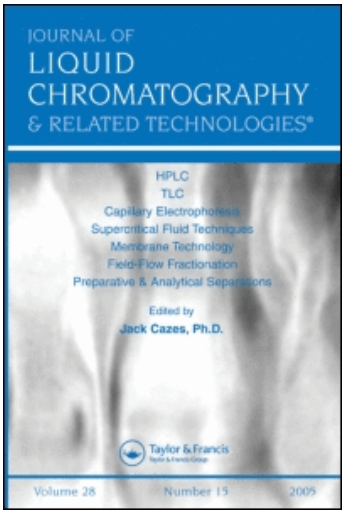

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

PHARMACOKINETICS OF TRIPTOLIDE. DEVELOPMENT AND APPLICATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATION OF TRIPTOLIDE IN PLASMA

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Online publication date: 05 November 1999

J. LIQ. CHROM. & REL. TECHNOL., 22(9), 1355–1366 (1999)

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ABSTRACT

In order to evaluate the bioavailability and study the pharmacokinetics of triptolide, an HPLC method was developed for the quantitative determination of this diterpenoid in plasma. The procedure for the plasma assay employed liquid - liquid extraction with chloroform followed by high speed centrifugation. The UV absorbance of the effluent was monitored at 218 nm. An internal standard (acetophenone) was used to calibrate injection and instrument reaction errors. The proposed methodology is sensitive, rapid, and reproducible. The limit of quantitation is 0.005 mg /L in plasma (0.05 mg /L in final solution) and a linear range of determination is observed over the concentration of 0.05 mg/L to 30 mg/L. The inter- and intraday coefficients of variation for the assay of triptolide in plasma were < 16.82 % at low concentration (0.005-0.076 mg/L) and < 8.05% at high concentration (0.152-5.000 mg/L). Recovery of triptolide in plasma is greater than 96.72%.

Triptolide was stable in plasma during 30 days of storage at -80°C, whereas degradation products appeared within 4 hours when it was dissolved in methanol at room temperature. The method was employed to determine the pharmacokinetics of triptolide in rat plasma.

After oral administration of a single dose of 840 μg/kg, triptolide was found to reach a peak concentration (Cmax) of 0.210 μg/mL in 19.5min. (Tmax). The AUC was 157.28 μg and the elimination half time was 50.60 min..

INTRODUCTION

Several different preparations of *Tripterygium willfordii Hook F* (TwHF) have been marketed in China for many years and widely used in the clinic as an immunosuppressive and anti-inflammatory agent.[1-4] An ethanol/ ethyl acetate (EA) extraction of TwHF made in the USA has finished phase I clinic testing and a phase II double blind clinical trial has been begun at the University of Texas Southwestern Medical Center at Dallas.

Based on the results of a variety of investigations, triptolide has been thought to be the most important therapeutic component of extracts of TwHF. Indeed, the content of triptolide has been used to evaluate the quality of preparations of extracts of TwHF.[5-8]

Several methods have been reported for the identification and quantitation of triptolide as a means to control the quality of preparations of extracts of TwHF. These include methods that involve gas-chromatography,[9] dual-wavelength thin-layer scanning methods,[10-13] and recently spectrophotometry,[14] HPTLC densitometry,[15] and reverse-phase HPLC coupled with ultraviolet monitoring.[16]

However, as triptolide is active at very low concentrations, detection of therapeutically relevant amounts of triptolide in biologic fluids remains a challenge. No previous methods have been employed and validated to be capable of measuring plasma triptolide concentrations in treated animals or humans.

This communication describes a rapid, sensitive, and reliable analytical method for the quantitation of triptolide in small amounts of plasma. The method has been validated according to Good Laboratory Practice guidelines and has been used successfully to assess the pharmacokinetics of triptolide.

EXPERIMENTAL

Materials and Chemicals

Triptolide was isolated from TwHF. Its structure was verified by UV, IR, MS, and NMR and compared with published data.[17] Acetophenone stock (0.5 mg/L) was prepared by dissolving an appropriate amount of acetophenone (Sigma Chemical Co. St. Louis, MO) in methanol, followed by storage at 4°C. Potassium phosphate monobasic sodium hydroxide buffer (pH 7.00) was purchased from Fisher Scientific (Pittsburgh, PA); Acetonitrile was HPLC grade (Aldrich Chemical Company, Milwaukee, WI); methanol and chloroform were reagent grade (E. M. Science Chemicals, Gibbstown, NJ); and water was Millipore pure prepared with a Millipore Milli-Q Plus device.

