Simultaneous HPTLC Analysis of Ursolic Acid, Betulinic Acid, Stigmasterol and Lupeol for the Identification of Four Medicinal Plants Commonly Available in the Indian Market as Shankhpushpi

Neeraj Kumar Sethiya* and Shrihari Mishra
Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The M. S. University of Baroda, Vadodara, Gujarat 390002, India

*Author to whom correspondence should be addressed. Email: nscognosy2006@gmail.com

Received 30 December 2013; revised 21 June 2014

In this study, we investigated a new, simple, sensitive, selective and precise high-performance thin-layer chromatography (HPTLC) fingerprint and quantitative estimation method for the analysis of ursolic acid, betulinic acid, stigmasterol and lupeol in Shankhpushpi botanicals. Linear ascending development was carried out in a twin trough glass chamber saturated with petroleum ether–ethyl acetate–toluene (7:2:1, v/v/v). The plate was dried, sprayed with anisaldehyde reagent and analyzed by CAMAG TLC scanner III at 580 nm. The system was found to give compact spots for ursolic acid (0.21), betulinic acid (0.29), stigmasterol (0.33) and lupeol (0.50). The relationship between the concentration of standard solutions and the peak response is linear within the concentration range of 100–600 ng/gpt for ursolic acid, betulinic acid, stigmasterol and lupeol. The concentration of 134.2 and 146.1 mg of ursolic acid per gram of Clitorea ternatea (CT) and Canscora decussata (CD); 110.6 mg of betulinic acid per gram of EA; 92.75, 154.95, 31.947 and 39.21 mg of stigmasterol per gram of Evolvulus alsinoides (EA), Convolvulus pluricaulis (CP), CT and CD; 30.12 mg of lupeol per gram of CT were found. The proposed HPTLC method may use for routine quality testing and identification of Shankhpushpi botanicals.

Introduction

The current usage of botanicals is quite different from their historical use, because scientists call that traditional knowledge is validated, that the results are reproducible and that the treatments are safe and effective (1, 2). This has led to the production of standardized botanical extracts from properly identified and controlled plant species (3). Shankhpushpi is an official drug of Ayurveda and has been utilized as Medhya Rasayana—meaning a drug that rejuvenates, maintains and potentiates intellect and memory (4). This herb is vigorously used in Indian industry as a source of raw material for development of ayurvedic and herbal formulation (bhasma, syrup and tablets) for enhancing memory and intellect (5). Shankhpushpi is a Sanskrit word, which means herb with conch (a musical instrument) like shape of flower (6). On the basis of flower shape, the following four botanicals Convolvulus pluricaulis Choisy. (CP) (Convolvulaceae), Evolvulus alsinoides Linn. (EA) (Convolvulaceae), Clitorea ternatea Linn. (CT) (Leguminosae) and Canscora decussata Schult. (CD) (Gentianaceae) were adopted as sources of Shankhpushpi by Indian practitioners (6–8). These plants proved their scientific potential in CNS depression, anxiolytic, tranquilizing, antidepres- sant, anti-stress, neurodegenerative, antiinmensive, antioxidant, hypolipidemic, immunomodulatory, analgesic, antifungal, antibacterial, antidiabetic, antiulcer, antacatonic and cardiovascular activity. These were reported to contain several types of alkaloids, flavonoids and coumarins as active chemicals that bring about their biological effects (6, 9–13).

Ursolic acid, betulinic acid and lupeol are pentacyclic triterpenic acids, while stigmasterol is a phytosterol (14). These are plant-based secondary metabolites, present in the form of free acids, sterols or saponins and were proven for their wide spectrum of pharmacological activities (15, 16). Among analytical methods, these were analyzed either alone or combination with one or two other secondary metabolites. The method includes separation of triterpenic acids and phytosterol by using high-performance liquid chromatography (HPLC) (17–23), evaporative light scattering (24), mass spectrometry (MS) (25), gas chromatography (GC) (26, 27), capillary supercritical fluid chromatography (28) and capillary electrophoresis (29, 30). The major limitation of these methods is need of sophisticated instrumentation and prolongs time for analysis. In the last two decades, high-performance thin-layer chromatography (HPTLC) has emerged as an efficient tool for the quantitative analysis of different compounds found within complex natural samples (31–33). Although there are several HPTLC methods were reported on the above-mentioned compounds either individually or combined with others (14, 34–36), these methods are not utilized for the present purpose because of their limitation to use in the quality control of controversial plants. So, there is still need to develop the TLC method, which could be utilized for identification and estimation of ursolic acid, betulinic acid, stigmasterol and lupeol to differentiate extracts of plants similar in common or local names. Keeping in view the utility of Shankhpushpi, and the lack of an appropriate simple TLC method for the simultaneous separation of triterpenic acids and phytosterol, it is proposed to develop a routine method of analysis for simultaneous qualitative and quantitative estimation of ursolic acid, betulinic acid, stigmasterol and lupeol (Figure 1) in botanicals of Shankhpushpi by HPTLC.

