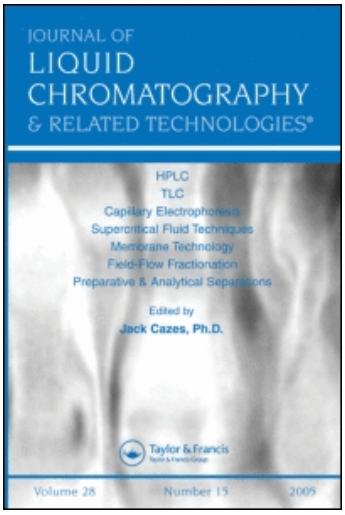

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High Performance Liquid Chromatographic Determination of Triptolide and Tripdiolide in an Ethyl Acetate Extract of *Tripterygium wilfordii Hook F.*

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRIPTOLIDE AND TRIPDIOLIDE IN AN ETHYL ACETATE EXTRACT OF *TRIPTERYGIUM WILFORDII HOOK F.*

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ABSTRACT

A new analytical method for the determination of triptolide and tripdiolide in ethyl acetate extracts of *Triterygium wilfordii Hook F.* is described. The procedure consists of preliminary enrichment by Sep-Pak alumina B cartridge chromatography followed by HPLC analysis. HPLC is performed with a stainless steel column packed with Nova-Pak C18, using acetonitrile-water (19 : 81) as a mobile phase for triptolide and acetonitrile-water (11 : 89) for tripdiolide. The effluent is monitored by ultraviolet detection at 214 nm. Quantitative analysis of triptolide is then carried out by comparison to an internal standard, and of tripdiolide by the external standard method. The amounts of triptolide and tripdiolide per 100 mg of the ethyl acetate extract were determined to be 19.88 ug and 9.58 ug respectively. The method is sufficiently sensitive and specific to assay the diterpenes found in *Tripterygium wilfordii Hook F.* accurately.

INTRODUCTION

Tripterygium wilfordii Hook F. (TWHF) is a medicinal plant that has been intensively studied[1]. The ethyl acetate extract of TWHF has been reported to be effective in the treatment of many autoimmune diseases, including rheumatoid arthritis[2,3]. Although the active ingredients of

TWHF have not been completely delineated, triptolide and tripdiolide are thought to be two of the more potent compounds, accounting for much of the efficacy and toxicity of this plant [4-9].

The techniques that have been employed to analyze the active components of TWHF involve thin-layer chromatograghy scanning densitometry and have quantitated triptolide only[10-12]. The TLC-Scanner procedure requires considerable expertise for its accurate application[17] and therefore, has not been widely applied. The total diterpene content of TWHF has been determined by a spectrophotometric method[13-15]. However, this method and TLC require visualizing the components with the Kedde reagent, that is usually not very stable[16]. Capillary gas chromatography (GC) has been used recently to the assess triptolide content of TWHF[18]. In contrast to high performance liquid chromatography, GC requires high temperature to evaporate the diterpene sample that may alter the components. In addition, it is difficult to use GC to prepare samples for biologic use. Since the extracts of TWHF are being more widely studied in clinical trials in both China and western countries, an accurate, sensitive and reliable method to separate and analyze its components is required. In this report, a new efficient and convenient method for the quantitation of the major active components of TWHF, triptolide and tripdiolide, using reverse phase high performance liquid chromatography (HPLC) is described.

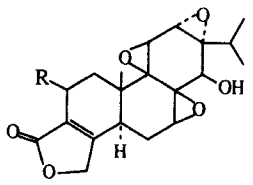
EXPERIMENTAL

Instruments

The Waters (Milford, MA) liquid chromatograph employed was configured with two Model 510 pumps, a Model U6K injector and a Model 441 UV detector set up at 214 nm. The data was processed with Millennium software, Version 1.10 (Waters Assoc.). The stainless steel column (150 mm x 3.9 mm I.D.) was packed with Nova-Pak C18, particle size 4 um (Waters Assoc.). An HPLC pre-column, with an insert packed with Nova-Pak C18, (Waters Assoc.) was used to extend the column life. The model ULTRAsonik 2QT / H ultrasonic water bath used in the solvent degassing and sample preparation was purchased from NEY Barkmeyer Division (Yucaipa, CA.).

