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A rapid and sensitive LC–MS/MS assay for the quantitation of deacetyl mycoepoxydiene in rat plasma with application to preclinical pharmacokinetics studies

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ABSTRACT

The purpose of this study was to develop and validate a high-performance liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method for analysis of the deacetyl mycoepoxydiene in rat plasma. The analyte and internal standard (I.S.), benorilate, were extracted from rat plasma by precipitation protein and separated on a C_{18} column using acetonitrile–0.5% formic acid as mobile phase. Detection was performed using a turbo-spray ionization source and mass spectrometric positive multi-reaction-monitoring-mode (+MRM) at an ion voltage of +4800 V. The assay was linear over the concentration range 5–5000 ng/mL with the lowest limit of quantification (LLOQ) of 5 ng/mL. The method also afforded satisfactory results in terms of the sensitivity, specificity, precision (intra- and inter-day, RSD < 5.8%), accuracy, recovery as well as the stability of the analyte under various conditions. The method was successfully applied to a preclinical pharmacokinetic study in rats after a single intravenous administration of deacetyl mycoepoxydiene 10 mg/kg.

1. Introduction

Deacetyl mycoepoxydiene (5-hydroxy-6-(8-methyl-9-oxa-bicyclo[4.2.1] nona-2,4-dien-7-yl)-5, 6-dihydropyran-2-one) is a novel antitumor agent extracted from a marine endophytic fungi *Phomopsis* sp. A123. It was first isolated in 2003 [1–5]. Appropriate analytical method is an urgent need to study its pharmacokinetics in vivo. The HPLC–UV approach for content determination has also been applied to a purity study of deacetyl mycoepoxydiene [6]. However, this method was not sensitive enough for pharmacokinetic studies of deacetyl mycoepoxydiene. LC/MS spectrometry based techniques are now the mainstay for such pharmacokinetic studies because of sensitivity, selectivity, speed and cost effectiveness [7]. To the best of our knowledge, there was no entirely validated HPLC–MS/MS method reported in the literature for quantification of deacetyl mycoepoxydiene in biological samples. This paper reports on a rapid and sensitive LC–MS/MS method for the determination of deacetyl mycoepoxydiene in rat plasma using benorilate as internal standard (I.S.). The method employs simple sample preparation and is completed in a run time of only 4 min. The method was successfully applied to a pharmacokinetic study of deacetyl mycoepoxydiene after intravenous administration in rats.

2. Experimental

2.1. Chemicals and reagents

Deacetyl mycoepoxydiene and benorilate were kindly supplied by Y.M. Shen (College of Chemistry and Chemical Engineering of Xiamen University, Xiamen, PR China), Benorilate (internal standard, I.S.) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), Formic acid (TEDIA, TEDIA company, Inc., USA), methanol and acetonitrile (DIMA Technology Inc., USA) were HPLC-grade. Distilled water was prepared from deionized water. All other chemicals and solvents were analytical grade and used as received. Blank rat plasma was prepared in laboratory animal center of Shandong University.

2.2. LC–MS/MS conditions

The HPLC–MS/MS system consisted of an Agilent 1100 series binary pump (Agilent Technologies, Palo Alto, CA, USA), an auto sampler connected to and an API 4000 mass spectrometer (Applied Biosystems Sciex, Ont., Canada) using a turbo-spray ionization

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source (ESI). The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.2 software.

Deacetyl mycoepoxydiene and I.S. was separated on a Venusil XBP C_{18} column (5 μm, 150 mm × 2.1 mm I.D., Agela. Science Inc., USA) through a 4 mm × 3 mm precolumn (Security Guard C_{18} cartridge, Phenomenex, Inc.) maintained 35 °C. The mobile phase consisted of acetonitrile–0.5% formic acid (50:50, v/v) (pH = 2.5) and was set at a flow rate of 0.3 mL/min.

The detector was operated at unit resolution in the positive multiple-reaction monitoring-mode (+MRM) mode using the transitions of the protonated molecular ions of deacetyl mycoepoxydiene (249.2 *m*/*z*; 145.1 *m*/*z*), I.S. (314.3 *m*/*z*; 272.2 *m*/*z*). MS parameters were optimized by syringe pump infusing of standard solution containing analyte and I.S. Optimized parameters were as follows: curtain gas, gas1 and gas2 (nitrogen) was 15, 50 and 40 psi respectively; dwell time was 200 ms; source temperature was 500 °C; ion spray voltage was 4800 V. Declustering potential and collision energy were 45 V and 15 V for deacetyl mycoepoxydiene, while 65 V and 11 V for I.S.