Experimental

Plant materials

Canscora decussata (CD) was collected from the Ninai ghat (Gujarat, India) and identified by Dr S.C. Agrawal (Department of Botany, CDRI, Lucknow, India). Clitorea ternatea (CT), Convolvulus pluricaulis (CP) and Evolvulus alsinoides (EA)
were collected from locality of Vadodara, Gujarat (India) and identified in the Botany Department, The M. S. University of Baroda, Vadodara, Gujarat (India). Voucher specimens of all four plants (No. Pharmacy/EA/09-10/10/NS, Pharmacy/CP/09-10/11/NS, Pharmacy/CT/09-10/12/NS and Pharmacy/CD/09-10/13/NS) have been deposited in the Herbal Drug Technology Lab, Pharmacy Department, The M. S. University of Baroda, Vadodara, Gujarat (India).

**Chemicals and reagents**

Ursolic acid, betulinic acid, stigmasterol and lupeol were obtained from Sigma Aldrich, Mumbai (India). Other solvents and chemicals used in study were procured from SD Fine Chemical, Mumbai (India) and all are of analytical grade. Precoated silica gel 60F254 TLC plates were purchased from Merck, Darmstadt (Germany).

**HPTLC fingerprinting**

**Instruments**

A Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland) was used as the spotting device. A 100-μL syringe was also used (Hamilton Bonaduz, Switzerland). The TLC chamber was a glass twin-trough chamber (20 × 10 × 4 cm) (Camag) and the densitometer was a TLC Scanner III linked to WINCATS software (Camag). The HPTLC plates were 20 × 10 cm, 0.2-mm thickness and precoated with silica gel 60 F254 (E. Merck, Darmstadt, Germany). The length of the chromatogram run was 8 cm. Subsequent to chromatographic development, TLC plates were dried in air with the help of a TLC plate dryer.

**Preparation of standard solutions**

A common stock solution (1 mg/mL) of ursolic acid, betulinic acid, stigmasterol and lupeol were prepared. Aliquots (1.06.0 mL) of the stock solution were transferred to 10 mL volumetric flasks and diluted to volume with methanol to furnish standard solutions containing 100, 200, 300, 400, 500 and 600 ng/μL (Figure 2).

**Preparation of sample solutions**

Each herb was shade dried at room temperature and coarsely powdered. Then, an accurately weighed 5 g of dried coarse powder of each CP, EA, CT and CD (whole herb) were extracted separately with methanol (3 × 50 mL) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and dried on a rotary evaporator and transferred to a 50-mL volumetric flask, and the volume was made up with methanol to get stock solution (10 mg/10 mL). The obtained extract from each plant was treated separately for the qualitative and quantitative estimation of phytochemicals (Figure 2).

**Figure 1.** Chemical structures of (A) ursolic acid, (B) betulinic acid, (C) stigmasterol and (D) lupeol.

**Figure 2.** Experimental protocol for chromatographic studies.

HPTLC Method for the Simultaneous Analysis of Ursolic Acid, Betulinic Acid, Stigmasterol and Lupeol in *Shankhpushpi*
Chromatographic studies
Thin layer chromatographic (TLC) studies were performed using various solvent systems, and finally, petroleum ether:ethyl acetate:toluene (7:2:1, v/v/v) were found to be suitable mobile phase for the proper separation of ursolic acid, betulinic acid, stigmasterol and lupeol in a single track. These markers were further fingerprint with various samples of Shankhpushpi to ascertain their presence (Figure 2).

Derivatization and densitometric scanning
The plates were derivatized with the use of anisaldehyde-sulfuric (AS) reagent. To prepare the reagent, 0.5 mL anisaldehyde is mixed with 10 mL glacial acetic acid, followed by 85 mL methanol and 5 mL of concentrated sulfuric acid. All the chemicals, used for the reagent preparation, were ice-cold. The plates were immersed in AS reagent for 1 s and then heated at 100°C for 10 min. The plates were densitometrically scanned (580 nm) with slit dimension 1 x 0.1 mm. After the development, bands in the extracts were identified by matching their RF values with those obtained for standards (Figure 2).

