Pharmacokinetic Study of Triptolide, a Constituent of Immunosuppressive Chinese Herb Medicine, in Rats

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Triptolide is a potential anti-immune agent, and has shown multi-organic toxicity, however its toxic mechanism remained undiscovered. This paper aimed at characterizing the pharmacokinetic profiles of triptolide in rats to provide the clue to approach the toxic mechanism. The absorption, distribution, metabolism and excretion of triptolide were investigated in male Sprague-Dawley rats after single doses of oral and i.v. administration. After oral administration of 0.6, 1.2 and 2.4 mg/kg, the concentration of triptolide in plasma reached the maximum within 15 min, and declined rapidly with an elimination half-life from 16.81 to 21.70 min. The triptolide kinetics was fitted into one compartment model after i.v. administration. Oral absolute bioavailability was 72.08% at the dose of 0.6 mg/kg. Triptolide was also rapidly distributed and eliminated in all selected tissues. Less than 1% triptolide of the dose was recovered from the bile, urine or feces as parent drug within 48 h. While triptolide could not be detected in tissues and plasma at 4 h post dose, rats in the group C (oral: 1.2 mg/kg) and D (oral: 2.4 mg/kg) showed obvious toxic response to triptolide and some of rats even died out. It was indicated that triptolide was metabolized extensively, eliminated rapidly, and also showed that the toxicity produced by the triptolide was lag behind the exposure concentration.

Key words triptolide; pharmacokinetics; LC/MS; HPLC

Tripterygium wilfordii Hook F (TWHF) is a herb used in traditional Chinese herb medicine for the treatment of rheumatoid arthritis and other autoimmune diseases. Triptolide (Fig. 1), a highly oxygenated diterpenoid triepoxide, is the major component responsible for the immunosuppressive and anti-inflammatory effects of TWHF and served as the quality control standard of TWHF. Clinical and animal experiments showed it an effective agent for the treatment of autoimmune diseases, inhibition of transplant rejection, and graft-versus-host disease. The inhibitory effect of triptolide on T-cell activation is even stronger than cyclosporine and FK-506 in vitro. Furthermore, it can induce apoptosis of activated T-cell. Unfortunately, it is well known that triptolide has small margin between the therapeutic and toxic doses and shows serious toxicity on digestive, urogenital and blood circulatory system when its level rises beyond the upper limitation. Ding H’ research also showed that the acute necrosis of liver was the main cause of death. However, the mechanism of hepatotoxicity of triptolide was not known up to today. It is well known that toxicity studies may be substantially supported by pharmacokinetic information. However, the pharmacokinetic of triptolide and its metabolic study have not been reported until now.

The objectives of this study were to describe the systemic exposure achieved in rats, and provide information for the subsequent nonclinical toxicity studies. This studies were undertaken (1) to develop and validate analytical methods for the measurement of triptolide in biological matrices, (2) to perform pharmacokinetic studies in rats that had received triptolide at a variety of doses and by i.v. or oral administration, and (3) to develop the histopathology studies after oral administration at the dose of 0.6, 1.2 and 2.4 mg/kg. Those studies form the basis of this report.

MATERIALS AND METHODS

Chemicals, Reagents and Apparatus Triptolide was kindly provided by Prof. Yun Chen (Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China). Prednisolone (I.S.) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetoniitrile and methanol were obtained from Fisher Scientific (Toronto, Canada). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, U.S.A.). Ethyl acetate and other chemicals and solvents used were of analytical grade.

Plasma, urine and feces samples analysis was performed on a Shimadzu 2010A liquid chromatographic/mass spectrometry system (LC/MS) with atmosphere pressure chemical ionization (APCI) interface, and Shimadzu LCMS solution workstation software (Ver 2.02) for the data processing. Tissue samples analysis was performed on a Shimadzu 2010C high performance liquid chromatography (HPLC) system including controller, degasser, low-pressure gradient formation (LPGE) unit, pump, static mixer, high-speed autosampler, column oven, and UV–VIS detector and CLASS-VP software was used for data processing.

Animals The animal care, use, and experimental protocols were approved by the animal care committee of China Pharmaceutical University. Male Sprague-Dawley rats (mean body weight ± S.D.: 215 ± 25 g) were obtained from the Laboratory Animal Center of China Pharmaceutical University and maintained under a 12 h light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22—24 °C) and rel-
ative humidity of 50±10%. All animals in this experiment were acclimated for one week prior to use. Food and water were available ad libitum. Animals utilized in the experiment were fasted overnight (approximately 16 h) prior to and four hours following dosing.

