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Determination of triptolide in root extracts of *Tripterygium wilfordii* by solid-phase extraction and reverse-phase high-performance liquid chromatography

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Abstract

Extracts of *Tripterygium wilfordii* roots have a long history of use in traditional Chinese medicine and have shown great promise in recent clinical trials as a treatment for rheumatoid arthritis. The major active component of *Tripterygium* root extracts is the diterpenoid triptolide. This paper describes a method for the determination of triptolide in root extracts that is suitable for the analysis of many small samples simultaneously. Extracts are applied to aminopropyl solid-phase extraction (SPE) tubes that are then eluted with dichloromethane–methanol (49:1, v/v). The eluate is chromatographed on a pentafluorophenyl HPLC column using an acetonitrile:water gradient. Triptolide is quantified by ultraviolet detection at 219 nm. Using this method, it was shown that smaller diameter roots with secondary growth contained higher triptolide concentrations than larger roots. This suggests that roots to be used for production of the drug extract could be harvested while still small, which would reduce the growing time necessary and thus be economically beneficial for the growers.

Keywords: Pharmaceutical analysis; *Tripterygium wilfordii*; Triptolide; Diterpenes; Plant materials

1. Introduction

Extracts of the roots of *Tripterygium wilfordii* Hook.f, a plant known as *lei kung teng* or *lei gong teng* (“Thunder God Vine”), have long been used in traditional medicine in China [1]. At present, root extracts are used in China to treat a number of autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, and skin diseases [1,2]. Western scientists have become interested in this plant in recent years because of its antiinflammatory, immunosuppressive, antitumor, antioxidant, and antiviral effects [1,3–7]. An ethanol–ethyl acetate extract is being developed as a botanical drug for rheumatoid arthritis. Two early stage clinical trials in the US [8,9] have shown very encouraging results, and a Phase II trial is in progress.

The major active constituent of *Tripterygium* root extracts is triptolide, a diterpenoid [10] (Fig. 1). It is anticipated that the botanical drug based on the *Tripterygium* extract and developed for rheumatoid arthritis will be standardized based on triptolide content. A method for quantifying triptolide in *Tripterygium* extracts is therefore required. The method must be suitable for analyzing many samples at once, and should use only instrumentation widely available in quality control labs.

Some previously published methods (e.g. [11]) entail separation by TLC followed by detection with Kedde reagent, which is unstable and fades quickly. A method using micellar electrokinetic capillary chromatography (MEKC) has been described [12], but this equipment is not as commonly available as HPLC or GC. A published GC method [13] requires derivatization of samples with both diazomethane and *N*-(trimethylsilyl)imidazole. It is also slow, requiring 70 min for each chromatographic run. An HPLC method with a solid-

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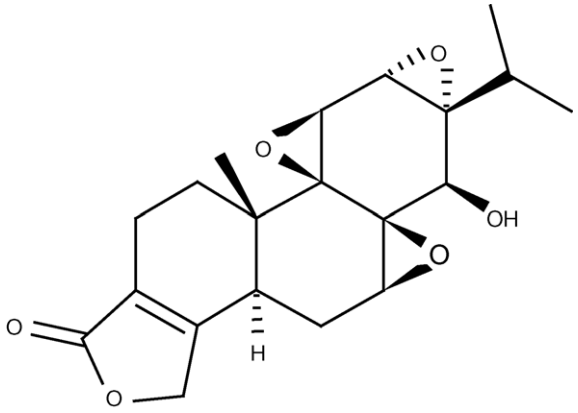

Fig. 1. Chemical structure of triptolide.

phase extraction (SPE) cleanup step has been used [14], but this method calls for a rather large (50 mg) sample of extract, and correspondingly large amounts of eluting solvent. The method described below uses HPLC and SPE, both common procedures in analytical labs, and is suitable for the analysis of many samples simultaneously.

2. Experimental

2.1. Solvents and chemicals

Dichloromethane, methanol, and acetonitrile were HPLC grade; ethyl acetate was reagent grade (Sigma-Aldrich, St. Louis, MO, USA or Fisher, Fair Lawn, NJ, USA). Ethanol (95%, Pharmco, Brookfield, CT, USA) was ACS/USP grade, and acetic acid (EM Science, Gibbstown, NJ, USA) was ACS grade. Water was from a Millipore (Billerica, MA, USA) Synergy™ 185 system and was degassed before HPLC.