Calibration of ursolic acid, betulinic acid, stigmasterol and lupeol and their analysis in different Shankhpushpi botanical extracts
Different concentrations (100–600 ng/spot) of 1:1:1:1 w/v mixture of ursolic acid, betulinic acid, stigmasterol and lupeol were plotted against the peak area to obtain a calibration plot. Further, 20 µL of the extract solution were applied. After applying the chromatography technique, the amounts of ursolic acid, betulinic acid, stigmasterol and lupeol present in the respective extracts were determined by means of the calibration plot.

Method validation
Accuracy and precision
The repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot (300 ng/spot) of ursolic acid, betulinic acid, stigmasterol and lupeol, which were expressed in terms of percentage relative standard deviation (% RSD) and standard error (SE). The intraday precision was determined at three different concentration levels of different markers, 100, 300 and 500 ng/spot, six times on the same day, and the interday precision was determined at three different concentrations of markers, 100, 300 and 500 ng/spot, six times on five different interval days over a period of 1 week (37).

Robustness of the method
By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions such as petroleum etherethyl acetate:toluene (8:2:1, v/v/v) and petroleum etherethyl acetate:toluene (7:3:1, v/v/v) were tried and the chromatograms were run. The amount of mobile phase, temperature and relative humidity were varied in the range of ±5%. The plates were prewashed by methanol and activated at 60°C ± 5 for 2, 5 and 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. The robustness of the method was done at three different concentration levels 100, 300 and 500 ng/spot (38).

Limits of detection and quantification
The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formulas: LOD = 3.3 (SD/S) and LOQ = 10 (SD/S). The SD of the response was determined based on the SD of y-intercepts of regression lines (39).

Table 1
<table>
<thead>
<tr>
<th>CP</th>
<th>EA</th>
<th>CT</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stigmasterol</td>
<td>Betulinic acid</td>
<td>Stigmasterol</td>
<td>Ursolic acid</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>Lupeol</td>
<td>Ursolic acid</td>
<td>Stigmasterol</td>
</tr>
</tbody>
</table>

Figure 3. TLC densitograms of standards. Ursolic acid standard (Peak 1; RF 0.21), betulinic acid standard (Peak 2; RF 0.29), stigmasterol standard (Peak 3; RF 0.33) and lupeol standard (Peak 4; RF 0.50). Scanned at 580 nm after derivatization with AS reagent using petroleum ether:ethyl acetate:toluene (7:2:1, v/v/v) as solvent system.

Sethiya and Mishra
Downloaded from https://academic.oup.com/chromsci/article/53/5/816/314000 by guest on 30 November 2022
Figure 4. TLC densitogram of four botanicals of Shankpushpi for the qualitative and quantitative estimation of ursolic acid, betulinic acid, stigmasterol and lupeol.

Figure 5. Calibration (regression via area) plot for (A) ursolic acid, (B) betulinic acid, (C) stigmasterol and (D) lupeol.
Specificity

The specificity of the method was ascertained by analyzing the standard drug and sample. The spots for ursolic acid, betulinic acid, stigmasterol and lupeol in sample were confirmed by comparing the Rf values and spectra of the spot with that of standard. The peak purity of ursolic acid, betulinic acid, stigmasterol and lupeol were assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot (40).

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 analyzed in a manner similar to that described for the assay (40–42). The methanol extract of CP, EA, CT and CD were used for recovery studies. These were preanalyzed by the method of standard addition.

The recovery of added standard was studied at three different levels, which were spiked with an extra 1:2 ratio, i.e., 100 and 200 ng of the respective standard of the ursolic acid, betulinic acid, stigmasterol and lupeol content of the respective sample, which were reanalyzed by the proposed method.

Results

TLC fingerprinting of ursolic acid, betulinic acid, stigmasterol and lupeol in Shankhpushpi botanicals

The Rf of ursolic acid, betulinic acid, stigmasterol and lupeol were found to be 0.21, 0.29, 0.33 and 0.50, respectively (Figure 3). The results obtained by fingerprinting of ursolic acid, betulinic acid, stigmasterol and lupeol in Shankhpushpi botanicals are shown in Table I and Figure 4.