**Pharmacokinetic Phase** Forty-eight rats were divided into four groups, A, B, C and D of 12 animals each. Group A received triptolide intravenously (i.v.) and the other groups (B, C and D) orally. The i.v. or oral triptolide solution was prepared by dissolving triptolide in 0.9% saline solution containing 8% ethanol. For i.v. administration (group A), each rat was given triptolide solution as bolus through a tail vein at the dose of 0.6 mg/kg weight. The oral dose of triptolide was delivered with a gavage needle at the dose of 0.6, 1.2 and 2.4 mg/kg weight for group B, C and D, respectively. In order to avoid too much strain on each individual animal, the sampling times were divided into two series. Consequently, 12 animals of each group were sampled alternatively to obtain six data sets at each time point. Around 400 μl blood was collected from venous plexus of eye socket in heparinized tubes at 0, 2, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min before and after dosing. 200 μl plasma was immediately separated by centrifugation at 8000 rpm for 5 min and stored in eppendorf tubes at −20 °C.

**Tissue Distribution** Thirty rats were divided into five groups (6 rats per group). Each group of rats was terminated by exsanguination from the abdominal aorta under isoflurane anaesthesia at 0, 5, 10, 15 and 60 min after oral administration of the dose of 1.2 mg/kg weight. The hearts, livers, spleens, lungs and kidneys were removed, rinsed with ice-cold phosphate-buffered saline (PBS), blotted and then stored in polypropylene tubes at 20 °C.

**Excretion and Metabolism** Six male SD rats were administered i.v. a single dose of 0.6 mg/kg. After dosing, the animals were housed in individual metabolism cages designed for separation and collection of urine and feces. Urine and feces were collected separately at the interval of 0—6 h, 6—12 h, 12—24 h, 24—48 h. The other six male SD rats were subject to ether anesthesia and a cannula was implanted surgically into their bile duct. The biliary output was collected separately between 0—2 h, 2—4 h, 4—8 h, 8—12 h, 12—24 h after i.v. administration of triptolide (0.6 mg/kg). The collected urine, feces and bile were stored at −20 °C until analysis.

**Sample Preparation** Triptolide concentration in samples (plasma, bile, urine, and feces) was determined by LC/MS. Feces was dried, weighed (1 g), gently ground with 2 ml PBS (pH 7.4, added slowly) and at last the feces homogenate was prepared. The plasma, urine and bile was taken out from the refrigerator and thawed at 37 °C. Then plasma, bile, urine or feces homogenate (0.2 ml) and the internal standard solution 20 μl (3 μg/ml) were added to eppendorf tubes. The sample was mixed and then extracted with 1.2 ml ethyl acetate using a vortex for 3 min, and centrifuged at 8000 rpm for 5 min. The top organic layer (1 ml) was transferred to another tube and evaporated to dryness in a SpeedVac System (Thermo Savant SPD 2010, Thermo Electron Corporation, U.S.A.). The residue was reconstituted in 0.1 ml of methanol, and centrifuged at 20000 rpm at 4 °C for 10 min. The supernatant (10 μl) was injected into the LC/MS system.

**LC/MS Analysis** Triptolide concentration in tissues had been found higher (>20 ng/ml) and found that the tissue complex matrix components could pollute and damage the APCI source during the preliminary experiment using LC/MS method. So the HPLC external standard method was developed to determine the triptolide concentration in tissues. The tissue sample was performed on the same column as the LC/MS analysis. The column temperature was 30 °C. The mobile phase was composed of methanol/acetonitrile/water (15/20/65, v/v/v %). The flow rate was 1.0 ml/min and the detected wavelength was 218 nm. The standard curve was established by fitting the areas of triptolide with the corresponding nominal concentrations. The retention time of triptolide was about 10.3 min.

**LC/MS Method Validation** Specificity was ascertained by analyzing drug-free plasma without adding internal standard to determine the interference with the analytes. Five sets of calibration curves ranging from 1 to 800 ng/ml for triptolide were constructed by plotting the peak-area ratios of target/internal standard versus concentrations in plasma on a single day. Assay precision was determined by intra-day and inter-day relative standard deviation (RSD) at four concentration (1.0, 3.0, 300.0, 600.0 ng/ml). The accuracy was determined by comparing the calculated concentration (obtained from the calibration curve) to the theoretical concentration of each sample and expressed as percent of nominal values. The
sample was built by adding the working solutions of triptolide to drug-free plasma in extractive recovery study, while the reference sample was the relative standards prepared in methanol. Only peak areas of triptolide were compared at three levels of concentration to provide recovery values.