Chemicals and reagents

Triptolide and tripdiolide were prepared from the ethyl acetate extract of TWHF by silica gel column chromatography successively with chloroform, chloroform-ether and chloroform-ethyl acetate as the eluents. The fractions containing triptolide and tripdiolide were purified on preparative HPLC with a Nova-Pak C18 column, 25 x 100 mm, using acetonitrile-water as the mobile phase. The compounds were recrystalized from n-hexane-dichloromethane. Triptolide was identified by UV, IR, proton NMR and mass spectrums. Tripdiolide was identified by HPLC, TLC and proton NMR and comparison with the known laboratory product provided by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, Connecticut). Acetonitrile was HPLC grade purchased from Aldrich Chemical Co. (Milwaukee, WI), water was Millipore pure, and other



Triptolide: R=H
Tripdiolide: R= β-OH

Fig. 1. Structures of triptolide and tripdiolide.

solvents were GR grade. The mobile phases were degassed by vacuum in conjunction with sonication just before use. The Sep-Pak Plus alumina B cartridge was purchased from Waters Assoc.(Milford, MA); Acetophenone, selected as an internal standard for the triptolide assessment, was purchased from Sigma Chemical Co. (St. Louis, MI). The chemical structures of triptolide and tripdiolide are shown in Fig. 1.

Preparation of the ethyl acetate extract of TWHF

The roots of TWHF were collected from Fujian province, China. The skin was removed from the roots and the woody portion of the roots was ground to coarse powder. 1000 g of the coarse powder was extracted with ethanol three times. The ethanol solutions were combined and evaporated under reduced pressure. The residue was then extracted with ethyl acetate. Concentration of the solution under reduced pressure yielded 22 g of the ethyl acetate extract.

Enrichment procedure

About 50 mg of the ethyl acetate extract was weighed accurately and dissolved in 10 ml of chloroform in an ultrasonic bath for 25 minutes. The extract solution was filtered and the residue was washed with 10 ml of chloroform-ethyl acetate (9 : 1). The washings combined with the original chloroform solution were applied to the Sep-Pak cartridge. 25 ml of chloroform-ethyl acetate (9 : 1) and 15 ml of ethyl acetate-methanol (9 : 1) were successively passed through the cartridge. The chloroform-ethyl acetate fraction, used for the determination of triptolide, was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved with 1.00 ml of acetophenone solution, that was prepared by dissolving acetophenone in methanol to obtain a solution having a concentration of 12.5 ug per ml. The dissolved residue was diluted with acetonitrile-water (19 : 81) to 2.00 ml. The ethyl acetate-methanol fraction was evaporated. The residue was dissolved in 1.00 ml of acetonitrile-water (11 : 89) solution and used to analyze for tripdiolide content.

Determination of diterpenes

A 10 ul volume of each purified sample solution was injected into the liquid chromatograph. The mobile phase for each separation is listed with the individual chromatogram.

Triptolide was determined by comparison to an internal standard. The reference solutions containing 1.83, 3.66, 7.32, 16.08 and 36.18 ng ul^{-1} of triptolide and 6.25 ng ul^{-1} of acetophenone for each solution were prepared in acetonitrile-water (19 : 81). The reference solutions of tripdiolide were prepared in acetonitrile-water (11 : 89) at the concentrations of 1.28, 2.55, 5.10, 10.20, 20.40, 30.60, and 40.80 ng ul^{-1}. Two replicates of each were injected into the HPLC system. The resulting chromatograms yielded data for the standard curves. The contents of triptolide and tripdiolide were calculated and expressed per 100 mg of the dried extract (drying at 80^0 C to a constant weight [19])