The triptolide used as a standard was obtained from Fujian Provincial Medicines and Health Products Import and Export Corp., Fuzhou, China. The compound and its solutions were stored at −20 °C in aluminum foil-wrapped glass vials. A sample of tripdiolide was also obtained from the same source. The identity of both compounds was confirmed by LC–MS using a Waters LC–MS Integrity™ System comprising a 717plus autosampler, 616 pump together with 600S pump controller, in-line degasser, 996 photodiode array detector, and two mass detectors: a Waters Thermabeam electron impact (EI) single quadrupole mass detector, and a Varian 1200L triple quadrupole mass detector with electrospray ionization interface (ESI), operated in positive ionization mode. Data from the EI and UV detectors were collected and compiled using the Waters Millennium 32 software package, complemented by the Wiley library of EI mass spectral data, 6th edition. Data from the Varian 1200L mass detector were collected and compiled using Varian's MS Workstation, v. 6.3, SP1. The HPLC column was a Luna® C_8 (Phenomenex®, Torrance, CA, USA), 250 mm × 4.6 mm, 5 μm particle size, 100 Å pore size, with a Phenomenex SecurityGuard™ C_8 guard cartridge. Samples were run using a gradient of acetonitrile with 0.5% acetic acid in water, at ambient temperature and a flow rate of 0.5 mL/min. The retention times, UV spectra, and EI fragmentation patterns matched those of authentic samples, and the molecular weights were confirmed using the electrospray ionization detector.

2.2. Plant material

Roots of *T. wilfordii* were obtained from plants grown hydroponically in a commercial greenhouse. A voucher specimen (Anita Brinker s.n., 9/2/04) was deposited in the Chrysler Herbarium, Rutgers University (CHRB). Root samples were also obtained from plants growing outdoors at Arnold Arboretum, Harvard University, Jamaica Plain, MA, and from plants grown in a field plot at Rutgers University, New Brunswick, NJ. Normally, only roots in which secondary growth had begun (i.e. roots that had become woody) were used. Roots with secondary growth are easily detected in this species because they are bright orange, due to the presence of the quinone methide celastrol in the root bark. The roots were peeled to remove the thin bark layer.

For the root age study, roots were harvested separately from five plants grown hydroponically. The roots were divided into three classes: those that had not yet begun secondary growth, smaller (diameter <3 mm) roots with secondary growth, and larger (diameter ≥3 mm) roots with secondary growth. Because the root samples were small, only one determination per sample was done. Only the roots with secondary growth were peeled.

2.3. Extract preparation

Roots were dried in a 54 °C oven for at least 16 h, and cut into small pieces. Ethanol was added at the rate of 12 mL/g dry weight of root pieces and the samples were refluxed for 2 h. The liquid fraction was removed and replaced with fresh ethanol (9 mL/g dry root weight), and the samples were heated again for 1 h. The combined extracts were evaporated to dryness under vacuum in a SpeedVac® model AES2010 (Savant, Holbrook, NY, USA) on the low heat setting. The dried solid was extracted with ethyl acetate by sonicating for 30 min in a Fisher Scientific (Pittsburgh, PA, USA) FS30 sonicator. The samples were centrifuged at 0.6 × *g* for 5 min, and the supernatant removed. The ethyl acetate extraction step was repeated on the solid twice more, and the combined supernatants were dried and weighed.

2.4. Solid-phase extraction

Samples of the ethanol–ethyl acetate extracts were dissolved in dichloromethane–methanol (49:1 v/v) at a concentration of 5 mg/mL with 10–15 min sonication. SPE tubes (Phenomenex Strata™ NH_2, 3 mL

with 500 mg packing) were equilibrated with 5 mL dichloromethane–methanol. Extract solution (600 μL) was loaded, and the triptolide-containing fraction was eluted with 1 mL dichloromethane–methanol. Flow through the tubes was by gravity. The eluates were dried in the SpeedVac, then redissolved in 60 μL ethanol for HPLC.

2.5. HPLC

The HPLC system consisted of a Waters (Milford, MA, USA) 717+ autosampler, 996 photodiode array detector, and three 510 pumps controlled by Millenium™ software, Version 2.10. The column was a Phenomenex Curosil™ PFP (pentafluorophenyl) column, 250 mm × 4.6 mm, 5 μm particle size, with a Phenomenex SecurityGuard™ phenylpropyl guard cartridge. Solvent A was water and solvent B was acetonitrile. The gradient program was as follows: begin with 73% A, linear gradient to 52% A over 4 min, hold at 52% A for 3 min, linear gradient to 5% A over 8 min, return to 73% A over 5 min, hold 10 min (30 min total run time). The system was run at ambient temperature and the flow rate was 1 mL/min. Sample injections were 20 μL. Triptolide was quantified based on peak areas at 219 nm, the maximum in the UV spectrum of triptolide. The identity of the peak could be confirmed by examining the UV spectrum.

