive et al., 2003). The extract of C. ternatea has been shown to improve learning ability, enhance memory, increase apical and basal dendritic branches, and increase acetylcholine content and acetylcholinesterase activity in rats (Rai et al., 2001; 2002; Taranalli and Cheermankuzhi, 2000).

The major phytoconstituents found in C. ternatea are the pentacyclic triterpenoids such as taraxerol (1) and taraxerone (Banerjee and Chakravarti, 1963, 1964). Taraxerol (1) is one of the major bioactive principle of C. ternatea and has been shown to have a number of pharmacological activities including anti-inflammatory and antimicrobial potentials (Singh et al., 2002; Naik et al., 2004), acetylcholinesterase inhibitory...
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(Lee et al., 2004), anti-carcinogenic, cytotoxic and cancer chemo preventive activities (Jang et al., 2004; Lin et al., 2001; Takasaki et al., 1999). A method based on UV spectrophotometry has been developed and reported for the quantitative determination of pentacyclic triterpenoids (Simonyan et al., 1974). A GC-MS technique was reported for the identification and determination of taraxerol and other triterpenoids (Gawronska-Grywacz and Krzaczek, 2007; Jaffe et al., 2006). GC-MS techniques have been reported for the identification and determination of triterpenoids in Hieracium pilosella L., finding the occurrence of taraxerol, α- and β-amyrin, taraxasterol and fern-7en-3β-ol. Based on quantitative analysis taraxasterol is distinctly predominant in this triterpenoid fraction (Grywacz and Krzaczek, 2007).

In recent years, the profiling of the relative amounts of various active ingredients (i.e. marker profiling) has been shown to be a convenient and effective method for standardisation and quality control of various herbal materials, especially when there is a lack of authentic standards for the identification of all the active components present in these complex natural products (Chen et al., 2006; Lianga et al., 2004). Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) are the methods commonly applied for the identification, assay and testing for purity, stability, dissolution or content uniformity of raw materials and formulated products. HPTLC is a powerful analytical technique due to its merits of reliability, simplicity and speed. Currently, HPTLC is becoming a routine analytical technique owing to its advantages of low operating cost, high sample throughput, need for minimum sample clean-up and being a widely accepted technique for its high accuracy, precision and reproducibility (Larsen et al., 2004; Di et al., 2003). HPTLC has no limitation on the choice of the mobile phase and, unlike HPLC, mobile phases having pH 8 and above can be employed. Direct application of suspensions or dirty or turbid samples is possible. Furthermore, it permits a simultaneous assay of several components in a multicomponent formulation or herbal extract (Abourashed and Mossa, 2004; Patravale et al., 2001).

The validation of analytical methods is largely recognised as the best safeguard against the generation of unreliable data and is becoming an absolute requirement in many fields. Depending on the objective of the analytical procedure, the typical validation characteristics, which can be considered through a statistical approach, are accuracy, precision, detection limit, quantification limit and linearity (Anonymous, 1993, 2002; Mukherjee, 2003). HPTLC combined with scanning densitometry has been adopted as a general method in the European Pharmacopoeia, allowing the use of planar chromatographic procedures for quantitative purposes in different stages of pharmaceutical research, development and production. HPTLC marker analysis of several plants used in Ayurveda has been reported from our laboratory (Kumar et al., 2006; Mukherjee et al., 2006, 2007; Rai et al., 2006). There is no reported analytical method for estimation of taraxerol in C. ternatea, which is a potent memory-enhancing plant used in Ayurveda. The objective of this study was to develop an accurate, specific, repeatable HPTLC method for the determination of taraxerol in root extract of C. ternatea. The proposed method was validated in compliance with ICH guidelines and the USP protocol (ICH, 1996, 2005; Anonymous, 2000).

EXPERIMENTAL

Materials and chemicals. The root parts of C. ternatea were collected from Udhagamandalam, Tamilnadu, India during the month of April and authenticated by Dr S. Rajan, Field Botanist, Government of India. A voucher specimen (SNPS-1032) of this plant has been retained at the School of Natural Products Studies, Jadavpur University, India. Analytical-grade solvents were obtained from E-Merck, Mumbai, India.

Preparation of sample. Freshly collected root of C. ternatea was dried under shade, powdered (10 g), extracted with alcohol (70%, 50 mL) and, after standing for 48 h at room temperature, the hydroalcoholic extract was drained off. This process of extraction at ambient temperature was repeated four times. The extraction of plant material was thoroughly monitored for exhaustive extraction of taraxerol by TLC analysis. The fourth alcoholic extract showed no taraxerol by TLC analysis. The combined hydroalcoholic extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator at 45°C and finally dried under high vacuum to furnish the final extract. A measured quantity of the dried hydroalcoholic extract of C. ternatea root was dissolved in methanol and filtered through Whatman qualitative filter paper no. 1, pore size 11 μm (Maidstone, UK) and the volume of the solution was adjusted with methanol in a volumetric flask to obtain a final concentration of 1 mg/mL. This solution was used for the HPTLC analysis.