RESULTS AND DISCUSSION

This study focused on the development of a reliable method to analyze the two major components of TWHF. The enrichment procedure and chromatographic separation as well as the selection of an internal standard are three major problems in HPLC analysis of crude plant extracts. Many enrichment procedures were investigated during the preliminary phases of this study. These included different absorbents, such as silica gel, alumina N, florisil, diol, aminopropyl NH_2, cyanopropyl CN, activated carbon and polyamide. In addition, different solvent systems were tested. Sep-Pak Plus alumina B cartridge was found to be an efficient and convenient purifying method that involved the minimum number of steps. HPLC was performed with a Nova-Pak C18 column using acetonitrile-water as a mobile phase system. This resulted in a better separation of triptolide, tripdiolide and acetophenone from other components of the plant than did the use of methanol-water as a mobile phase. A detective wavelength of 214 nm was employed because of the α,β-unsaturated lactone ring in the diterpene structures. Acetophenone was found to be the most suitable internal standard for the determination of triptolide. Because of interference from other components, attempts to use an internal standard in the determination of tripdiolide were unsuccessful. Fig.2 illustrates the chromatogram of triptolide and acetophenone. The retention times of the two compounds were 11.35 min. and 8.15 min. respectively. Fig. 3 shows the chromatogram of tripdiolide. The retention time was 10.3 min.

The separation of triptolide, acetophenone and tripdiolide from the extracts of TWHF by HPLC was achieved using the method described above. This approach provided a good quantitative and reproducible recovery. Fig. 4 depicts a typical chromatogram of the extract for the determination of triptolide after addition of acetophenone. It is apparent that the other components present in the extract did not alter the internal standard peak. Fig. 5 shows a typical chromatogram of the extract for the determination of tripdiolide.

The peak purity was tested by collecting the fractions corresponding to both of the compounds and analyzing them by HPLC on the same column using methanol-water (30 : 70) as a mobile phase and adjusting the flow rate to 1.0 mL per min. The results indicated that a single component with the retention times corresponding to triptolide (5.1 min.) or tripdiolide (16.7 min.) had been isolated.

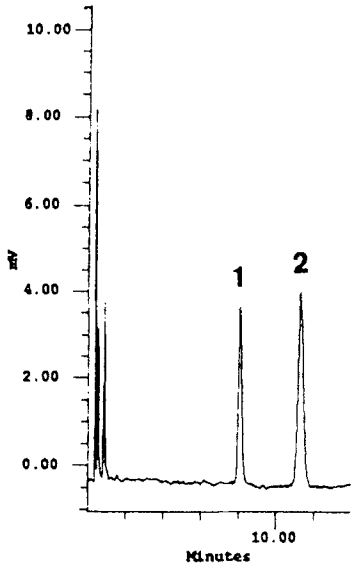


Fig. 2. Chromatogram of triptolide and acetophenone. Peaks: 1 = acetophenone; 2 = triptolide. Conditions: Nova-Pak C18 stainless steel column (150 mm x 3.9 mm I.D.); mobile phase, acetonitrile-water (19 : 81); flow-rate, 1.5 ml/ min.; UV monitor at 214 nm; sample volume 10 ul.

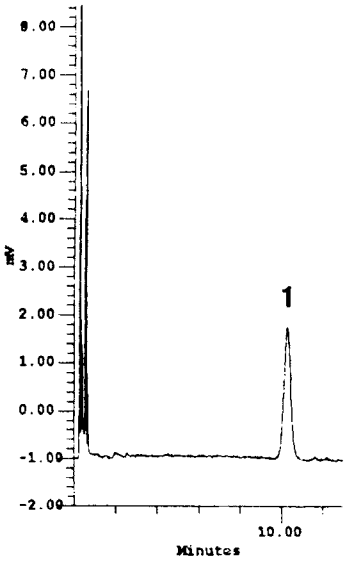


Fig. 3. Chromatogram of tripdiolide. Peak: 1 = tripdiolide. Mobile phase acetonitrile-water (11 : 89); flow-rate, 2.0 mL/ min. Other conditions as in Fig. 2.

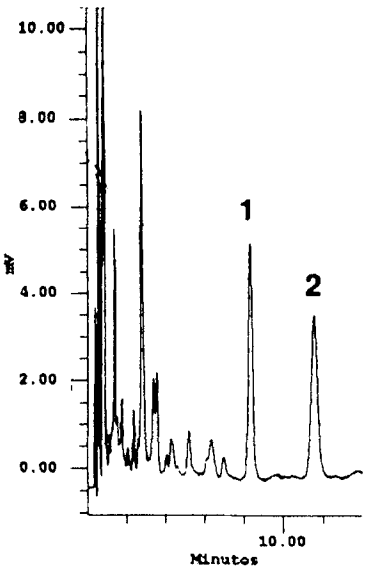


Fig.4. Chromatogram of the extract of Tripterygium wilfordii Hook F. with the internal standard for the determination of triptolide. Peak: 1 = acetophenone; 2 = triptolide. Conditions as in Fig. 2.

