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Determination of Triptolide in Root Extracts of *Tripterygium wilfordii* by Solid-phase Extraction and Reversed-phase High-performance Liquid Chromatography

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Abstract

Extracts of *Tripterygium wilfordii* Hook F. have been widely used in China to treat a variety of autoimmune and inflammatory diseases. The diterpenoids triptolide and triptolide are two major active components in the *T. wilfordii* ethyl acetate extract. An efficient solid-phase extraction and high-performance liquid chromatography (SPE-HPLC) method to measure triptolide content in the extract has been previously reported. However, a suitable means of triptolide quantification is not available because of interfering compounds in the extract that co-elute with triptolide. Therefore, this paper describes a method wherein triptolide content can be measured from a small amount of the extract. The extract solution (600 μ L) was applied into an aminopropyl SPE tube. Triptolide was eluted with dichloromethane:methanol (1 mL, 49:1 v/v), followed by triptolide elution with dichloromethane:methanol (3 mL, 17:3 v/v). The triptolide eluate was analysed by HPLC using an isocratic solvent system and was quantified by measuring the peak area at 219 nm. The contents of triptolide and triptolide in the extract were determined to be 807.32 ± 51.94 and 366.13 ± 17.21 μ g/g of extract, respectively. Since triptolide is biologically active and makes up a considerable portion of the extract, for extract quality control and standardisation purposes, it should be measured along with triptolide using the proposed SPE-HPLC method.

Keywords

SPE; HPLC; triptolide; triptolide; *Tripterygium wilfordii*

INTRODUCTION

Extracts of *Tripterygium wilfordii* Hook F. have been widely used in China to treat autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, and Behcet's disease (Lipsky and Tao, 1997). The ethyl acetate extract is one of the most popular preparations of *T. wilfordii* for the treatment of rheumatoid arthritis. Both phase I and II studies of the extract in rheumatoid arthritis patients in the USA have showed it to be safe and clinically beneficial (Tao *et al.*, 2001, 2002). A 6

month, double-blind, phase II clinical trial involving 120 patients with moderate to severe rheumatoid arthritis showed exceptional stand-alone American College of Rheumatology efficacy results for the extract (Goldbach-Mansky *et al.*, 2006). Phase III development plans are underway.

Previous studies have shown that the anti-inflammatory and immunosuppressive effects of the extracts could be accounted for by the presence of two diterpenoid epoxides, triptolide and triptolide (Tao *et al.*, 1991, 1995; Fig. 1). Triptolide and triptolide contents were measured in the extract, and then the anti-inflammatory and immunosuppressive activities of the extract were compared with the activities of triptolide and triptolide standards. An efficient method for determining triptolide in the extracts has already been developed (Brinker and Raskin, 2005), but a similar method for triptolide determination does not exist. Using the HPLC conditions described for triptolide, other compounds co-elute with triptolide. Triptolide and triptolide contents have been used to evaluate the quality of preparations of the extracts (Tao *et al.*, 1991, 1995). Therefore, since triptolide is a major active component in the extract, it was necessary to develop a suitable method for efficiently quantifying triptolide.

Other techniques have been used to measure triptolide including thin-layer chromatography (TLC; Kutney *et al.*, 1981), solid-phase extraction and high-performance liquid chromatography (SPE-HPLC; (Cai *et al.*, 1994), micellar electrokinetic capillary chromatography (MEKC; Song *et al.*, 2003), capillary gas chromatography (CGC; Rao *et al.*, 2005), and HPLC coupled with atmospheric pressure chemical ionisation mass spectrometry (HPLC-APCI-MS; OuYang *et al.*, 2007). However, there are significant limitations to each of these techniques. TLC shows very poor accuracy when used for quantitative determination (Nie *et al.*, 1994). MEKC and HPLC-MS equipment is not as commonly available as HPLC. The published CGC method (Rao *et al.*, 2005) requires laborious sample pretreatment for derivatisation of triptolide with trifluoroacetic anhydride, which is not convenient for large numbers of samples. In addition, GC requires high temperatures to evaporate the sample that may damage the active components. The published SPE-HPLC method (Cai *et al.*, 1994) uses large amounts of extract (50 mg) and large amounts of eluting solvents (25 and 15 mL). This method is also not convenient for large numbers of samples. Here we describe a streamlined SPE-HPLC method for determination of triptolide in the extracts that does not require derivatisation of triptolide, uses commonly available equipment and is suitable for large numbers of samples.

EXPERIMENTAL

Solvents and chemicals

HPLC-grade solvents acetonitrile, dichloromethane and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water from a Millipore (Billerica, MA, USA) Synergy 185 system was degassed before it was used for HPLC. The triptolide standard was obtained from Fujian Provincial Medicines and Health Products Import and Export Corp. (Fuzhou, China). Triptolide and its solutions were stored at -20°C in aluminium foil-wrapped glass vials. The identity of the triptolide standard was confirmed by comparing its ^1H - and ^{13}C -NMR data with published data (Kutney and Han, 1996). ^1H - and ^{13}C -NMR

spectra were recorded using a Bruker Avance AV-300 NMR spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) at 300 MHz (^1H) and 75 MHz (^{13}C) respectively. The triptolide standard was measured in CDCl_3 .