Isolation and characterisation of taraxerol. The shade-dried plant material (250 g) was extracted four times with ethanol (750 mL) for 5 h at 60°C and filtered. The filtrate was evaporated in vacuo to give a dark brownish residue. The resultant ethanolic extract (15.6 g) was suspended in water and sequentially extracted using hexane (200 mL), chloroform (200 mL), ethyl acetate (150 mL) and n-butanol (150 mL). The combined hexane and chloroform fraction (6 g) was subjected to chromatography on a silica gel column, using chloroform–methanol (from 100:0 to 0:100, v/v) as an eluent,

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yielding five fractions (A–E). Fraction A (2.5 g) was subjected to chromatography on a silica gel column, using hexane–ethyl acetate (80:20, v/v) as an eluent, to yield compound 1 (0.071 g). The isolated compound was analysed by IR, MS, 1H and 13C NMR and identified by comparison of their spectral data with that in the literature (Banerjee and Chakravarti, 1963; Ogihara et al., 1987; Sakurai et al., 1987).

**Instrumentation and chromatographic conditions.** A Camag (Muttenz, Switzerland) HPTLC system including a Linomat IV sample applicator, a Camag twin-trough plate development chamber, Camag TLC Scanner 3 and WinCats integration software was used. Aluminium-backed HPTLC plates 10 × 10 cm with 0.2 mm layers of silica gel 60 F254 (E. Merck), prewashed with methanol, were used. Taraxerol was used as the marker, and a working standard (100 μg/mL) was prepared. Different concentrations of this solution were applied by means of a Linomat IV sample applicator to the plates about 1 cm above the edge using a bandwidth of 10 mm and distance between tracks of 5 mm. The chromatogram was developed up to 80 mm under chamber saturation conditions with hexane and ethyl acetate (80:20 v/v) as the mobile phase in a Camag twin-trough chamber. The plate was then dried with an air dryer and sprayed as the mobile phase in a Camag twin-trough chamber. Camag TLC Scanner 3 using a detector wavelength of 420 nm. The plate was then scanned with a Camag TLC scanner 3 using a detector wavelength of 420 nm.

**Calibration of taraxerol and analyses of taraxerol in C. ternatea extract.** Different concentrations of taraxerol were plotted against peak area to obtain a calibration plot. A 10 μL aliquot of the extract solution (1 mg/mL) was applied along with 10 μL of standard solution (100 μg/mL). After chromatography, the amount of taraxerol present in the extract was determined by means of the calibration plot.

**Recovery studies.** To study the accuracy and precision of the method, recovery studies were performed by the method of standard addition. The recovery of added standard was studied at three different levels, each being analysed in a manner similar to that described for the assay. The hydroalcoholic extract of Clitoria ternatea root used for recovery studies was pre-analysed by the developed method as mentioned in the Experimental section and found to contain 12.4 mg of taraxerol per gram of extract. Thus, 25 μg of the same pre-analysed extract contained 310 ng/spot of taraxerol, which was used for the recovery studies. The pre-analysed extract samples were spiked with an extra 100, 200 and 300 ng/spot of the standard taraxerol and the mixtures were reanalysed by the proposed method. The experiment was conducted six times to check for the recovery of the taraxerol.

**Precision.** Repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot (400 ng/spot) of taraxerol and was expressed in terms of percentage relative standard deviation (% RSD) and standard error (SE). The intra-day precision was determined at three difference concentration levels of taraxerol 400, 600 and 800 ng/spot three times on the same day and inter-day precision was determined at three difference concentrations of taraxerol (400, 600 and 800 ng/spot) for three times on five different days over a period of one week.

**Limits of detection and quantification.** Three different levels (100, 200 and 300 ng/mL) of the standard stock solution (25 μg/mL) of taraxerol were prepared and used accordingly. Blank methanol was spotted six times following the same method as explained in instrument and chromatographic conditions and the signal-to-noise ratio was determined. The limit of detection (LOD) was considered as 3:1 and LOQ as 10:1. The LOD and limit of quantitation (LOQ) were experimentally verified by diluting the known concentration of taraxerol until the average responses were approximately 3 or 10 times the standard deviation of the response for six replicate determinations.

**RESULTS AND DISCUSSION**

**Isolation and characterisation of taraxerol**

The isolated compound showed a single spot in TLC with hexane–ethyl acetate (80:20, v/v), Rf 0.53. Compound 1 was recrystallised from benzene, giving a melting point of 282°C. To identify the structure of this compound the IR, MS, 1H and 13C NMR were recorded. The EIMS of isolated compound 1 showed m/z (rel. int.) = 426 [M]+ (22), 411(15), 302(41), 287(41), 269(16), 218(28), 204(100), 133(30). HREI-MS gave a molecular at m/z = 426. 6014 [M]+ (calcd 426.7174),
and the corresponding molecular formula was C_{30}H_{50}O. The structure of taraxerol (1) was confirmed by comparing physicochemical and spectral data (UV, FTIR, NMR and MS) with those in the published literature (Banerjee and Chakravarti, 1963; Ogihara et al., 1987; Sakurai et al., 1987).

