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Article

# Development and validation of an LC-MS/MS method for pharmacokinetic study of lobetyolin in rats

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A simple and selective liquid chromatography tandem with mass spectrometry (LC-MS/MS) method for quantification of lobetyolin in rat plasma was developed and validated. Chromatographic separation was achieved on a Thermo ODS $C_{18}$ reversed-phase column using 0.1% aqueous formic acid-methanol (50:50, v/v) in an isocratic elution mode at a flow rate of 0.4 $mL.min^{-1}$. LC/MS performance was done in a positive ion ESI mode and the MS/MS transitions were monitored at *m/z* 419.3 $[M+Na]^+ \rightarrow$ *m/z* 203.1 for lobetyolin and *m/z* 394.9 $[M+Na]^+ \rightarrow$ *m/z* 231.9 for IS, respectively. The assay exhibited a linear dynamic range over 1.0–500 $ng.mL^{-1}$ for lobetyolin in plasma. Both the precision (%RSD) and accuracy (RE%) were within acceptable criteria (<15%). Recoveries ranged from 87.0% to 95.6%, and the matrix effects were from 91.0% to 101.3%. After oral administration, the peak plasma concentration of lobetyolin was obtained as 60.1 $ng.mL^{-1}$ at 1.0 h. The proposed LC-MS/MS method could be applied to a pharmacokinetic study employing 66 samples from 6 Wistar rats.

**Keywords**Lobetyolin. LC-MS/MS. Pharmacokinetic study.

## INTRODUCTION

Dang-shen, the dried roots of *Codonopsis pilosula*, is a well-known traditional Chinese medicine that is used to suppress blood pressure, enhance immune system, improve memory and attenuate gastrointestinal function (Lin, Tsai, Kuo, 2013; Ji *et al*., 2019; Yang *et al*., 2019). Furthermore it is commonly combined with other herbs into formulae to improve Qi deficiency that is the underlying cause of chronic obstructive pulmonary disease (COPD) during remission stage (Shergis *et al*., 2015). Besides *Codonopsis pilosula* (Franch.) Nannf., other Codonopsis species are recorded in Chinese Pharmacopoeia (2015), such as *C. pilosula* var. modesta (Nannf.) L. T. and *C. tangshen* Oliv.

Lobetyolin, a critical polyacetylene compound of *Codonopsis pilosula*, was reported to exert anti-inflammatory, antitumor, antiviral, antioxidant, and xanthine oxidase inhibiting properties (He *et al*. 2020; Yoon, Cho, 2019). A recent study indicates that lobetyolin can promote angiogenesis and cause vascular malformations during the early embryonic development of transgenic zebrafish, and shows low toxicity with strong neuroprotective and nerve growth-promoting effects (Wang *et al*., 2020). Therefore, lobetyolin pharmacokinetics must be investigated to explain efficacy and predict toxicity. Moreover, lobetyolin is used as a phytochemical marker for TLC identification of *Codonopsis pilosula* in the Chinese Pharmacopoeia (2015).

Several analytical methods, including near infrared spectroscopy, high-speed counter-current chromatography, high-performance liquid chromatography with ultra-violet or dyode-array detection, liquid chromatography coupled with mass spectrometry, and ultrahigh-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (Hou *et al*., 2020; Wang *et al*., 2019; Zhang, Li, 2019; Meng, Liu, Hu, 2019; Qiao *et al*., 2007; Chen *et al*., 2018; Qi, Tian, Ran, 2019; Choi *et al*., 2018), have been used for the assay of lobetyolin in raw materials

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and related traditional Chinese prescriptions. Recently, an UPLC-MS/MS assay is retrieved for comparative pharmacokinetic study of lobetyolin in rats after oral administration of lobetyolin and *Codonopsis pilosula* extract (Dong *et al*., 2021). This reported method has a relative long run time (4 min) with gradient elution. In our study, an liquid chromatography tandem with mass spectrometry (LC-MS/MS) is quantified for lobetyolin in rats within 2 min for each ananlysis.