The stability was assessed at two concentration levels (30.0, 600.0 ng/ml). The freeze and thaw stability study samples at two concentrations were stored at −20 °C and subjected to three freeze-thaw (37 °C) cycles. For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during the routine sample preparation (<12 h); Additionally, the stability of plasma aliquots were also assessed during storage in the autosampler at 4 °C by repeated injection every 2 h for a period of 12 h. The long-term stability of triptolide in plasma was assessed at two concentration levels after storage at −20 °C for 4 weeks.

HPLC Method Validation The specificity was tested by analyzing the blank tissues sample (heart, liver, spleen, lung, kidney) obtained from six sources. Each blank sample was analyzed for exclusion of potential interference within the time range of analysis under the HPLC condition through the extraction procedure. Five sets of calibration curves ranged from 25 to 400 ng/ml for triptolide in each tissue were constructed by plotting the area of triptolide with the nominal concentration on a single day. Assay precision and accuracy was determined by relative standard deviation (RSD) and percent of nominal values, respectively, at two concentrations (50 ng/ml, 200 ng/ml). The extraction recovery of triptolide was assessed by comparing the area of spiked tissue sample with the area of standard solution at the same concentration not carried by extraction procedure.

Evaluation of Toxicity Rats in group B, C and D were observed for signs of toxicity during the pharmacokinetic experiments. All information was recorded including the clinical features and weather the rats died or not. Three rats were randomly taken out from the group B, C or D, respectively at 24 h after dose. At the same time the three blank rats were chosen as the control. Necropsy was performed on these rats. Samples of heart, liver, spleen, lung, kidney and adrenal gland were collected and fixed in 10% buffered formal saline. After processing, wax-embedded sections (5 μm) were cut, stained with hematoxylin and eosin, and examined by light microscopy.

RESULTS

LC/MS Method Validation Under the acquisition of negative SIM (selective-ion monitoring) mode, blank plasma yielded relative clean chromatograms without co-eluting interference peaks at the retention of triptolide and I.S. Typical chromatograms of the blank and spiked plasma are given in Fig. 2. The representative peaks had the same m/z values from standard samples. The retention time of triptolide and I.S. were about 3.2 and 4 min respectively. Signal suppression or enhancement may occur due to co-eluting endogenous components of sample matrix and so it was important to evaluate the matrix effect in any LC-MS method. No matrix effect and interferences from endogenous components were detected for plasma from six different sources of rats (Table 1).

Five set calibration curves were constructed in the range of 1—800 ng/ml for triptolide in plasma. Non-weighted least-squares linear regression analysis was used. The mean regression equations and their correlation coefficients (r²) were calculated as follows: y=0.0045x+0.0018, r=0.9993. The mean recoveries for triptolide were 76.8±5.7%, 78.5±5.4%, and 83.2±2.3% at the 3, 30 and 600.0 ng/ml, respectively (n=5).

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the standard curve that can be measured with acceptable precision and accuracy, and was found to be 1 ng/ml in rat plasma which is sufficient to support its pharmacokinetic studies. The intra-day precision for triptolide at LLOQ was 13.1% and the accuracy was 112.0% (Table 2). The inter-day precision for triptolide 14.7% and the accuracy was 112.0%. The middle and upper levels of triptolide ranged from 3.0 to 600.0 ng/ml in rat plasma. For the intra-day experiments the precision ranged from 1.0 to 7.0% and the accuracy from 101.1 to 105.1% (Table 2). For the inter-day experiments the precision and accuracy for the analytes also met the acceptance criteria (±15%). These results indicated that the present method has a satisfactory accuracy, precision and reproducibility. Extracted recovery results were more than 70%, indicating the ethyl acetate extraction efficiency was satisfied.

Table 3 displays stability for triptolide under the following conditions.

![Fig. 2. Chromatograms of Triptolide Using LC/APCI/MS](image)

(a) Blank plasma, (b) plasma sample obtained from a rat at 5 min after i.v. administration of 0.6 mg/kg triptolide. 1. triptolide, 2. prednisolone (I.S.).