Sample preparation

Plasma was stored in 0.3 - 0.5 mL aliquots in 1.5 mL polypropylene tubes at -80°C until analysis. After thawing, 1 mL chloroform was added to each tube. All tubes were mixed by vortexing for ten seconds and centrifuged at 7000 x g for 8 minutes so that a membrane layer was formed at the interface of plasma and chloroform. A hole was then made in the bottom of the tube and the chloroform layer was collected. The chloroform layer was dried gently and the dried sample was stored at -20°C until analysis. Acetophenone stock was added to the dried samples to obtain a final volume equivalent to 1/5 the volume of plasma. The samples were sonicated and, afterward, 20 μL of each sample solution was injected into the chromatographic system.

Chromatographic conditions

The HPLC used to quantitate triptolide in plasma was equipped with two model 510 pumps, an U6K injector, a 486 detector (Waters Assoc., Milford, MA.) and a Nova-Pak C18 stainless steel column (300 cm x 3.9 mm i.d., particle size 4μm; Waters). The mobile phase was acetonitrile : water (32 : 68), flow rate was 1.0 mL / min and the column eluate was monitored at 218 nm. All the procedures were carried out at room temperature. The chromatographic data were calculated by Millennium Chromatography Manager software version 2.01 (Waters).

Instrument calibration

Standard curves for the assay of triptolide in plasma were prepared by adding various amounts of triptolide to control rat plasma over the concentration range of 0.05 - 30 mg/L (0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 4.00, 8.00, 15.0 and

30.0 mg/L). All of the standards were treated concurrently in the same manner as the plasma samples from dosed animals (method described above). The linear calibration of triptolide was obtained by computing the ratio of the peak areas of triptolide to the internal standard.

Limit of Determination (LOD) and Limit of Quantitation (LOQ)

The LOQ was the lowest concentration that could be detected with a coefficient of variation less than 15% for three independent sources of samples,[18] each of which was assayed in 6 replicates. To determine the analytical error in the LOQ, known concentrations of triptolide were added to control rat plasma and assayed as described above. The LOD was defined as the concentration at which the signal-to- noise ratio was equal to 3.[19] Methanol solutions of triptolide were used for the LOD test, with 6 replicate determinations carried out for each concentration.

Stability in the final methanol solution

Methanol solutions containing a known concentration of triptolide (0.1 mg/L and 10.0 mg/L) were used to define the stability of triptolide in final solution. Analyses were carried out at hourly intervals.

Stability of quality control (QC) samples

QC samples (0.02 mg/L and 2.0 mg/L) were stored in polypropylene tubes at -80°C along with experimental samples to determine the influence of storage. The aliquotted QC samples were randomized to be analyzed on day 0, 3, 10, 17, 24, and 30. The peak area of triptolide was compared with that at the initial time.

Specificity

0.5 mL aliquots of drug-free plasma were assessed to determine whether endogenous material in plasma co-eluted with triptolide. The retention times of plasma components were compared with those of QC samples. Three independent sources of plasma were assayed in duplicate.

Recovery

Triptolide (0.05, 0.5 and 5.0 mg/L) was added to control plasma or to phosphate buffered saline (pH 7) and assessed to determine the efficiency of the extraction procedure. 0.3 mL and 0.5 mL aliquots of these samples were evaluated to determine the relative recovery (extraction efficiency) by computing the ratio of the amount of triptolide extracted from plasma to the

amount extracted from the buffer solution. For this analysis, each sample was assessed in duplicate. To detect the influence of other factors on extraction efficiency, different concentrations of triptolide were assessed and different ratios of plasma to chloroform used to extract triptolide were compared.