## EXPERIMENTAL

### Chemical and reagents

Lobetyolin (>98% purity) and syringin (>98% purity)–used as internal standard (IS, Figure 1) were obtained through Chengdu Ruifensi Biotechnology Co., Ltd (Chengdu, China). Methanol and formic acid were provided by Merck (Darmstadt, Germany). Ultra-pure water was obtained from a Milli-Q water system (Milford, MA, USA).

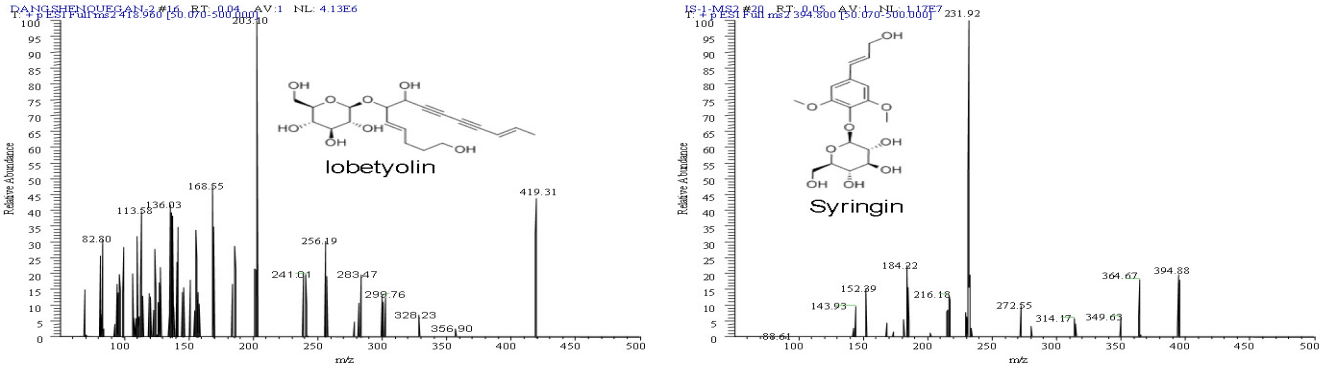


**FIGURE 1 -** The structures and product ion mass spectra of lobetyolin and IS.

### Instrumentation

Chromatographic separation was performed using a Thermo ODS $C_{18}$ reversed-phase column (50mm×2.1mm, i.d., 5μm) maintained at 35 °C. The LC-MS/MS system consisted of an Ultimate 3000 HPLC system coupled with a Thermo Scientific Quantum Access triple quadrupole mass spectrometer. The mobile phase consisted of 0.1% aqueous formic acid and methanol (50:50, v/v) with a flow rate of 0.4 mL.min$^{-1}$. The MS conditions were optimized using positive ESI mode as follows: capillary voltage, 3.0 kV; sheath gas, 50 arbitrary units; auxiliary gas, 10 arbitrary units; collision gas, 1.5 mTorr; vaporizer temperature, 400 °C. The dwell time was 200 ms per transition. Selected reaction monitoring (SRM) transitions were selected for quantification as follows: *m/z* 419.3 $[M+Na]^+ \rightarrow$ *m/z* 203.1 for lobetyolin and *m/z* 394.9 $[M+Na]^+ \rightarrow$ *m/z* 231.9 for IS (syringin). Collision energies were 25 and 27 eV for lobetyolin and IS, respectively.

### Preparation of calibration and quality control (QC) standards

Stock standard solutions of lobetyolin and the IS were prepared in methanol at a concentration of 0.1 mg.mL$^{-1}$ and stored at 8 °C. The plasma calibration standards for lobetyolin were prepared at concentrations of 1.0, 2.0, 5.0, 20.0, 50.0, 200 and 500 ng.mL$^{-1}$ by adding appropriate aliquots of working solutions to blank plasma. Low, medium and high concentration QC samples were prepared in blank rat plasma at concentrations of 2.5, 30.0 and 450 ng.mL$^{-1}$. The working solution of IS was prepared at 50.0 ng.mL$^{-1}$. All calibration standards were performed in triplicates and QC samples were prepared in six replicates.