**Calibration of taraxerol and analysis of taraxerol in**

**C. ternatea** **extract**

The selected mobile phase resolved taraxerol efficiently from other components of C. ternatea. The $R_f$ of taraxerol was found to be 0.53. The calibration plot (Fig. 1) was linear in the range 100–1200 ng of taraxerol, and the correlation coefficient of 0.9961 was indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation $y = 7.86x + 1318.5$, where $y$ is the response as peak area and $x$ is the concentration. The developed TLC plate with extract of CT root and taraxerol is shown in Fig. 2 and the HPTLC chromatograms obtained from taraxerol and the hydroalcoholic extract of C. ternatea roots are shown in Fig. 3(A, B), respectively. The taraxerol content was found to be 12.4 mg/g of hydroalcoholic extract of CT root.

**Accuracy and precision**

To ascertain the effectiveness of the method, a suitability test was performed on a freshly prepared standard stock solution of taraxerol spiked with pre-analysed C. ternatea extract. The results of recovery studies are listed in Table 1. The recovery values

<table>
<thead>
<tr>
<th>Excess taraxerol added to extract (ng)</th>
<th>Measured content of taraxerol in mixture (ng)</th>
<th>Average amount of taraxerol found (ng)</th>
<th>Average recovery (%)</th>
<th>Overall average recovery (%)</th>
<th>% RSD</th>
<th>SE</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>310</td>
<td>309.08</td>
<td>99.70</td>
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<td>0.157</td>
<td>0.198</td>
</tr>
<tr>
<td>100</td>
<td>410</td>
<td>408.57</td>
<td>99.65</td>
<td>99.66</td>
<td>0.070</td>
<td>0.117</td>
</tr>
<tr>
<td>200</td>
<td>510</td>
<td>508.68</td>
<td>99.74</td>
<td></td>
<td>0.106</td>
<td>0.220</td>
</tr>
<tr>
<td>300</td>
<td>610</td>
<td>607.98</td>
<td>99.67</td>
<td></td>
<td>0.223</td>
<td>0.554</td>
</tr>
</tbody>
</table>

To determine the accuracy of the method, a suitability test was performed on a freshly prepared standard stock solution of taraxerol spiked with pre-analysed C. ternatea extract. The results of recovery studies are listed in Table 1. The recovery values...
obtained were in the range 99.65–99.74%, showing the reliability and reproducibility of the method. The overall average percentage recovery was found to be 99.69%. A percentage relative standard deviation of <1% for intra- and inter-day analysis was found and it is depicted in Table 2. Intra-day precision (%RSD) on the basis of content of taraxerol was between 0.034 and 0.019. Inter-day precision (%RSD) on the basis of content of taraxerol was between 0.036 and 0.023.
Limit of detection and quantification

The LOQ and LOD were calculated from the equations LOD = 3xN/B and LOQ = 10xN/B, where N is the SD of the peak area of the standard (n = 3), taken as a measure of the noise, and B is the slope of the corresponding calibration curve. The LOQ was found to be 105 ng and the LOD was 31 ng.

Herbal drugs, singularly and in combinations, contain myriad compounds in complex matrices in which no single active constituent is responsible for the overall efficacy. This creates a challenge in establishing quality control standards for raw materials and the standardisation of finished herbal drugs. Currently, there is a common practice in natural product analysis of selecting one or more markers for purposes of identification and quality assessment. Estimation of the content of marker in Ayurvedic botanicals is of the utmost importance in evaluating the phytochemical entity of the herb. *Clitoria ternatea* is used traditionally for various ailments. Roots, seeds and leaves of *C. ternatea* are commonly used in Ayurveda. This plant extract is used in ‘Medhya Rasayana’ as a rejuvenating recipe for treatment of neurological disorders and is considered to enhance the intellect. The current study was performed to develop an HPTLC method for the determination of taraxerol in roots of *C. ternatea*. The developed HPTLC method is precise, specific and accurate for the determination of taraxerol, a selected marker for this plant. Since the proposed mobile phase effectively resolves taraxerol, the method can be used for quantitative analysis of taraxerol in herbal extracts used for Ayurvedic formulation.

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Table 2  Intra- and inter-day precision of HPTLC (n = 6)

<table>
<thead>
<tr>
<th>Amount (ng/spot)</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean area</td>
<td>SD</td>
</tr>
<tr>
<td>400</td>
<td>4581.67</td>
<td>1.57</td>
</tr>
<tr>
<td>600</td>
<td>5629.10</td>
<td>1.53</td>
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<tr>
<td>800</td>
<td>7498.58</td>
<td>1.45</td>
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REFERENCES


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