Precision and Accuracy

Various concentrations of triptolide (0.005 - 5.0 mg/L) were added to control rat plasma and the concentration of each sample was determined 5 times for intraday variation during the same day. For inter-day reproducibility, various concentrations of triptolide (0.01, 0.06, 0.08, 0.16, 1.6, and 3.2 mg/L) were stored at -80°C and were assayed at different days over a 6 day period and the values obtained compared to standard curves prepared on the day of assay. The accuracy and precision were expressed as percent deviation of the experimental concentration from the expected concentration.

Pharmacokinetics Assay

A pharmacokinetics assay was carried out using a male Lewis rat weighing 485 g. 0.407 mg of triptolide was dissolved in 0.1 mL ethanol (Midwest Grain Products of Illinois, Pekin, Illinois) and 0.2 mL dimethyl sulfoxide (Sigma Chemicals Co., St. Louis MO) and diluted with water to a volume of 2.0 mL. After a single dose oral administration of this material, blood samples (0.6 - 1.0 mL) were taken from the tail vein 4, 8, 15, 30, 45, 75, 120, 240, 420, and 600 minutes later and analyzed in duplicate. The plasma concentration time curve was analyzed by the software Rstrip (MicroMath Scientific Software, Salt Lake City, Utah).

RESULTS AND DISCUSSION

HPLC was employed to detect triptolide because of its sensitivity and reliability for trace or chemically unstable compounds. Chloroform was used for liquid-liquid extraction of samples because of its easy application to plasma and the known solubility and stability of triptolide in chloroform.[20] A C18 cartridge method for triptolide purification from the EA extract was reported previously[16] and, therefore, this method was compared with chloroform extraction.

The cartridge method is effective in enriching the materials that are found at trace levels, but it required more time and steps to treat samples so that the overall recovery of triptolide was less than with chloroform extraction.

Table 1

Percentage Recovery of Triptolide in Plasma

Conc. in Plasma mg/L	Plasma Volume mL	Final Conc. mg/L	Conc. Found (Mean ± SD) mg/L	CV (%)	Recovery (%)
0.05	0.3	0.25	0.04869±0.002308	4.74	97.37
0.05	0.5	0.25	0.05077±0.003138	6.18	101.54
0.50	0.3	2.50	0.4748±0.02065	4.35	94.96
0.50	0.5	2.50	0.4740±0.005451	1.15	95.40
5.0	0.3	25.0	4.8391±0.07210	1.49	96.78
5.0	0.5	25.0	4.6715±0.03831	0.82	93.43
					X = 96.58
					SD = 2.804
					CV = 2.903

Percentage Recovery of Triptolide in Buffer

Conc. in Buffer mg/L	Buffer Volume mL	Final Conc. mg/L	Conc. Found (Mean ± SD) mg/L	CV (%)	Recovery (%)
0.05	0.3	0.25	0.05012±0.0004185	0.835	97.37
0.05	0.5	0.25	0.04969±0.0005004	1.007	99.38
0.50	0.3	2.5	0.49955±0.003397	0.680	99.91
0.50	0.5	2.5	0.5028±0.0002916	0.058	100.55
5.0	0.3	25.0	4.9745±0.02358	0.474	99.49
5.0	0.5	25.0	4.9753±0.04935	0.992	99.51
					X = 99.85
					SD = 0.4715
					CV = 0.4722

Other liquid-liquid extraction methods such as those using acetonitrile and ethyl acetate, were also compared with the chloroform extraction method. Both required more steps to purify samples. Besides being simpler, chloroform extraction yielded the best recoveries.

Acetophenone exhibited a retention time and absorptivity at 218 nm that is similar to that of triptolide. However, it could not be used as a surrogate of triptolide to calibrate the error of the purification procedure because of its volatility which resulted in its loss during the sample preparation. Therefore,

Table 2

Intraday Reproducibility of the Analysis of Triptolide

Conc. Added (mg/L)	Conc. Found (Mean±SD) (mg/L)	CV (%)	Mean Recovery (%)
0.005	0.004623±0.00-514	11.12	92.46
0.008	0.007931±0.0006246	7.88	99.14
0.016	0.01670±0.0006635	3.97	104.36
0.032	0.03186±0.0003349	1.05	99.54
0.076	0.08020±0.003852	4.80	105.53
0.152	01567±0.003758	2.40	103.07
0.304	0.2988±0.004994	1.67	98.30
0.608	0.6093±0.01925	3.15	100.22
1.216	1.1885±0.05182	4.36	97.73
2.432	2.3587±0.08298	3.52	96.99
5.000	4.7553±0.05521	1.16	95.12
		Mean recovery = 99.32%	
		SD of recovery = 3.901%	
		CV of recovery = 3.93%	