### Sample preparation

50 μL plasma sample (or calibration standard or QC sample) was added with 25 μL IS working solution and 200 μL cold methanol. After vortexing for 3 min and centrifuging at 10000 rpm for 5 min, 100 μL of supernatant was collected and transferred to Ultimate HPLC vials. A 3 μL of the supernatant was injected into the LC-MS/MS system for the analysis. Blank sample (without analyte and IS) and zero sample (blank plus IS) were also prepared, and 3 μL of the supernatant was injected for the LC-MS/MS detection.

### Pharmacokinetic application

The developed assay was applied to a rat pharmacokinetic study following oral administration of lobetyolin at a dose of 10 $mg.kg^{-1}$. The rats were fasted for 12h but free access to water before dosing and further fasted for 2 h postdose. Blood was taken from retro-orbital puncture with a glass capillary at 0, 0.083, 0.17, 0.33, 0.67, 1, 2, 4, 6, 9 and 12 h after oral administration and collected into heparinized plastic tubes. The separated plasma after centrifugation (4000 rpm at 4 °C for 10 min) was stored frozen at -20 °C until analyzed.

## RESULTS AND DISCUSSION

### Selection of IS

An ideal IS should be a stable isotope-labeled compound (Deng *et al*., 2020; Yang *et al*., 2011) however isotope-labeled lobetyolin was unavailable commercially. Syringin was used as internal standard for lobetyolin analysis in this method considering its physicochemical properties, ionization and retention times. In addition, syringin was optimized in the positive ion mode as with lobetyolin, and it showed perfect peaks in the Thermo ODS $C_{18}$ reversed-phase column under the described above chromatography condition.

### Mass spectrometry condition optimization

For the optimization of mass transitions (*m/z*), the analyte and IS solutions at 200 ng/mL were infused into MS system by a syringe pump. The MS signal was acquired in full scan mode in the *m/z* range of 100–500 under positive ionization mode. The best signal of $[M+Na]^+$ ions for lobetyolin at *m/z* 419.3 and $[M+Na]^+$ ion for IS at *m/z* 394.9 were selected. After fragmentation, the analytical SRM at *m/z* 419.3→203.1 for lobetyolin and *m/z* 394.9→231.9 for IS were selected since they were the most abundant transitions.

### Validation procedures

The validation experiment was performed according to the Guidance on Bioanalytical Method Validation (The Pharmacopoeia Committee of China, 2020).

#### *Selectivity*

Selectivity was investigated by comparing the chromatograms of blank plasma with those spiked with lobetyolin and IS, as well as real plasma samples from treated rats. Figure 2 represents the MRM chromatograms of blank plasma, plasma spiked with lobetyolin at the LLOQ, and a rat plasma sample collected at 1.0 h after oral administration. No significant interference was detected at the retention times of both compounds.