2.3. Preparation of calibration standards and quality control samples

A stock solution of deacetyl mycoepoxydiene was prepared in methanol at a concentration of 1.0 mg/mL. Standard solutions (20, 100, 500, 2000, 5000, 10,000, 20,000 ng/mL) were prepared by serial dilution of the stock solution with methanol–water (50:50, v/v). Low, medium and high concentration quality control (QC) solutions (50, 5000, 16,000 ng/mL) were prepared in the similar way. The stock solution of I.S. (1.0 mg/mL) was also prepared in methanol and then diluted with methanol–water (50:50, v/v) to a final concentration of 100 ng/mL. All solutions were stored at 4 °C and used within one month after preparation. Calibration standards and QC samples were prepared by adding 25 μL standard and 25 μL I.S. or QC solution to 100 μL blank rat plasma.

2.4. Sample preparation

An aliquot (100 μL) of rat plasma was transferred to a 2.0 mL Eppendorf tube, 25 μL methanol–water (50:50, v/v) or 25 μL standard of deacetyl mycoepoxydiene and 25 μL I.S. solutions were added. The mixture was vortexes for 30 s, and then added 200 μL acetonitrile. After vortex-mixed for 1 min, the mixture was centrifuged at 12,000 rpm for 10 min at room temperature. Then the supernatant was transferred to an auto sampler vial and 5 μL was injected into the HPLC–MS/MS system.

2.5. Method validation

Three independent calibration curves and five replicates of QC samples (12.5, 1250, 4000 ng/mL, respectively) were analyzed on three different days. Linearity was analyzed by weighted least-squares linear regression ($1/x^2$) of calibration curves based on peak area ratios of analyte to I.S., $y = ax + b$ (x = concentration).

Intra- and inter-day precision (as relative standard deviation, R.S.D.) and accuracy (as relative error, R.E.) were based on assay of five replicates of QC samples analyzed on three consecutive days use independently calibration curves.

The LLOQ was defined as the lowest concentration in the calibration curve at which both precision and accuracy were less than or equal to 20%, and signal/noise (S/N) >10.

Recovery of deacetyl mycoepoxydiene was determined by comparing peak areas of QC samples with peak areas of corresponding concentration QC solutions dissolved in the supernatant of the processed blank rat plasma.

The matrix effects were evaluated by comparing the peak areas of deacetyl mycoepoxydiene of corresponding concentration QC solutions dissolved in the supernatant of the processed blank rat plasma with those of the standard solutions, which were prepared in the same way as QC samples except water substituted for drug free rat plasma.

Stability of deacetyl mycoepoxydiene was established by analysis of five replicates of QC samples under the following conditions: Storage at room temperature for 6 h; processed samples in auto sampler vials for 6 h; storage for 7 and 30 days at −20 °C; two freeze–thaw cycles.

Validation of the method was performed according to the Chinese guidelines on pharmacokinetics testing in animals [8].

2.6. Pharmacokinetic study

Six rats (3 males and 3 females, Laboratory Animal Center of Shandong University, Jinan, PR China), weight 200–250 g, were involved in the pharmacokinetic study approved by the Institutional Animal Ethics Committee of Shandong University. After a 12 h fast, all rats were administered a single dose of deacetyl mycoepoxydiene (10 mg/kg) by the intravenous routes. Blood samples (approximately 0.4 mL) were collected into heparinized tubes before the dose and at 0.03, 0.08, 0.25, 0.50, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0 h after the dose. Rat plasma was prepared by centrifugation at 3000 rpm for 10 min and stored at −20 °C until analysis.

3. Results and discussion

3.1. LC/MS/MS conditions

The LC–MS/MS operation parameters for determination of deacetyl mycoepoxydiene and I.S. were carefully optimized. Benorilate was selected as the I.S. because its chromatographic behavior and extraction efficiency were similar to those of analyte. Both analyte and I.S. responded best to the positive ionization mode, with the protonated molecular ions $[M+H]^+$ as the major species. Product ion spectra of deacetyl mycoepoxydiene and I.S. were shown in Fig. 1. The MRM acquisitions were performed at unit resolution using the transition 249.2 → 145.1 *m*/*z* for deacetyl mycoepoxydiene and 314.3 → 272.2 *m*/*z* for I.S. respectively. The transition 249.2 → 145.1 *m*/*z* was used for quantification of deacetyl mycoepoxydiene because of its stabilized ions response and no endogenous interference. The mass parameters were optimized by observing the maximum response obtained for the product ions.

