LC-ESI-MS/MS analysis and pharmacokinetics of jolkinolide B, a potential antitumor active component isolated from *Euphorbia fischeriana*, in rat plasma

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**Abstract**

A simple, specific and reproducible liquid chromatography–electrospray ionization mass spectrometry was developed and validated for the determination of jolkinolide B, a potential antitumor active component isolated from *Euphorbia fischeriana*, in rat plasma. Chromatographic separation was achieved on a Venusil MP-C18 column using an isocratic elution. Jolkinolide B and osthole (internal standard) were monitored by positive electrospray ionization in the selected reaction monitoring mode. Good linearity \((r^2 > 0.996)\) was achieved by a weighted \((1/x^2)\) linear least-squares regression over a concentration range of 6.50–2600 ng/mL. The accuracy and precision of the assay were satisfactory and the method proved to be applicable to pharmacokinetics following a single intravenous bolus injection of jolkinolide B to rats. Copyright © 2013 John Wiley & Sons, Ltd.

**Keywords:** jolkinolide B; antitumor component; LC-ESI-MS/MS; rat plasma; pharmacokinetics

**Introduction**

The dried root of *Euphorbia fischeriana*, known as *lang-du* in traditional Chinese medicine, has been used in China as a remedy for cancer, edema and ascites (Jiangsu New Medical College, 1977). Many chemical investigations of *E. fischeriana* have revealed that a variety of diterpenoids exist in the plant (Che *et al*., 1999; Wang *et al*., 2006). Jolkinolides are the main abietane-type diterpenoids isolated from the root of *E. fischeriana*, which exhibit significant inhibitory effects against several tumor lines (Luo and Wang, 2006; He *et al*., 2012).

Jolkinolide B, a typical *ent*-abietane-type diterpenoid, has been proved to exhibit significant cytotoxic activities against tumor cell lines like prostate LNCaP cancer cell lines (Liu *et al*., 2002), and induce apoptosis in human ovarian carcinoma SKOV-3 cell and Leukemic U937 cells (Wang *et al*., 2011). Additionally, *in vitro* studies indicated that jolkinolide B inhibits cell proliferation and induces the apoptosis of human breast cancer cell lines (Lin *et al*., 2012; Xu *et al*., 2013). Molecular analysis demonstrated that jolkinolide B induces the apoptosis of cancer cells through the phosphoinositol-3-kinase (PI3K)/Akt signaling transduction pathway, suggesting that the diterpenoid has therapeutic applications in the treatment of breast cancer.

Several methods for analyzing jolkinolides in herbal materials have been reported, including high-performance liquid chromatography (HPLC) with ultraviolet detection, liquid chromatography with evaporative light-scattering detection, and liquid chromatography tandem mass spectrometric (LC-MS/MS) detection (Su *et al*., 2003; Qiao *et al*., 2006; Tang *et al*., 2012). However, the previous HPLC methods did not provide satisfactory lower limits of quantification (LLOQ): 810 ng/mL and 16 μg/mL (Qiao *et al*., 2006; Tang *et al*., 2012, respectively). An LC-MS/MS was developed for the identification and characterization of the Chinese herb *lang-du* using a gradient mobile phase with a long run time of 60 min (Su *et al*., 2003), but this method was not suitable for a methodological study. To date, little data is available on the pharmacokinetics of *E. fischeriana* components. Given that jolkinolide B is abundant in *E. fischeriana* extract, it was deemed necessary to develop and validate a simple, specific and reproducible LC-MS/MS for accurately determining jolkinolide B in rat plasma.

**Experimental**

**Chemicals and reagents**

Jolkinolide B and osthole (internal standard, IS) with purity >98% were obtained from Must Bio-Technology (Chengdu, China). HPLC-grade chemicals and reagents were obtained from Must Bio-Technology (Chengdu, China).

**Supporting information**

Supporting information can be found in the online version of this article.

**Abbreviations used:**

- ESI, electrospray ionization.
methanol was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA), and analytical-grade formic acid was from Tedla Company Inc. (Fairfield, OH, USA). Deionized water (>18 mΩ) was obtained from a Milli-Q water purification system (Bedford, MA, USA).

Instrumentation and analytical conditions

The assay was achieved on an Agilent 6410 LC–MS/MS system equipped with an electrospray ionization (ESI) source. The system was controlled with MassHunter workstation software for data acquisition and analysis (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was carried out on a Venusil MP-C18 column (50 × 2.1 mm, 5 μm; Agela Technologies Inc., Wilmington, DE, USA) with a Security-Guard C18 guard column (3.0 × 4.0 mm, 5 μm; Phenomenex Inc., Torrance, CA, USA), maintained at 30 °C. The mobile phase was methanol–water–formic acid (70:30:0.1, v/v/v) at a constant flow rate of 0.3 mL/min and the injection volume was 4 μL.