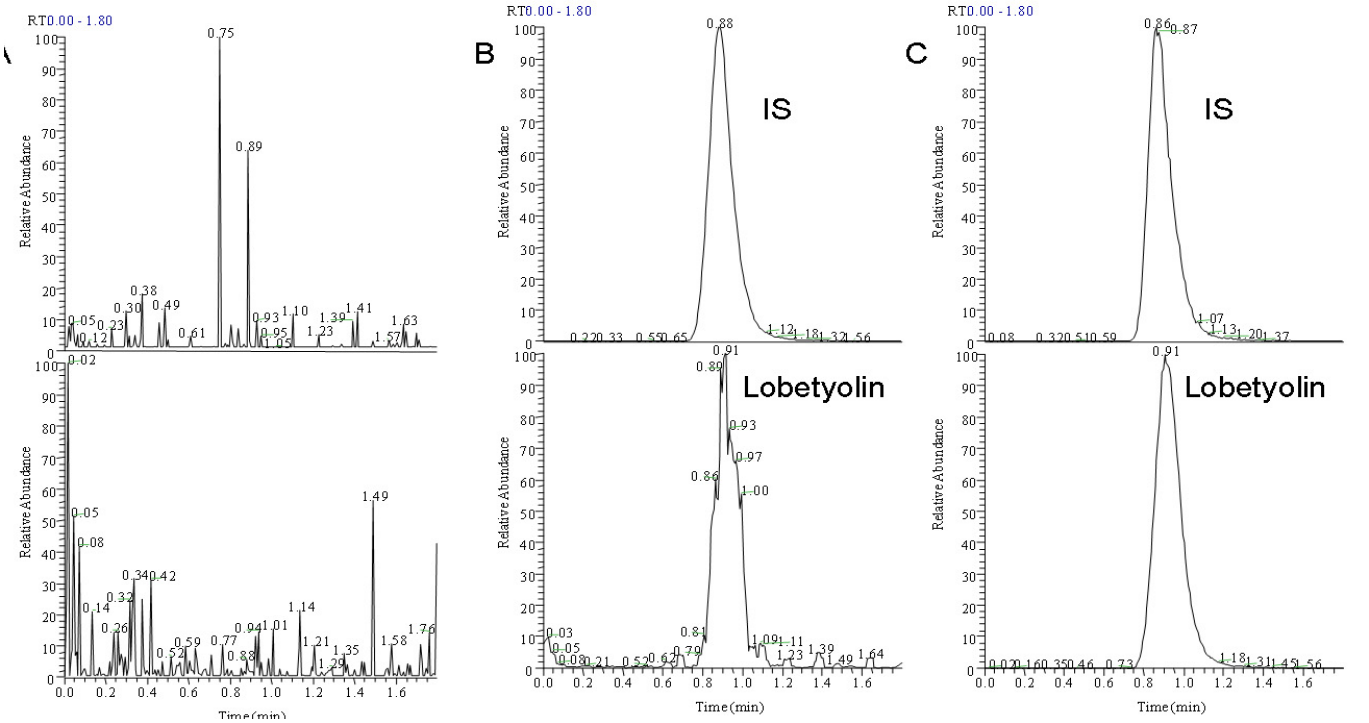


**FIGURE 2 -** Representative MRM chromatograms (A) blank rat plasma; (B) blank rat plasma spiked with lobetyolin and IS; (C) real plasma sample 1 h after oral dose of 10 mg/kg.

*Assay performance and reproducibility*

The assay performance was tested by conducting analysis of variance calculations using the QC samples of all the analytical runs (Fontana *et al.*, 2019). Intra- and inter-day precision (defined as pecent relative standard deviation, %RSD) and accuracy (defined as percent relative error, %RE) were calculated by determining lobetyolin QC concentrations in plasma in six replicates on three consecutive days. The resulting data showed that intra- and inter-day precision and accuracy were within ±15% (Table I).

**TABLE I -** Assay performance of lobetyolin QC samples in rat plasma

| Mean observed conc. | LLOQ (1.0 ng.mL-1) | LQC (2.5 ng.mL-1) | MQC (30.0 ng.mL-1) | HQC (450 ng.mL-1) |
|---|---|---|---|---|
| | **1.02±0.05** | **2.17±0.14** | **34.4±3.4** | **452.4±5.8** |
| Accuracy (%RE) | 1.9 | –13.3 | 14.5 | 0.5 |
| Intra-day precision (%RSD) | 2.2 | 8.4 | 6.0 | 2.1 |
| Inter-day precision (%RSD) | 5.1 | 6.4 | 9.8 | 1.3 |
| n | 18 | 18 | 18 | 18 |
| Numbers of runs | 3 | 3 | 3 | 3 |

*Linearity and sensitivity*

Linear calibration curves were plotted at lobetyolin concentration range of 1.0–500 ng.mL$^{-1}$ in rat plasma, with a good correlation coefficient ($r^2$) of >0.99 in the three analytical runs. The LLOQ was determined at 1.0 ng.mL$^{-1}$ of lobetyolin in plasma, at which the S/N ratio was higher than 10. The accuracy of ≤±15% was considered tot be acceptable, excluding for the LLOQ where accuracy of ±20% was acceptable.