Furthermore, the mobile phase system was optimized through several trials to achieve satisfactory chromatographic behavior and the ionization responses of deacetyl mycoepoxydiene and I.S. The ionization of deacetyl mycoepoxydiene and I.S. was affected by the composition of mobile phase. In the preliminary experiments, methanol, acetonitrile, ammonium acetate and formic acid in various proportions were tested. Acetonitrile, rather than methanol, was chosen as the organic modifier because it lead to the good peak shape of deacetyl mycoepoxydiene and satisfactory retention time. When ammonium acetate was added in the mobile phase, the response of the analyte was distinctly decreased. Both the analyte and IS were found to have higher response and better peak shapes in the mobile phase containing 0.5% formic acid. Finally, in terms of peak shape, retention time and sensitivity, we employed acetonitrile–0.5% formic acid (50:50, v/v), as the mobile phase.

3.2. Assay validation

The representative chromatograms of blank rat plasma (Fig. 2A), blank rat plasma spiked with deacetyl mycoepoxydiene at the

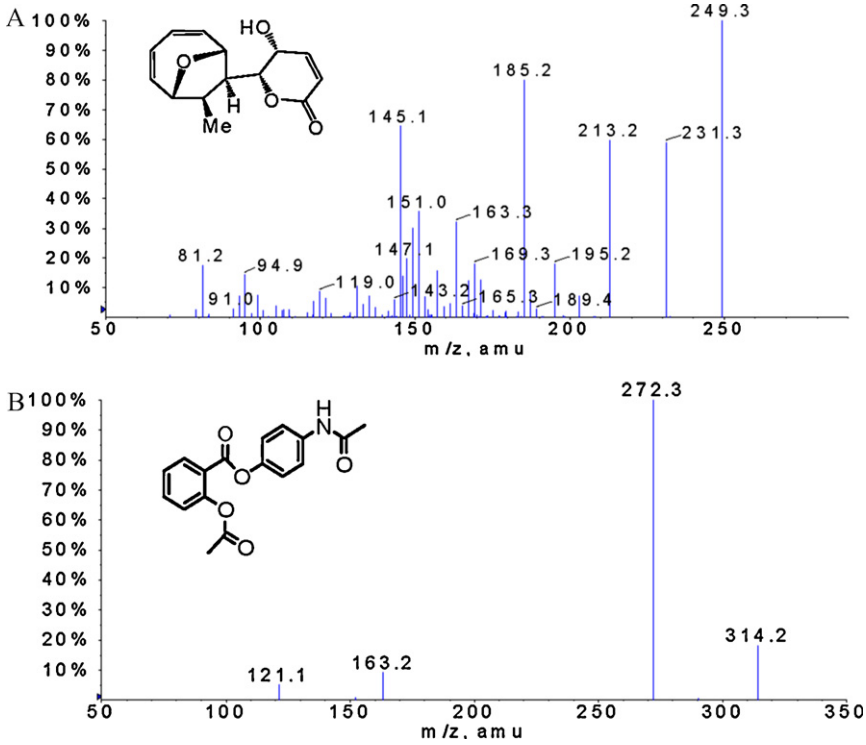

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ for (A) deacetyl mycoepoxydiene and (B) benorilate.