2.6. Standard curve

The triptolide concentrations in the standard curves and the sample addition experiments were chosen based on the results of root analyses done by another method. The highest concentration found among several dozen root samples was about 6 μg triptolide/mg extract, although the majority of samples tested had values lower than 1500 ng/mg extract.

A stock solution of triptolide was made by dissolving the compound in ethanol in a volumetric flask. Various amounts of the stock solution were added to sample vials, dried in the SpeedVac, then redissolved in 60 μL ethanol to give concentrations ranging from 5 μg to 250 mg/L. Twenty microliters of each solution was injected into the HPLC; there were three replicate injections for each concentration (1.25, 2.5, 6.25, 10, 17.5, 35, 60, 100, 175, 250 mg triptolide/L for the first two experiments; 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 mg/L for the third).

2.7. Standard addition

For the standard addition experiments, aliquots of triptolide stock solution (three replicates per concentration; final concentrations of added triptolide 0, 2.5, 17.5, 35, 60, 100, 175, 250 mg/L in the first experiment; 0, 1.25, 2.5, 5, 12.5, 25, 37.5 mg/L in the second) were added to small vials and dried; then 600 μL of a solution of root extract (5 mg/mL in dichloromethane–methanol, 49:1 v/v) was added to each vial. The spiked samples were analyzed by the SPE-HPLC method.

2.8. Stability studies

To confirm that drying samples in the SpeedVac did not cause loss of triptolide, aliquots (10 μL) of a stock solution of triptolide were diluted tenfold with ethanol. Half the samples were analyzed immediately by HPLC; the rest were dried in the SpeedVac and then redissolved in 100 μL ethanol for HPLC.

3. Results and discussion

3.1. Stability of triptolide in solutions

Because the analytical method entails removal of solvent using low vacuum and low heat, it was confirmed that this process did not lead to loss of triptolide. Although the variances among samples dried in the SpeedVac were greater than among the samples not dried (RSD values 14.7% versus 10.9%), a *t*-test ($\alpha = 0.05$) indicated the means were not significantly different.

Mao et al. [15] evaluated the stability of triptolide in various solvents at room temperature and higher, but did not study the compound's stability in organic solvents at lower temperatures. Standard solutions of triptolide in 95% ethanol stored at −20 °C remained stable over periods of up to 6 months. However, ethanolic extract solutions lost 30–60% of the triptolide within 5 months of incubation at −20 °C, indicating that long-term storage of extracts as solutions is not advisable.

3.2. HPLC method

Chromatography of *T. wilfordii* root extracts on C_{18} and C_8 columns showed that triptolide eluted from reverse-phase columns before most other components of the extracts, indicating that reverse-phase chromatography was preferable in this case. Several published methods [14,16–19] for the analysis of triptolide in plant material or plant extracts used C_{18} columns and acetonitrile–water or methanol–water solvent systems (the latter with addition of phosphate or phosphoric acid in many cases). However, we found that a pentafluorophenyl column gave sharper peaks and better separation of the compounds in the extract than a C_{18} column. Acetonitrile was used as the organic component of the HPLC solvent in preference to methanol because of methanol's higher absorbance at 219 nm and higher back pressure. Addition of acetic or phosphoric acids to the solvent system was tested but gave no benefit over water alone. Using the pentafluorophenyl column and acetonitrile–water as the solvent mixture, we then developed the gradient described in this paper to keep the run time reasonably short while maintaining a good separation and good peak shape of triptolide.

3.3. Solid-phase extraction method

HPLC alone was not sufficient to resolve triptolide from the other components of the extract, so an additional separation step was required. Solid-phase extraction was chosen as being relatively simple and suitable for use with large numbers of samples. Because the HPLC step used a reverse-phase system, a normal-phase SPE step was desirable, as being a complementary separation method. Of the five normal-phase SPE sorbents tried, aminopropyl (NH_2) gave the best separation of triptolide from the other components. Reverse-phase sorbents were also tested, but did not give adequate separation of triptolide from compounds of similar polarity.

In earlier work involving TLC of extracts on silica, dichloromethane–methanol mixtures had given good separations, so mixtures with varying dichloromethane:methanol ratios were tested as eluting solvents. The optimum solvent for separation of triptolide was dichloromethane–methanol, 49:1 v/v. Dichloromethane alone was tested for dissolving extract samples and equilibrating the SPE tubes, but gave comparable results to dichloromethane–methanol, 49:1 for sample dissolution, and caused triptolide to elute later when used to equilibrate the tubes. Therefore, dichloromethane–methanol, 49:1 was used for all steps. A pre-equilibration conditioning wash with methanol provided no benefit, so this step was eliminated.