*Matrix effect and recovery*

Matrix effect was evaluated by comparing the peak area of post-extraction spiked QC samples with that of the corresponding standard solutions (Meyer, Shah, 2019; Veerman *et al.*, 2019). The extraction recovery was investigated by comparing the peak area of extracted spiked samples with that of the post-extraction spiked samples (Wu *et al.*, 2021). The matrix effect for lobetyolin at concentrations of 2.5, 30.0 and 450 ng.mL$^{-1}$ in rat plasma was determined to be 101.3±2.7, 91.3±4.7, and 91.0±6.9% (n=5), respectively. The absolute recoveries for lobetyolin of plasma samples at three QC levels were determined to be in the range of 87.0–95.6% (data not shown). Results demonstrate that the analyte loss was negligible during sample preparation (n=5).

*Carryover*

Carryover study was checked by measuring the peak areas for the analyte by injecting blank plasma samples sequentially after an ULOQ injection. Carryover was performed to ensure whether it affects the accuracy and precision of the present LC-MS/MS assay. No carryover effect from analyte and IS come across injecting blank plasma sample given subsequent to ULOQ sample.

*Stability*

The stability was evaluted by determining three concentration levels of QC samples at 22 ºC for 6 h, –20 ºC storage for 23 days, 10 ºC for 24 h in autosampler trials, and three freeze-thaw cycles in matrix. The stability data under different storage conditions are summarized in Table II. Results met well within the acceptable limit during the entire process.

**TABLE II -** Stability of lobetyolin QC samples in rat plasma (n=5)

| Storage condition | Conc. added (ng.mL$^{-1}$) | Conc. measured (ng.mL$^{-1}$) | Accuracy (%RE) | Precision (%RSD) |
|---|---|---|---|---|
| Short-term (6 h at 22 °C | 2.5 | 2.40±0.21 | –3.9 | 9.0 |
| | 30.0 | 26.3±1.6 | –12.4 | 6.2 |
| | 450 | 431.9±48.7 | –4.0 | 11.3 |
| Long-term (23 days at −20 °C) | 2.5 | 2.77±0.06 | 10.9 | 2.2 |
| | 30.0 | 26.8±0.4 | –10.7 | 1.5 |
| | 450 | 486.4±59.3 | 8.0 | 12.2 |
| Three freeze−thaw cycle | 2.5 | 2.37±0.05 | –5.4 | 2.1 |
| | 30.0 | 27.1±2.5 | –9.6 | 9.0 |
| | 450 | 431.9±36.8 | –4.0 | 8.5 |
| Autosampler trials (24 h at 10 °C) | 2.5 | 2.46±0.33 | –1.7 | 13.4 |
| | 30.0 | 29.0±1.9 | –3.2 | 6.7 |
| | 450 | 418.1±9.2 | –7.1 | 2.2 |

**Pharmacokinetic application**

In order to acquire the main pharmacokinetic parameters of lobetyolin, the concentration-time curves were analyzed by DAS 2.0 Software. Data were expressed as mean±SD. The mean plasma concentration–time profile is shown in Figure 3 and the corresponding parameters of lobetyolin are listed in Table III. The present assay allowed for the quantification of lobetyolin up to 12 h (last sampling time). As shown in Figure 3, the plasma concentrations of lobetyolin reached its maximum plasma concentration ($C_{max}$, 60.1±33.1 ng.mL$^{-1}$) at the peak time ($T_{max}$, 1.0±0.6 h), followed by a relative long time (12 h) decreasing to the LLOQ, with the elimination time ($t_{1/2}$) of 2.2±1.1 h. The $MRT_{0-t,}$ $AUC_{0-t}$ and $AUC_{0-\infty}$ values in rats were 2.8 ± 1.0 h, 212.4 ± 172.9 ng h.mL$^{-1}$, and 253.8 ± 192.6 ng h.mL$^{-1}$ for lobetyolin. The pharmacokinetic properties of lobetyolin ($C_{max}$, $T_{max}$, AUC and $t_{1/2}$) were comparable with those of the previously reported data (Dong *et al.*, 2021).