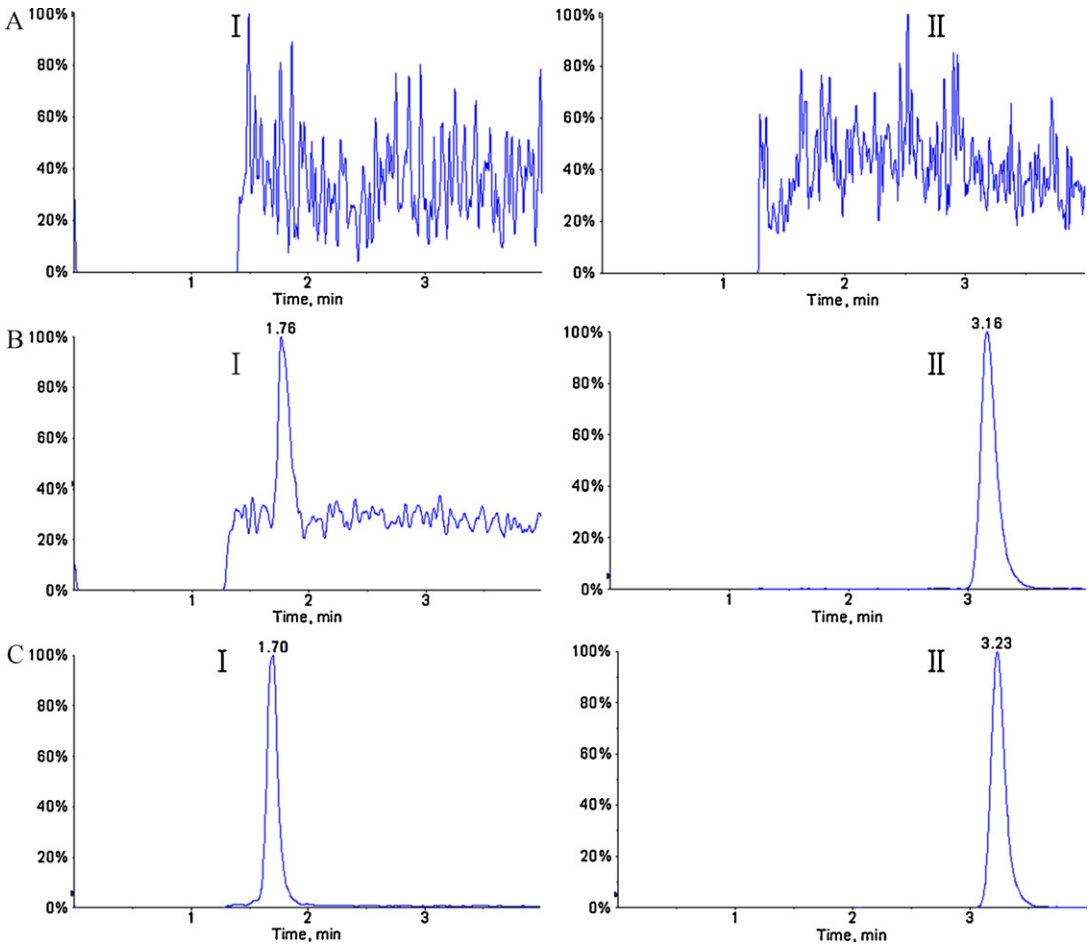

Fig. 2. Representative MRM chromatograms of (A) blank plasma; (B) plasma spiked with deacetyl mycoepoxydiene at lower limit of quantitation (5 ng/mL) and (C) a study sample containing deacetylmycoepoxydiene. Peak I, deacetyl mycoepoxydiene; Peak II, benorilate.

Table 1
The matrix effects, recovery, precision and accuracy of the assay method.

Concentration (ng/mL)	Matrix effects (n = 5)		Absolute recovery (n = 5)		Intra-day (n = 5)		Inter-day (n = 15)	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Precision RSD (%)	Accuracy (bias %)	Precision (RSD %)	Accuracy (bias %)
12.5	95.6	3.6	85.4	2.7	3.0	4.3	1.7	12.0
1250	98.7	0.9	84.7	1.0	4.5	−4.9	2.0	9.4
4000	95.5	1.7	84.3	1.2	5.8	6.5	3.0	−6.7

Table 2
Stability of deacetyl mycoepoxydiene in rat plasma under various conditions at three QC levels (n = 5).

Storage conditions	Parameter	QC concentration (ng/mL)		
		12.5	1250	4000
In rat plasma at room temperature for 6 h	Calculated concentration (ng/mL)	12.5	1280	4078
	RDS (%)	2.2	4.7	8.2
In processed samples at room temperature for 6 h	Calculated concentration (ng/mL)	13.3	1296	4180
	RDS (%)	7.5	8.4	4.1
In rat plasma after one freeze-thaw cycles	Calculated concentration (ng/mL)	12.4	1144	3778
	RDS (%)	6.4	5.9	5.7
In rat plasma after two freeze-thaw cycles	Calculated concentration (ng/mL)	4.6	724	2130
	RDS (%)	17.9	6.1	4.7
In rat plasma for 7 days at −20 °C	Calculated concentration (ng/mL)	11.5	1204	3914
	RDS (%)	3.6	4.5	6.0
In rat plasma for 30 days at −20 °C	Calculated concentration (ng/mL)	13.1	1090	3586
	RDS (%)	4.8	0.9	5.0

LLOQ of 5 ng/mL and I.S. (Fig. 2B) and a male rat plasma sample at 0.5 h after a single intravenous administration of 2.26 mg (10 mg/Kg weight) of deacetyl mycoepoxydiene (Fig. 2C) mean that no endogenous interferences were present at the retention times of 1.7 min (deacetyl mycoepoxydiene) and the 3.2 min (I.S.). The run time was only 4 min.