An experiment evaluating recovery of triptolide from extract applied at different concentrations between 25 and 0.05 mg/mL found that 5 mg/mL was optimal. When 600 μL of such a solution was loaded onto a 500 mg SPE tube, the triptolide was eluted completely by 1 mL of eluting solvent and did not appear in the solvent that ran through the tube while the sample was being loaded. When a larger (750 μL) sample was used, a small amount of triptolide was detected in the solvent that ran through during sample loading. A chromatogram of the eluate from a root extract is shown in Fig. 2.

3.4. Validation of the method

For both triptolide standard and root extract samples spiked with different amounts of triptolide, the graphs of peak area versus triptolide concentration were linear over the ranges tested. However, the variability among replicates increased with triptolide concentration, which violates one of the statistical assumptions of regression. Log transformation of both x and y values usually solved this problem. For the standard curve with 1.25–250 mg triptolide/L, the resulting equation was $\log_{10} y = 0.981(\log_{10} x) + 3.338$ with $R^2 = 0.9993$, where y is peak area and x = ng triptolide injected(mg triptolide/L × 20 = ng/injection). Transforming the data for the lower concentrations of triptolide standard reduced but did not entirely eliminate the correlation between variation and triptolide concentration. For values from 0.02 to 5 mg triptolide/L, the resulting line had the equation $\log_{10} y = 1.029(\log_{10} x) + 3.239$ ($R^2 = 0.9935$). For triptolide-spiked root extract, the results were similar; over a range of 16.2–266.2 mg triptolide/L, the regression line had the equation $\log_{10} y = 0.964(\log_{10} x) + 3.467$ ($R^2 = 0.9958$). In this particular case, log transformation of the data was not necessary to even out the variances, but did improve the fit of the regression line. Lower concentrations of triptolide in extract (1.25–37.5 mg/L) gave a regression line with equation $\log_{10} y = 0.8415(\log_{10} x) + 3.595$ ($R^2 = 0.9794$).

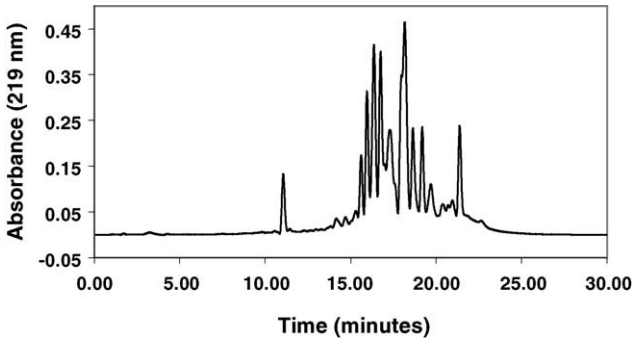

Fig. 2. HPLC chromatogram obtained after SPE of an extract of roots of a *T. wilfordii* plant grown hydroponically. The peak at approximately 11 min is triptolide.

Mean recovery values in the first standard addition experiment ranged from 96.6% to 107.8% (average = 101.8%) with RSD values ranging from 1.02% to 10.6% (average = 4.2%) for all but the lowest concentration tested (17.5–250 mg triptolide/L). For the lowest concentration (2.5 mg triptolide/L), the recovery was anomalously high (126.9% with a RSD of 73.1%). In the second experiment, in which the triptolide concentrations were lower (1.25–37.5 mg triptolide/L), mean recovery values ranged from 62% to 81% (average = 72.2%) with RSD values ranging from 9.7% to 27.4% (average = 19.6%). In both experiments, regression analysis indicated that triptolide level was not correlated with the RSD or with percent recovery.

For triptolide standard, between-day and within-day variabilities were similar. Between-day RSD values (at least three replicates) for triptolide levels of at least 1 mg/L ranged from 1.2% to 11.8% and were not correlated with triptolide concentration. However, for triptolide amounts less than 1 mg/L the RSD values were greater than 10% and generally increased with decreasing triptolide concentration. The within-day RSD (three replicates) was 7.5%. Between-day variability for extract samples ranged from 0.96% to 23.1% and tended to be higher for lower triptolide concentrations.

The limit of detection (LOD) and limit of quantitation (LOQ) for triptolide, calculated by the signal to noise method (3:1 and 10:1, respectively) using the data from the standard curve with the lowest triptolide concentrations, were 0.028 and 0.094 ng triptolide injected, respectively. In practice, however, injections of 0.1 and 0.2 ng triptolide produced peaks that were difficult to detect.