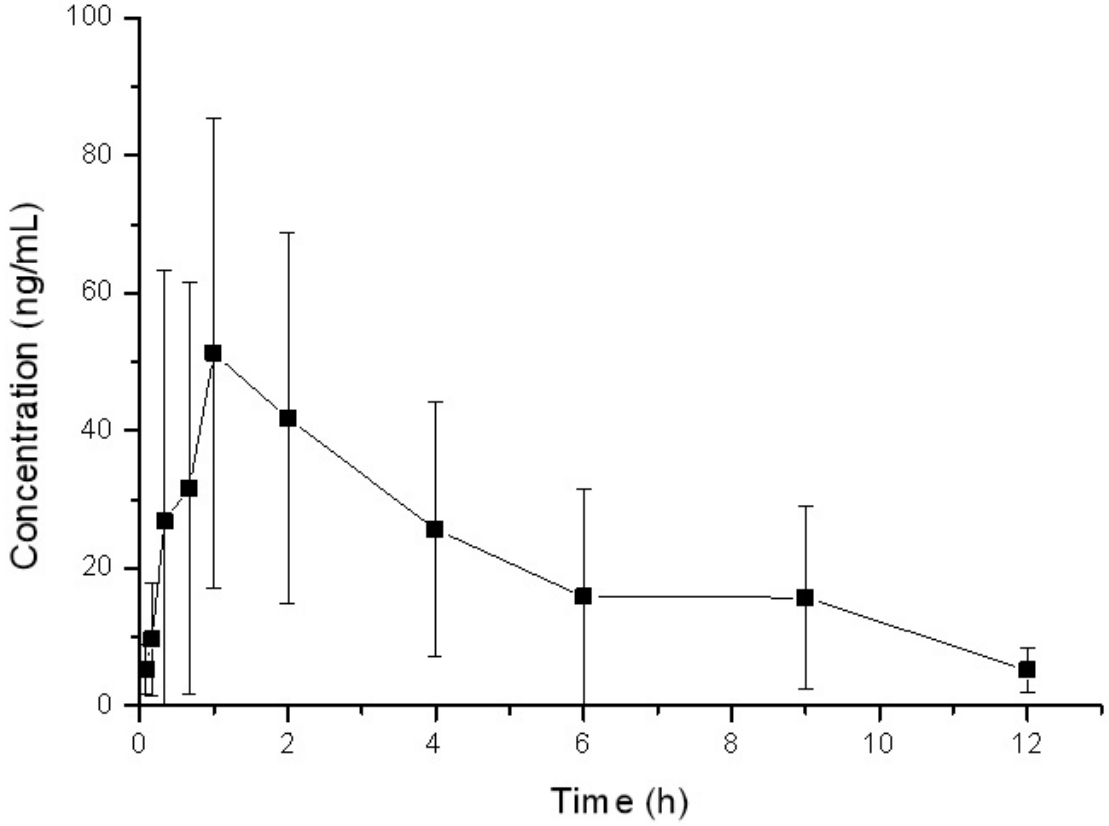


**FIGURE 3 -** Mean plasma concentration–time profile of lobetyolin in rats (n=6).

**TABLE III -** Pharmacokinetic parameters of lobetyolin after an oral administration of lobetyolin (10 mg.kg$^{-1}$) to rats (n = 6)

| Pharmacokinetic parameter | Lobetyolin |
|---|---|
| $C_{max}$ (ng.mL$^{-1}$) | 60.1 ± 33.1 |
| $T_{max}$ (h) | 1.0 ± 0.6 |
| $T_{1/2}$ (h) | 2.2 ± 1.1 |
| $AUC_{0-t}$ (ng h.mL$^{-1}$) | 212.4 ± 172.9 |
| $AUC_{0-\infty}$ (ng h.mL$^{-1}$) | 253.8 ± 192.6 |
| $MRT_{0-t}$ (h) | 2.8 ± 1.0 |
| $MRT_{0-\infty}$ (h) | 4.1 ± 1.9 |

## CONCLUSIONS

A rapid and sensitive LC-MS/MS assay was established to quantify lobetyolin concentrations in rat plasma. It allowed to achieve good accuracy and precision and to perform the pharmacokinetics of lobetyolin administered to Wistar rats. The present *in vivo* pharmacokinetic study can give helpful information for the rational usage of *Codonopsis pilosula*.

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