Calibration of ursolic acid, betulinic acid, stigmasterol and lupeol and their analysis in different Shankhpushpi botanical extracts

The calibration plots (Figure 5) were linear in the range 100–600 ng, and the correlation coefficient (r) of 0.9825 (ursolic acid), 0.9689 (betulinic acid), 0.9915 (stigmasterol) and 0.9893 (lupeol) were indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation $y = 3581 + 1.585x$, $y = 4862 + 0.6321x$, $y = 71.25 + 0.4862x$ and $y = 2626 + 0.7194x$ for ursolic acid, betulinic acid, stigmasterol and lupeol, respectively (where $y$ is the response as peak area and $x$ is the concentration). Among Shankhpushpi botanicals, 1 g extract of CT and CD was found to contain 134.2 and 146.1 mg of ursolic acid; 92.75, 154.95, 31.947 and 39.21 mg of stigmasterol per gram of CT and CD; 110.6 mg of betulinic acid per gram of EA; 92.75, 154.95, 31.947 and 39.21 mg of stigmasterol per gram of EA, CP, CT and CD and 30.12 mg of lupeol per gram of CT, respectively.

Method validation

Accuracy and precision

To ascertain the effectiveness of the method, suitability tests were performed on a freshly prepared mixture of standard

---

**Table II**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean area (ng)</th>
<th>SD %</th>
<th>RSD %</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ursolic acid</strong></td>
<td>2353.07</td>
<td>3.18</td>
<td>0.12</td>
<td>1.29</td>
</tr>
<tr>
<td><strong>Betulinic acid</strong></td>
<td>2931.76</td>
<td>0.37</td>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Stigmasterol</strong></td>
<td>7142.97</td>
<td>0.71</td>
<td>0.01</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Lupeol</strong></td>
<td>7142.97</td>
<td>0.65</td>
<td>0.01</td>
<td>0.27</td>
</tr>
</tbody>
</table>

---

**Table III**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD % of peak area</th>
<th>RSD % of peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ursolic acid</strong></td>
<td>1.62</td>
<td>1.24</td>
</tr>
<tr>
<td><strong>Betulinic acid</strong></td>
<td>1.01</td>
<td>1.12</td>
</tr>
<tr>
<td><strong>Stigmasterol</strong></td>
<td>0.97</td>
<td>1.26</td>
</tr>
<tr>
<td><strong>Lupeol</strong></td>
<td>0.65</td>
<td>0.87</td>
</tr>
</tbody>
</table>

---

*Average of three concentration 100, 300 and 500 ng per spot.*
stock solutions of ursolic acid, betulinic acid, stigmasterol and lupeol spiked with preanalysed with identified extract of Shankhpushpi botanicals. The repeatability of sample application and measurement of peak area were expressed in terms of RSD % and the % RSD s for intra- and interday analysis are depicted in Table II. Intraday precision (% RSD) on the basis of content of ursolic acid, betulinic acid, stigmasterol and lupeol were found to be 0.02–0.10, 0.004–0.65, 0.0003–0.1 and 0.007–0.04, respectively, whereas interday precision (% RSD) on the basis of the content were found to be 0.005–0.12, 0.009–0.01, 0.006–0.02 and 0.003–0.03, respectively.

Robustness of the method
The low values of RSD % as shown in Table III indicate the robustness of the method.

Limit of detection and quantification
The LOQ and LOD were calculated from the equations LOD = 3.3 (SD/S) and LOQ = 10 (SD/S). The LOQ and LOD are shown in Table IV.

Specificity
The peak purity of ursolic acid, betulinic acid, stigmasterol and lupeol was assessed by comparing the spectra of standard at peak start, peak apex and peak end positions of the spots, i.e., \( r(\text{start, middle}) = 0.9973 \) and \( r(\text{middle, end}) = 0.9979 \). Good correlation \( (r = 0.9994) \) was also obtained between the standard and sample.

Recovery studies
The results of content estimation and recovery studies of ursolic acid, betulinic acid, stigmasterol and lupeol from botanicals of Shankhpushpi extracts after spiking it with 100 and 200 ng/spot of additional standards are listed in Table V.

Discussion
Shankhpushpi is an ayurvedic herb and utilized as raw material for the herbal formulation (mainly syrup, tablet and bhasma) development in various parts of India for treatment of nervous debility. The major ambiguity reflected with this plant is that there is adaptation of four herbs CP, EA, CT and CD because of similarity in morphological appearance of flower as a source of Shankhpushpi (43). So, there is need of method related to their routine quality control. The simplicity of the sample preparation and the possibility of analysing several samples of herbal products simultaneously in a short time make HPTLC the method of choice (44, 45). There are some HPTLC methods related to quality control of individual herbs (8, 46). The literature also reveals the presence of other validated methods for quantification of ursolic, oleanolic and betulinic acids and quantification of lupeol and stigmasterol in plant extracts and formulation (34–36). These methods are simple and robust for identification of single herbs but lacking in purpose of differentiation among Shankhpushpi botanicals due to the limitation of markers selection. So, there is no report of simultaneous quantification of ursolic acid, betulinic acid, stigmasterol and lupeol, which can be utilized for purpose of differentiation among the four Shankhpushpi botanical extracts. Hence we developed a simple and precise method for quantification of these marker compounds.