Plant material

The extracts of *T. wilfordii* were obtained from BioVectra DCL (Charlottetown, Prince Edward Island, Canada). Debarked roots of *T. wilfordii*, cultivated in Southern China, were shipped to BioVectra DCL and extracted with ethanol (10:1 v/w) at room temperature. Ethanol was reduced by approximately 90% *in vacuo*, and the amount of water equivalent in volume to the remaining extract was added. The resulting extract was partitioned with an equivalent volume of ethyl acetate. The ethyl acetate fraction was dried *in vacuo* to give the ethyl acetate extract of *T. wilfordii*.

Solid-phase extraction

About 5 mg of the extract was dissolved in dichloromethane:methanol (49:1 v/v) at a concentration of 5 mg/mL with 10–15 min sonication. A 3 mL SPE tube with 500 mg Strata aminopropyl (NH_2) packing (Phenomenex, Torrance, CA, USA) was equilibrated with 3 mL of dichloromethane:methanol (49:1 v/v). The extract solution (600 μL) was loaded into the SPE tube. An aliquot (1 mL) of dichloromethane: methanol (49:1 v/v) and 3 mL of dichloromethane: methanol (17:3 v/v) were successively applied into the SPE tube, and the two eluates were collected. The dichloromethane:methanol (49:1 v/v) eluate was collected for the determination of triptolide using the previously published HPLC method (Brinker and Raskin, 2005), and the dichloromethane:methanol (17:3 v/v) eluate was collected for the determination of triptolide using the HPLC method described below. The two eluates were dried in a SpeedVac (Savant Instruments Inc., Holbrook, NY, USA), then re-dissolved in 60 μL methanol, respectively, for HPLC analysis. This entire procedure was replicated four times to yield four 60 μL triptolidecontaining eluate solutions for HPLC analysis, and therefore, four sets of data for analysis.

HPLC analysis

HPLC analysis was performed on an HPLC system consisting of three Waters (Milford, MA, USA) model 510 pumps interfaced with a Waters Pump Control Module, Waters 717 plus auto-sampler, and a Waters 996 photodiode array detector (PAD), controlled by Empower 2 software, using a Curosilpentaffluorophenyl (PFP; Phenomenex, Torrance, CA, USA) column (250×4.6 mm i.d.; 5 μm particle size). The mobile phase consisted of water (A) and methanol: acetonitrile (1:1 v/v; B). The system was run with an isocratic solvent system consisting of 79:21 (A:B) and a 1 mL/min flow rate, at room temperature, and a 30 min running time. The sample injection volume was 20 μL . Triptolide was quantified based on its peak area at 219 nm, the wavelength of maximum absorbance of triptolide in the UV spectra. The identity of its peak can be confirmed by examining its UV spectra.

Calibration curve

A stock solution (1 mg/mL) of triptolide standard was prepared by dissolving the compound in HPLC-grade methanol. The stock solution was diluted with HPLC grade

methanol into 11 two-fold serial solutions. The calibration curve was established on the concentrations of the following 12 two-fold serial solutions: 0.488, 0.977, 1.953, 3.906, 7.813, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL.

Limits of detection and quantitation

The 0.488 µg/mL triptolide standard solution was further diluted with HPLC-grade methanol into a series of two-fold solutions for the limit of detection (LOD) and limit of quantitation (LOQ) test. The LOD and LOQ of triptolide for HPLC-PAD were determined by analysis of the peak height vs the baseline noise at a signal-to-noise ratio of 3:1 and 10:1, respectively.

Recovery test

For the recovery test, 200 µL triptolide standard solutions (1.5625, 3.125 and 6.25 µg/mL) were added to small glass vials (three replicates for each concentration) and dried in a SpeedVac. The final amounts of added triptolide were 312.5, 625 and 1250 ng, respectively. The extract solution [600 µL of 5 mg/mL in dichloromethane:methanol (49:1 v/v)] was added to each vial. The spiked samples were analysed by the described SPE-HPLC method.

Stability test

To test the stability of triptolide in methanol, two aliquots of triptolide standard solution (125 µg/mL) were prepared in HPLC grade methanol. One sample was stored at room temperature; the other sample was stored at -20°C. Triptolide content was measured weekly using the described HPLC method.