<table>
<thead>
<tr>
<th>Concentration (ng/ml); n=5</th>
<th>Peak area of triptolide</th>
<th>Peak area of I.S.</th>
<th>Peak area ratio of triptolide/I.S.</th>
<th>Matrix effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Set 1</td>
<td>Set 2</td>
<td>Set 1</td>
<td>Set 2</td>
</tr>
<tr>
<td>30</td>
<td>36432</td>
<td>36528</td>
<td>235880</td>
<td>237309</td>
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<td>732680</td>
<td>782560</td>
<td>236552</td>
<td>244300</td>
</tr>
</tbody>
</table>

Set 1: triptolide or I.S. in pure methanol; Set 2: triptolide or I.S. spiked into post-extracted blank plasma; matrix effect expressed as the mean peak area of triptolide spiked post-extracted (set 2) to the mean peak area of triptolide standard (set 1) multiplied 100.
conditions: (1) short term stability of triptolide plasma aliquot at room temperature for at least 12 h, (2) stability of triptolide in plasma through at least three freeze-thaw cycles, (3) stability of triptolide in rat plasma for at least 12 h at 4°C in the autosampler, (4) stability of triptolide stored at 20°C for at least 4 weeks. As a result, triptolide showed a very good stability under the four conditions.

**HPLC Method Validation** No interference peak was observed in all tissue matrices at the retention time of triptolide. Typical chromatograms of the blank tissues and the standard of triptolide are given in Fig. 3. The method showed good linear response over the selective concentration range of 20—400 ng/ml in tested tissues from rats after the linear regression analysis. The regression equations and their corre-

<table>
<thead>
<tr>
<th>Sample concentration (ng/ml); n=5</th>
<th>Concentration found (mean±S.D.) (ng/ml)</th>
<th>Precisiona (%)</th>
<th>Accuracya (%)</th>
<th>Concentration found (mean±S.D.) (ng/ml)</th>
<th>Precisiona (%)</th>
<th>Accuracya (%)</th>
</tr>
</thead>
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<tr>
<td>Short-term stability for 12 h in plasma</td>
<td>30</td>
<td>30.9</td>
<td>5.9</td>
<td>102.9</td>
<td>30</td>
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<tr>
<td>600</td>
<td>606.1</td>
<td>1.8</td>
<td>101.0</td>
<td></td>
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<tr>
<td>Three freeze/thaw cycles</td>
<td>30</td>
<td>30.5</td>
<td>5.2</td>
<td>101.7</td>
<td>30</td>
<td>30.5</td>
</tr>
<tr>
<td>600</td>
<td>603.2</td>
<td>2.7</td>
<td>100.5</td>
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<tr>
<td>Autosampler stability for 12 h</td>
<td>30</td>
<td>30.7</td>
<td>5.9</td>
<td>102.4</td>
<td>30</td>
<td>30.7</td>
</tr>
<tr>
<td>600</td>
<td>605.2</td>
<td>1.9</td>
<td>100.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stability for 4 weeks at −20°C</td>
<td>30</td>
<td>29.7</td>
<td>4.0</td>
<td>99.1</td>
<td>30</td>
<td>29.7</td>
</tr>
<tr>
<td>600</td>
<td>596.3</td>
<td>1.5</td>
<td>99.4</td>
<td></td>
<td></td>
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a) Expressed as R.S.D. %: (S.D./mean)×100. b) Calculated as (mean concentration found/nominal concentration)×100%.

**Pharmacokinetic Phase** The plasma triptolide concentration–time profiles in rats following i.v. administration at the dose of 0.6 mg/kg and oral administration at the dose of 0.6, 1.2, 2.4 mg/kg, are shown in Figs. 4 and 5, respectively. Pharmacokinetic parameters were calculated using a DAS software package (Drug and Statistics, ver 1.0, Chinese Pharmacological Society), according to the principle of the best correlation between the observed and computed concentrations and the lowest value of AIC. A good fit of observed concentrations to time points with one-compartment was obtained following i.v. administration. The pharmacokinetic parameters are shown in Table 6, with the $T_{1/2\text{ke}}$ of 15 min. At the dose of 0.6 mg/kg, oral absolute bioavailability ($F$) of triptolide was calculated using the equation:

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Intercept</th>
<th>Slope</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>−170.42</td>
<td>56.843</td>
<td>0.9998</td>
</tr>
<tr>
<td>Liver</td>
<td>−615.88</td>
<td>53.904</td>
<td>0.9992</td>
</tr>
<tr>
<td>Spleen</td>
<td>−293.96</td>
<td>59.306</td>
<td>0.9997</td>
</tr>
<tr>
<td>Lung</td>
<td>−534.17</td>
<td>49.364</td>
<td>0.9994</td>
</tr>
<tr>
<td>Kidney</td>
<td>−303.25</td>
<td>34.304</td>
<td>0.9997</td>
</tr>
</tbody>
</table>

Table 4. The Linearity Parameters of Triptolide in Tissues, n=5
As a result, the F was about 72.08%, which indicates an efficient absorption in gastrointestinal tract.