MS/MS was operated in positive mode under the following operating parameters: gas temperature, 300 °C; gas flow, 10 L/min; nebulizer pressure, 35 psi; capillary voltage, 4.5 kV. Quantitative analysis was performed using selected reaction monitoring (SRM) of the transitions of m/z 331.2 → 165.1 for jolkinolide B and m/z 245.1 → 131.0 for IS, with a scan time of 0.20 s per transition (Fig. S1 in the Supporting Information).

Preparation of standards and quality control samples

Stock solutions of jolkinolide B and IS were prepared in methanol at concentrations of 1.3 and 1.0 mg/mL, respectively. A set of jolkinolide B standard solutions and quality control (QC) solutions were obtained by successive dilutions of the stock solutions with methanol. A 20 μL aliquot of jolkinolide B standard solution was spiked into 180 μL of blank plasma, resulting in nine nonzero calibration standards with jolkinolide B concentrations of 6.50, 13.0, 32.5, 65.0, 130, 325, 650, 1300, and 2600 ng/mL. QC samples with concentrations of jolkinolide B at 13.0, 130, and 2340 ng/mL were also prepared in blank plasma. A working IS solution of 1000 ng/mL was prepared by appropriate dilution of the IS stock solution in methanol.

Sample preparation. Plasma samples (50 μL) were spiked with 50 μL of IS solution (1000 ng/mL). The mixture was precipitated with 250 μL of methanol by vortexing for 2 min. After centrifugation at 11,000 g for 5 min, the supernatant was transferred into a clean glass tube and evaporated until dryness under a gentle stream of nitrogen gas at 40 °C. The resulting residue was reconstituted with 150 μL of mobile phase, and a 4 μL aliquot was injected into the LC–MS/MS system.

Method validation

The method was validated in terms of specificity, sensitivity, calibration curve, matrix effect, recovery, precision, accuracy, dilution integrity and stability mainly according to the FDA guideline of bioanalytical method validation (US Food and Drug Administration, 2001).

Application

Eighteen male Wistar rats (200–220 g) were fasted overnight before drug administration and randomly assigned to three groups (n = 6). Blood samples (approximately 0.2 mL) were collected from the ocular vein into heparinized tubes at 0 (pro-drug), 5, 10, 20 and 40 min, and 1, 1.5, 2, 3, 5, 8, 12, and 24 h after intravenous bolus injection at 1.09, 3.27 and 9.81 mg/kg jolkinolide B. Blood samples were placed in heparinized Eppendorf tubes and the plasma samples were immediately separated by centrifugation at 11,000 g for 10 min. The obtained plasma was stored at −20 °C until LC–MS/MS analysis.

Results and discussion

Recovery and chromatographic conditions optimization

Protein precipitation or liquid–liquid extraction using ethyl acetate was evaluated for recovery efficiency. Our initial analysis showed that liquid–liquid extraction of plasma samples yielded low recovery for the analyte and the IS. Methanol and acetonitrile were tested as protein precipitation reagents for checking endogenous interference. No endogenous interference was observed for the analyte and IS using methanol. When protein precipitation method was adopted, the extraction efficiency of jolkinolide B was found to be very high and significant increase was observed in the extraction efficiency using methanol. The overall recoveries by determining triplicates of QC samples at low, medium and high concentrations of jolkinolide B in rat plasma were 86.5 ± 4.3, 89.3 ± 6.7 and 90.1 ± 2.5%, respectively; the recovery for IS was 88.6 ± 7.2% (n = 3).

Various mobile phases were evaluated for chromatographic retention times, peak shapes and the ionization responses of jolkinolide B and IS. Methanol presented higher responses than acetonitrile, and formic acid promoted the ionization of analytes and improved peak shapes. Eventually, the best response and peak shape were achieved on a Venusil MP-C18 column with an isocratic mobile phase consisting of methanol–water–formic acid (70:30:0.1, v/v/v). The overall chromatographic run time was completed within 3.7 min.

Method validation

Specificity. Figure 1 shows the typical chromatograms for blank plasma, blank plasma spiked with IS, blank plasma spiked with jolkinolide B of 6.50 ng/mL and IS, and the plasma samples obtained at 10 min after intravenous administration of 3.27 mg/kg jolkinolide B to rats. The retention times of jolkinolide B and IS were 3.0 and 2.2 min, respectively.

Linearity and lower limit of quantitation. Calibration curves provided good linearity ranging from 6.50 to 2600 ng/mL for jolkinolide B. Typical equations for the calibration curves were: y = 7.323 × 10⁻⁶x + 1.140 × 10⁻⁵, r² = 0.9968, where y indicates the analyte/IS peak area ratio and x represents the concentration of jolkinolide B in plasma. The lower limit of quantitation (LLOQ) was 6.50 ng/mL for jolkinolide B, at which the precision was <3.0% and the accuracy was 1.1% (relative standard deviation, RSD); Table S1 in the Supporting Information).