The effective range of the method is determined not only by the LOQ but also by the linearity, accuracy, and

variability. Factors such as the UV detection limit also affect the practical lower limit of the method. In both standard addition experiments, the samples with the lowest amounts of added triptolide (1.25 mg/L in one experiment, 2.5 in the other) gave particularly high or low recovery values with high variability. Variability was also high (RSD > 10%) for concentrations of triptolide standard lower than 1 mg/L, particularly for the two lowest concentrations (5 and 10 μg/L). Furthermore, with the HPLC system used, triptolide amounts less than 1 mg/L gave UV spectra that were too undistinguished to be useful for confirming compound identity. Therefore, 1 mg/L is the lower limit of the useful range of this method. The upper limit appears to be at least as high as the highest triptolide concentration tested (250 mg/L).

The ability of the method to distinguish triptolide from the other components of the extracts was tested by chromatographing tripdiolide, a structurally similar compound also found in *T. wilfordii* root extracts, and by analyzing extracts from 14 plants, seven of which contained triptolide as determined by another method, and seven of which did not. The HPLC system cleanly separated standards of triptolide and tripdiolide from each other (retention time of triptolide = 11.1 min; tripdiolide = 8.5 min). Retention times of other compounds related to triptolide could not be determined because standards were not available. The seven root extracts that did not contain triptolide also did not contain triptolide when analyzed by the present method. This method did detect triptolide in the seven extracts that were previously found to contain it.

The seven extracts in which triptolide was not detected did produce two small, overlapping peaks that eluted between about 10.7 and 11.7 min. The retention times of these peaks do not match that of triptolide, but they would overlap a triptolide peak. For the three extracts made from greenhouse-grown plants, the area of these two peaks combined was equivalent to 0.19–0.425 mg triptolide/L. The other four extracts were from plants grown outdoors; the peak areas in these extracts were more variable, ranging from the equivalent of zero to 3.47 mg triptolide/L.

3.5. Effect of root age on triptolide content

It has been found with other plant species that the composition of secondary metabolites varies in tissues of different developmental stages [20]. Since *Tripterygium* root extract is standardized based on triptolide content, it is necessary for commercial growers to know whether root age and developmental stage affect triptolide levels. The method described above was used to evaluate triptolide levels in roots that had not yet begun secondary growth, and in large (diameter ≥3 mm) and small (diameter <3 mm) roots with secondary growth (Fig. 3). The roots that had not yet begun secondary growth contained low levels of triptolide. However, small roots with secondary growth had higher concentrations of triptolide than large roots.

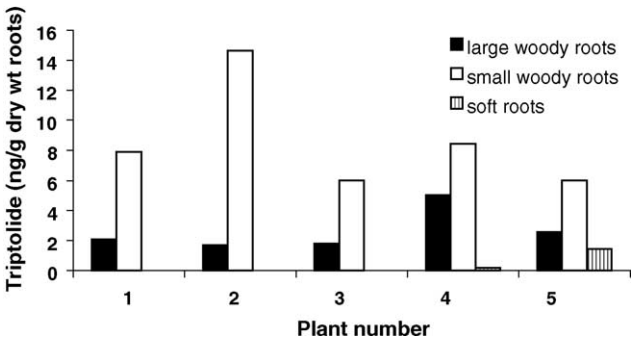

Fig. 3. Triptolide levels in large (diameter ≥3 mm) and small (diameter <3 mm) woody roots (roots with secondary growth) and, for plants 4 and 5, "soft" roots (roots without secondary growth) from *T. wilfordii* plants grown hydroponically. Due to small sample sizes, there was only one determination made per sample.

4. Conclusions

The method described in this paper should prove useful for monitoring triptolide levels when the simultaneous analysis of numerous plant samples is required. It uses smaller amounts of solvent than previous methods, and requires only 3 mg extract per determination, equivalent to as little as 200 mg fresh weight of root.

The results indicate that the method is less reliable for extracts containing triptolide at levels lower than about 20 ng/mg extract. Also, some background peaks that might co-elute with triptolide were observed. In practice, however, the root samples of greatest interest are those containing the highest levels of triptolide – several hundred to over 1000 ng triptolide/mg extract. At such high levels, the contribution of the background peaks is negligible.

Triptolide concentrations were higher in smaller diameter (i.e. younger) roots with secondary growth than in larger roots. Although the mass of smaller roots is less than that of larger roots, the higher triptolide concentrations may be enough to compensate for this. If so, roots could be harvested at a younger age, which would be economically beneficial for growers.

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