The standard curves showed good linearity over the concentration range of 5–5000 ng/mL with an LLOQ of 5.0 ng/mL for deacetyl mycoepoxydiene in rat plasma. A typical $1/x^2$ weighted linear regression equation was $y = 0.000261x + 0.00177$, $r = 0.9969$.

The intra-day and inter-day precision (RSD%, n = 5) for deacetyl mycoepoxydiene were satisfactory at the three concentrations studied.

The absolute recoveries of deacetyl mycoepoxydiene from the rat plasma at the three concentrations were about 85%.

In terms of matrix effects, the ratios of the peak responses for deacetyl mycoepoxydiene were 95.6%, 98.7%, and 95.5% at 12.5, 1250, and 4000 ng/mL, respectively. The results indicated that co-eluting endogenous substances hardly inhibited the ionization of deacetyl mycoepoxydiene, and the ion suppression from rat plasma matrix was consistent for this analytical method and would not interfere with the measurement of deacetyl mycoepoxydiene. Data on precision, accuracy, matrix effects and recovery are shown in Table 1.

Table 3
The pharmacokinetic parameters (mean ± S.D.) of deacetyl mycoepoxydiene In rats following intravenous administration at dose of 10 mg/kg (n = 6).

Parameters	Mean ± S.D.
C_{Max} (μg/L)	4726.67 ± 312.52
T_{Max} (h)	0.06 ± 0.03
$t_{1/2}$ (h)	2.09 ± 0.68
AUC_{0-t} (μg h/L)	3376.27 ± 544.46
$AUC_{0-\infty}$ (μg h/L)	3420.60 ± 541.76
CL_Z/F (L/h/kg)	2.99 ± 0.51
V_Z/F (L/kg)	9.11 ± 3.38

Table 2 summarizes all the results of stability of deacetyl mycoepoxydiene in rat plasma. From the results, freeze–thaw stability of deacetyl mycoepoxydiene in rat plasma was found an issue in relation to more than two cycles.

3.3. Pharmacokinetic study

The method was able to detect deacetyl mycoepoxydiene in rat plasma for 8 h after intravenous administration of deacetyl mycoepoxydiene at a dose of 10 mg/kg. The rat plasma concentration–time profiles are shown in Fig. 3 and pharmacokinetic parameters are listed in Table 3. Our results

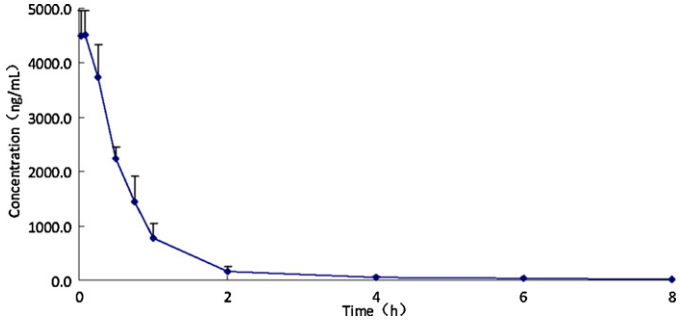

Fig. 3. Mean (±S.D.) plasma concentration-time curve of deacetyl mycoepoxydiene after intravenous administration 10 mg/kg to rats (n = 6).

indicate that peak concentration of deacetyl mycoepoxydiene was in the range 4320–4980 ng/mL after dosing. Further studies of the absorption, distribution, metabolism and excretion of deacetyl mycoepoxydiene in rat and dog are underway in our laboratory.

4. Conclusions

In the study, a HPLC–MS/MS method was developed and validated for the determination of deacetyl mycoepoxydiene in rat plasma. The method was rapid, selective and highly sensitive with a LLOQ of 5 ng/mL for deacetyl mycoepoxydiene after the rat plasma protein was simply removed by acetonitrile precipitation. The method has been successfully applied to a preclinical pharmacokinetic study following intravenous administration to rats. This is the first report of the use of LC–MS/MS to evaluate the pharmacokinetics of deacetyl mycoepoxydiene.

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