Table 3

Interday Reproducibility of the Analysis of Triptolide

Conc. Added (mg/L)	Conc. Found (Mean±SD) (mg/L)	CV (%)	Mean Recovery (%)
0.010	0.01077±0.00182	16.82	107.70
0.060	0.05924±0.00274	4.63	98.73
0.080	0.07857±0.00374	4.26	98.21
0.160	0.15964±0.01285	8.05	99.78
1.60	1.5491±0.08969	5.79	96.81
3.20	3.0625±0.1246	4.07	95.70

acetophenone could only be used to calibrate the injection and instrument response errors. The standard calibration curve for triptolide in plasma determined by computing the peak area ratio of triptolide to that of the internal standard was linear over the concentration range of 0.05 to 30 mg/L ($y = 0.22269 + 1.0279x$, the r^2 value was 0.999).

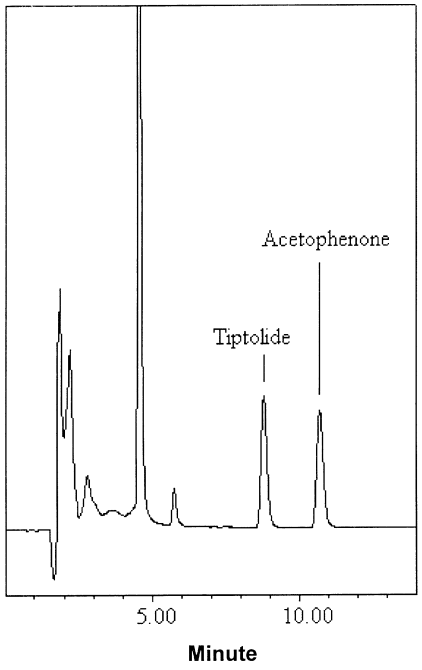

Figure 1. Plasma to which triptolide and acetophenone have been added.

$$\text{Rel. Recov.} = \frac{\text{\% Recov. Triptolide in Plasma}}{\text{\% Recov. Triptolide in Buffer}} = \frac{96.58\%}{99.85\%} = 96.72\%$$

The mean relative recovery of triptolide in plasma at concentrations of 0.05, 0.5 and 5.0 mg/L was 96.72 %. The ratio of plasma volume and the chloroform volume used to extract triptolide from plasma was in the range of 3 : 10 to 5 : 10 (Table 1). The intraday reproducibility tests indicated an average recovery of 99.73 $\pm$ 3.85% in the range of 0.005 mg/L to 5.0 mg/L plasma (Table 2).

The intraday and inter-day reproducibility of the triptolide assays in rat plasma are listed in Table 2 and 3, respectively. The intraday coefficient of variation of triptolide was in the range of 1.67% to 11.12% over the concentration of 0.005 to 5.000 mg/L (Table 2); The inter-day coefficients of variation were in the range of 4.26% to 16.82% over the concentration range of 0.01 to 3.20 mg/L (Table 3).

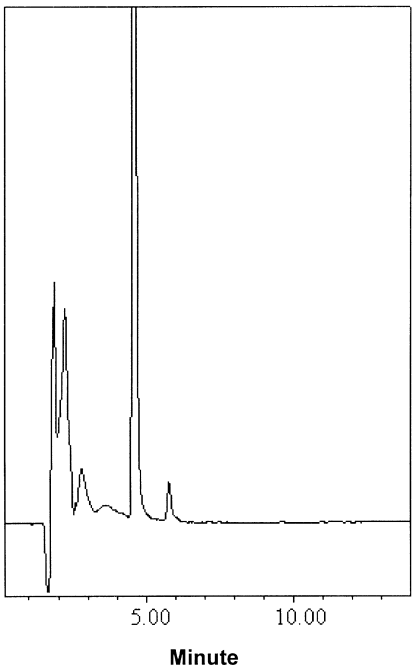

Figure 2. Control plasma.