In this method, ursolic acid, betulinic acid, stigmasterol and lupeol were quantified from Shankhpushpi botanical extracts by the TLC densitometric method using HPTLC. The TLC densitometric method was validated in terms of precision, repeatability, and accuracy. The selected mobile phase (petroleum ether:ethyl acetate:toluene; 7:2:1, v/v/v) well resolved ursolic acid, betulinic acid, stigmasterol and lupeol. AS reagent was

<table>
<thead>
<tr>
<th>Table IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>Ursolic acid</td>
</tr>
<tr>
<td>Betulinic acid</td>
</tr>
<tr>
<td>Stigmasterol</td>
</tr>
<tr>
<td>Lupeol</td>
</tr>
</tbody>
</table>

*Correlation coefficient.

<table>
<thead>
<tr>
<th>Table V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>Ursolic acid</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

CD, Canscora decussata; CT, Cilantro temetan; DP, Convolvulus pluricaulis; EA, Evulexus altior;
used to derivatize the chromatograms. Two modes of derivatization were checked: spraying and dipping. More reproducible results were obtained in the latter case. These markers solve the purpose of differentiation as evidence from Table I and Figure 3.

The linearity in the range of 100–600 ng for ursolic acid, betulinic acid, stigmasterol and lupeol, with correlation coefficient of 0.9825, 0.96899, 0.9915 and 0.9893, was indicative of good linear dependence of peak area on concentration, respectively.

The TLC densitometric method was found to be precise with RSD for intraday in the range of 0.02–0.10, 0.004–0.065, 0.0003–0.1 and 0.007–0.04 and for interday in the range of 0.005–0.12, 0.009–0.01, 0.006–0.02 and 0.003–0.03 for different concentrations of ursolic acid, betulinic acid, stigmasterol and lupeol, respectively.

The SD of peak areas was calculated for each parameter and RSD % was found to be <2%, which shows robustness of the method. This indicates that the proposed method was precise and reproducible.

The LOD values of ursolic acid, betulinic acid, stigmasterol and lupeol were found to be 0.1 and 0.007–0.04 and for interday in the range of 0.005–0.12, 0.009–0.01, 0.006–0.02 and 0.003–0.03 for different concentrations of ursolic acid, betulinic acid, stigmasterol and lupeol, respectively.

The peak purity of individual ursolic acid, betulinic acid, stigmasterol and lupeol were assessed by comparing the spectra at the peak start, peak apex and peak end positions of the spot, i.e., $r_{(\text{start, middle})} = 0.9991$ and $r_{(\text{middle, end})} = 0.9993$. We found a good correlation ($r = 0.9994$) of peak between the standard and sample.

The average percent recoveries at two different levels were found in the range of 99.36–100.35%, showing the reliability and reproducibility of the method.

Conclusion

The TLC method developed here for the fingerprinting and quantification of ursolic acid, betulinic acid, stigmasterol and lupeol is simple, rapid, cost-effective and easily adaptable for the routine quality control of Shankhpushpi botanicals. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities, which allows the determination of variations in the botanicals of Shankhpushpi.

Acknowledgments

Neeraj Kumar Sethiya is thankful to University Grant Commission, New Delhi (India), for providing Junior Research Fellowship for the project. Neeraj Kumar Sethiya and Shrihari Mishra are thankful to Anchorm HPTLC Technologists, Mumbai (India), for providing the facilities for technical advice regarding instrument handling.

References

3. Sethiya, N.K., Nahata, A., Dixit, V.K.; Simultaneous spectropho-41

...metric determination of scopoletin and mangiferin in a methanolic extract of *Canscora decussata* Schult.; *Asian Journal of Traditional Medicines*, (2008); 3: 224–229.
8. Sethiya, N.K., Nahata, A., Dixit, V.K.; Comparative thin layer chromatographic investigations on commercial sources of Shankhpushpi in India; *Pharmacognosy Journal*, (2009); 1: 224–226.
19. Zachigna, M., Cateni, F., Faudale, M., Sosa, D., Dellaloggia, R.; Rapid HPLC analysis for quantitative determination of the two isomeric triterpenic acids, oleanolic acid and ursolic acid, in *Planteago major*; *Scientia Pharmaceutica*, (2009); 77: 79–86.