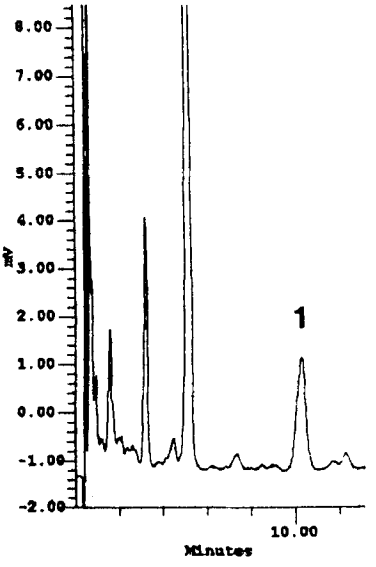


Fig. 5. Chromatogram of the extract of Tripterygium wilfordii Hook F. for the determination of tripdiolide. Peak 1 = tripdiolide. Conditions as in Fig. 3.

A linear calibration graph for triptolide was obtained by plotting the ratio of the peak area of triptolide to the internal standard (y) versus the amount of triptolide (x, ng). The regression equation and correlation coefficient (r) were $y = 0.025x - 0.049$, $r = 0.9999$, $n = 5$. The linear calibration graph of tripdiolide was obtained by plotting the peak area response of tripdiolide (y) versus the amount of tripdiolide (x, ng). The regression equation and correlation coefficient were $y = 744.2x - 2123$, $r = 0.9998$, $n = 7$. The range of the calibration curve was from 18.3 ng to 361.8 ng for triptolide and from 12.8 ng to 408.0 ng for tripdiolide.

The detection limit[20,21] was determined at very small concentrations using the described method. The minimum detectable amounts of triptolide and tripdiolide were 4.77 ± 0.66 ng ($n = 4$) and 9.05 ± 0.66 ng ($n = 3$) respectively.

The recovery test was carried out by adding pure triptolide and tripdiolide to the extract and assaying with the same procedure described above. The recoveries (mean % $\pm$ SD) of triptolide was 98.34 ± 1.54 ($n = 4$) and tripdiolide was 95.85 ± 1.49 ($n = 4$).

The assay results are displayed in table 1. Each term is the mean of two injections. The contents of triptolide and tripdiolide in 100 mg of the extract of TWHF were 19.88 and 9.58 ug respectively.

TABLE 1

CONTENTS OF TRIPTOLIDE AND TRIPDIOLIDE IN THE EXTRACT OF *TRYPTERYGIUM WILFORDII HOOK F.* DETERMINED BY HPLC.

Diterpenes	Amount in individual Determinations (ug per 100 mg of extract)	Mean $\pm$ SD	Relative Standard Deviation (%)
Triptolide	18.45 20.90 21.05 18.95 19.82 20.12	19.88 $\pm$ 1.04 n = 6	0.052
Tripdiolide	10.30 10.03 10.17 9.64 9.72 10.14 10.14 9.51 9.12 8.75 8.98 9.01 8.98 9.11 10.06	9.58 $\pm$ 0.54 n = 15	0.056

The values from individual experiments are the amounts per 100 mg of dry ethyl acetate extract.

In conclusion, this report describes a new accurate, sensitive and reliable method for the determination of triptolide and tripdiolide in an extract of TWHF. The pretreatment of samples with the Sep-Pak alumina B cartridge before HPLC represents a fast, simple and effective enrichment procedure with a very satisfactory recovery of the compounds. The successful employment of the internal standard greatly improved the accuracy and reproducibility for the triptolide assay. Triptolide and tripdiolide have been thought to be two of the major diterpene compounds contained in TWHF. This study provides the first quantitative data about the tripdiolide content in TWHF. Combined with the capacity to analyze triptolide, the approach makes it possible to evaluate the efficacy and toxicity of the TWHF extract and control the quality and safety of the preparation of this material for clinical trials and animal experiments.

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