**Tissue Distribution** After oral delivery at a dose of 1.2 mg/kg to rats, triptolide was rapidly distributed to all selected tissues in 5 min post dose (Fig. 6). Triptolide concentration detected in all selected tissues increased and declined in parallel with those measured in plasma, and the concentrations of triptolide found in livers exceeded those observed in other tissues at all four sampling-time points. Concentrations in the spleens and lungs were intermediate, with relatively low concentrations in kidneys and hearts.

**Excretion** Cumulative fecal, biliary and urinary excretions of triptolide were as low as 0.03%, 0.43% and 0.06% of the administrated dose (0.6 mg/kg) within 48 h, respectively,
which indicated that triptolide was extensively metabolized after i.v. administration.

**Clinical Observation and Histopathology** Compared with the control, rats in group B were observed normal according to clinical features. But the rats in the group C (orally: 1.2 mg/kg) or group D (orally: 2.4 mg/kg) showed dose-dependent tic, somnolence, kolyphremia, anorexy or nothing by mouth from 4 to 24 h post dose, and three rats died during experiment in group D. The histology of the involved organs displayed the dose-dependent harm of the rats in the group C or D. Cardiac muscle cell necrosis and matrix lysis with minimal inflammation was observed as well. The histology of the liver revealed remarkable degeneration of the liver cells with nuclear pycnosis and grey cytoplasmic instead of eosinophilic stain. Cell necrosis was sometimes a feature in liver toxicity. Necrosis of the spleen was characterized by the destruction of the splenic corpuscle. Within the collapse of lymphocyte and hyperplasia of macrophage can be noticed. Within the lung, local abscess formation and bacterial colony may be prominent. Kidney was also damaged of which epithelial cells of renal tubular was swollen and subsequently the lumina shrink. The bleed of adrenal gland zona reticularis medulla was observed.

**DISCUSSION**

The development of sensitive and specific assay of a drug is crucial to the study of drug pharmacokinetics. The LC/APCI/MS method,\(^2\) which had been successfully applied in monitoring the concentration of triptolide in the dog plasma in our lab, has been optimized for determination of the triptolide in the rat plasma and has been validated to be robust and sensitive to investigate pharmacokinetics of triptolide in rats. For reducing the damage and pollution of the complex tissue matrix to the APCI source of LC/MS apparatus, HPLC method was also developed and has been validated to determine the concentration of triptolide in tissues.

Triptolide was rapidly absorbed by rats with the maximum plasma concentration achieved within 10 min after dosing (Table 6). The kinetic properties were fit to the one-compartment model after rats given i.v. administration. The elimination \(T_{1/2}\) was 15—21 min, which shows that triptolide was also rapidly eliminated. By comparison of the plasma peak levels and the \(AUCs\) among the three oral dose levels it was found that the \(C_{\text{max}}\) and \(AUC\) did not increase in parallel with the dose. The less proportional elevation of \(C_{\text{max}}\) and \(AUC\) could show a non-linear kinetics for triptolide to be absorbed or eliminated over the dose range from 0.6 to 2.4 mg/kg. The oral absolute availability was 72.08%, which showed that triptolide was provided with a higher degree of absorption from the gastrointestinal tract. In order to obtain more characteristic of triptolide in rats, we studied the excretion of triptolide besides analyzing the plasma-concentration of triptolide. The urine, feces and bile was collected within 24 h. As a result, less than 1% triptolide of dose was detected in the bile, urine or feces as the parent drug. The tissue distribution of triptolide in main organ was also evaluated in order to understand whether there was tissue accumulation of triptolide or not. It was showed that triptolide was rapidly distributed in these main organs and the tissue concentration of triptolide was vibrated along with the level in plasma, which indicated that there was not tissue accumulation of drug in rats (see Fig 6).

It was concluded in this study that triptolide was rapidly absorbed, distributed and eliminated in rats and there was little parent triptolide in urine, feces and bile. Therefore, it was believed that the majority of triptolide was eliminated by metabolism. On the other hand, the rats showed obvious toxicity following oral administration of triptolide after 10 to 24 h post dose, while the concentration of triptolide had been below the detection limitation at 4 h post dose. It was a complex process for a drug to produce the toxic effect, and it was also complicate for the reason that the toxic effect lagged behind the exposure concentration. However, considering the pharmacokinetic characteristic of triptolide, we may conduct further experiments to elucidate the metabolites and the pathway for investigate whether the toxic principle of triptolide was involved in its metabolism.

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