The representative chromatograms from control plasma and plasma to which standard had been added are shown in Figure 2 and 1, respectively. The retention time of triptolide was 8.87 minute and that of acetophenone was 10.77 minute. No other peaks interfered at these retention times.

The method outlined in this report has sufficient sensitivity to characterize the pharmacokinetics of triptolide in rat plasma. The LOQ of triptolide in plasma from three independent plasma sources was 0.005 mg/L (Table 4). The LOD was approximately 0.00125 mg/L.

A previous stability study demonstrated that the $K_{25°C}$ (reaction speed of constant at 25°C) of triptolide in water solution was 8.0843×10^{-5}, whereas triptolide dissolved in chloroform or dry triptolide crystal is rather stable even when kept at room temperature for two years.[20] Based on these results and the fact that sample preparation usually was completed in 20 minutes, two stability tests were done for triptolide dissolved in plasma and triptolide dissolved in the final methanol solution. The data indicated that triptolide in plasma is stable at -80°C for 30 days.

Table 4

Determination of the Limit of Quantitation*

Conc. Added mg/L	Theoretical Peak Area	Experimental Peak (Mean±Sd) mg/L	CV (%)	Recovery (%)
0.004	744	684±76.33	11.16	91.93
0.004	744	780±127.17	16.30	104.86
0.004	744	829±116.97	14.26	111.42
0.005	930	1051±74.66	7.11	112.90
0.005	930	935±51.81	5.54	100.53
0.005	930	860±95.63	11.12	92.46

* (n = 6).

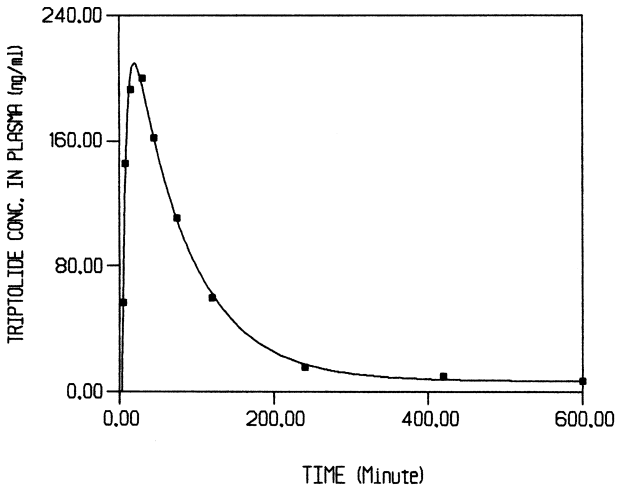

Figure 3. Pharmacokinetics of triptolide after oral administration of 407 μg (840 μg/kg) to a male.

Statistical analysis of 6 determinations on different days over 30 days did not show a significant difference. The CV was 6.65 %. In contrast, triptolide was rather unstable in methanol solution, with degradation products detected after 4 hours at room temperature for both 0.1 and 10.0 mg/L methanol solutions.

Fig. 3 demonstrates the application of this method for the pharmacokinetic assessment of triptolide. Following a single 407 μg (840 μg/kg) oral dose of triptolide, the maximal plasma concentration (Cmax) was 0.210 μg/mL at 19.56 min (Tmax) after administration. The AUC integrated to the last time point (600 min.) was 22.387 μg, integrated to infinity was 157.275 μg. After a lag-time of 3.447 min, the absorption phase of triptolide exhibited a half-life of 6.89 min.; The elimination and excretion phases had half-time of 50.06 min. and 13746 min., respectively.

CONCLUSION

A very simple, sensitive validated HPLC method for the quantitative determination of triptolide in plasma was developed. It allows quantitation of the triptolide concentration in plasma in the range of 0.005 - 5.0 mg/L using a minimum sample volume of 0.3 mL.

For correct determinations, the QC sample can be stored in - 80°C for 30 days. The purified dry samples, however, should not be dissolved in methanol until immediately before injection into the HPLC instrument.

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Received September 25, 1998
Accepted October 18, 1998
Manuscript 4918

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