Precision and accuracy. Precision and accuracy were determined by analyzing QC samples in six replicates at three concentration levels (13.0, 130 and 2340 ng/mL) on three validation days. The intra- and inter-run precisions by means of the RSD ranged from 2.3 to 7.9%, and the accuracy by means of percentage error was between −0.4 and 2.9% in plasma.

Dilution integrity. Dilution integrity was assessed by six replicate analysis of QC samples at 5 times the upper limit of quantitation (ULOQ), that is, 13,000 ng/mL. These samples were diluted 10-fold with blank plasma prior to analysis. The dilution integrity precision of the QC samples (1:10 dilution) was <5.2%, and the accuracy was in the range from −5.7 to 8.3%. The results suggest that plasma samples whose concentrations above ULOQ can be determined by appropriate dilution.
Matrix effects. The matrix effects of jolkinolide B at the three QC levels were 96.5 ± 2.4, 93.8 ± 8.1 and 97.2 ± 6.8%, respectively. These results suggest that no significant matrix effect was observed interfering with jolkinolide B determination in rat plasma via this LC-MS/MS method (n = 3).

Stability. The predicted concentrations for jolkinolide B at 13.0 and 2340 ng/mL samples deviated within ±15% of the nominal concentrations over all steps of the determination. The results demonstrate the good stability of jolkinolide B during the entire process. The method is therefore proved to be applicable for routine analysis (Table S2 in the Supporting Information).

Figure 1. Representative SRM chromatograms of (A) blank rat plasma, (B) blank plasma spiked with IS (1000 ng/mL), (C) blank plasma spiked jolkinoside B (6.50 ng/mL) and IS, and (D) real sample at 10 min after intravenous injection of 3.27 mg/kg jolkinolide B. Peak I, jolkinolide B; peak II, IS.
Table 1. Pharmacokinetic parameters estimated by non-compartmental model analysis following a single intravenous bolus injection of jolkinolide B to rats (n = 6 per dosage)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dosage of jolkinolide B (mg/kg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1.09</td>
</tr>
<tr>
<td>AUC0–t (h ng/mL)</td>
<td>789.1 ± 260.3</td>
</tr>
<tr>
<td>AUC0–∞ (h ng/mL)</td>
<td>819.2 ± 253.0</td>
</tr>
<tr>
<td>C5min (ng/mL)</td>
<td>739.5 ± 78.9</td>
</tr>
<tr>
<td>MRT0–t (h)</td>
<td>1.76 ± 0.97</td>
</tr>
<tr>
<td>MRT0–∞ (h)</td>
<td>2.35 ± 1.12</td>
</tr>
<tr>
<td>t1/2z (h)</td>
<td>3.30 ± 1.90</td>
</tr>
<tr>
<td>AUMC0–t (mg h2/mL)</td>
<td>1595.6 ± 1498.8</td>
</tr>
<tr>
<td>AUMC0–∞ (mg h2/mL)</td>
<td>2101.8 ± 1537.9</td>
</tr>
</tbody>
</table>

AUC, area under plasma concentration; C5min, plasma concentration at 5 min; MRT, mean residence time; t1/2z, elimination half-life; AUMC, area under the first moment curve.

Application

The major pharmacokinetic parameters of jolkinolide B are summarized in Table 1. The ratio of mean plasma concentration or area under the concentration (AUC) values approached 1:3:9, while the dose increased in the ratio of 1:3:9, which suggested that the increases in the plasma concentration and AUC were proportional to the increase in dose. Moreover, the elimination half-life (t1/2z) of jolkinolide B in rats was 3.30 ± 1.90, 2.71 ± 0.95 and 3.83 ± 2.32 h, respectively, which relate with the elimination rate constant (ke). There were no significant differences (p > 0.05) in the t1/2z values among the three dosages by analysis of variance. These results suggest that the pharmacokinetics of jolkinolide B in rats was linear after intravenous administration of the three dosages examined (Fig. S2 in the Supporting Information). The present pharmacokinetics study of jolkinolide B in rats will be helpful for the development of suitable dosage and beneficial for application of this herb in clinical therapy. To the authors’ knowledge, this is the first pharmacokinetic report on jolkinolide B in rats.

Conclusion

A simple, specific and reproducible LC-MS/MS method for quantifying jolkinolide B in rat plasma was developed and validated. This method provided adequate sensitivity, linearity, precision and accuracy. The present assay was successfully applied to the pharmacokinetic studies of intravenously administered jolkinolide B using the rat as an animal model.

Supporting Information

Supporting information can be found in the online version of this article.

References

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