RESULTS AND DISCUSSION

Method development

In order to develop a method for determining triptolide that would be compatible with the method for determining triptolide (Brinker and Raskin, 2005), we used the same SPE tube, HPLC column, and solvent system. For SPE, we continued using a NH₂ SPE tube and the dichloromethane:methanol solvent system with a few changes in solvent ratios and volumes. Triptolide standard solution (1 mL of 100 µg/mL in methanol) was dried in a SpeedVac, re-dissolved in dichloromethane: methanol (49:1 v/v; 600 µL), and applied to the NH₂ tube pre-equilibrated with 3 mL of dichloromethane: methanol (49:1 v/v). To simulate triptolide elution as if the extract had been applied, 1 mL of dichloromethane: methanol (49:1 v/v) was passed through the SPE tube. Next, different ratios of dichloromethane:methanol were used to elute the triptolide from the SPE tube. We found triptolide could be eluted completely using 1 mL of dichloromethane:methanol (17:3 v/v). Therefore, this solvent mixture was determined to be the optimum eluant for triptolide. However, when 600 µL of 5 mg/mL extract solution in dichloromethane:methanol (49:1 v/v) was loaded onto the SPE tube, the triptolide in the extract could not be eluted completely using the described method. Instead, we found that 3 mL of dichloromethane:methanol (17:3 v/v) was needed to elute all the triptolide in the extract from the SPE tube.

We used the same HPLC method for triptolide (Brinker and Raskin, 2005) to determine the triptolide contents in the triptolide-containing eluate. Unfortunately, the UV spectra for the triptolide peak in the HPLC chromatogram showed that other compounds were co-eluting with triptolide, so an accurate determination could not be made. Several HPLC solvent systems were tested with different ratios of acetonitrile: water, methanol:water, etc. and with different isocratic and gradient solvent systems. Finally, baseline separation was achieved; triptolide was eluted as a single peak at 26.5 min (Fig. 2) using the following conditions: water (solvent A) and methanol:acetonitrile (1:1 v/v) (solvent B) were run isocratically at room temperature, with a ratio of 79:21 (A:B), 1 mL/min flow rate, 30 min run time, monitored at 219 nm, while using the same HPLC column as for triptolide. With this method, the UV spectra for the triptolide peak revealed no interferences by co-eluting compounds.

Method validation

The described SPE-HPLC method was validated with respect to specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and stability. The method specificity was assured by checking the peak purity of triptolide in the HPLC chromatogram; no interferences with the triptolide peak by other compounds were detected.

A calibration curve was established by plotting the peak areas of triptolide standards (y) vs. their injection amounts (x , ng) on 12 data points covering an injection range from 9.76 to 2000 ng, with a linear regression equation of $y = 1770x + 11588$. The correlation coefficient (r^2) was 0.9997, showing good linearity.

The recoveries of standard addition were $110.75 \pm 4.47\%$ (1250 ng addition, $n = 3$), $98.81 \pm 4.50\%$ (625 ng addition, $n = 3$) and $95.93 \pm 12.05\%$ (312.5 ng addition, $n = 3$), respectively, showing good accuracy.

In order to check the method precision, intra-day and inter-day experiments of HPLC analysis were conducted using the triptolide-containing eluate obtained from the SPE method described above. The intra-day injections were at 2 h intervals ($n = 4$), and the interday injections were at 24 h intervals ($n = 4$). The intraday and inter-day relative standard deviation (RSD) values were 0.23 and 0.43%, respectively, showing good precision. The LOD and LOQ of triptolide by HPLC-PAD were 4.88 and 9.77 ng, respectively.

A stability test was conducted using 125 $\mu\text{g/mL}$ of triptolide standard solution in HPLC-grade methanol. Triptolide standard solution remained stable at room temperature for at least one week, demonstrating short-term stability, and remained stable at -20°C for at least 6 months, demonstrating long-term stability.

Contents of triptolide and triptolide in the extract

The contents of triptolide and triptolide in the extract have been determined using the previously published SPE-HPLC method (Brinker and Raskin, 2005) and the described SPE-HPLC method. An aliquot (1 g) of the extract contained $807.32 \pm 51.94 \mu\text{g}$ of triptolide

and 366.13 ± 17.21 μg of triptolide ($n = 4$). Triptolide content was about 45% of triptolide content. A previous study showed triptolide and triptolide had similar anti-inflammatory and immunosuppressive activities (Ma *et al.*, 2007). By comparison of their contents in the extract, we can conclude that triptolide plays a major role in the therapeutic effects of the extract, with some contributions of triptolide.

CONCLUSIONS

In this study, we have developed an accurate, sensitive and reliable SPE-HPLC method for the determination of triptolide in the extracts of *T. wilfordii*. Combined with the method developed previously for the determination of triptolide (Brinker and Raskin, 2005), we can determine the two major active components, triptolide and triptolide, in the extracts of *T. wilfordii* from only 5 mg of extract. These methods can be widely used in laboratories with HPLC facilities to evaluate the efficacy and toxicity of the extracts of *T. wilfordii*, and will be useful in quality control, clinical trials and toxicological studies. These methods can also be used to measure other diterpenoid epoxides in other plant extracts.

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Figure 1.
Chemical structures of triptolide (1) and tripdiolide (2).

Figure 2.
HPLC chromatogram of the tripdiolide-containing extract eluate after SPE (219 nm). Peak
1, tripdiolide.