LC-MS/MS Assay Validation for a New Immune Modulator, JHL45, and its Major Metabolite in Plasma: Application to Pharmacokinetic Studies in Rats

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JHL45, a novel immune modulator for anti-atopic dermatitis and allergic airway disease, was synthesized from decursin isolated from *Angelica gigas*. In order to conduct a pharmacokinetic study of JHL45, an analytical method, ideally one that uses a minimal amount of biological sample must first be validated. In this study, a HPLC-MS/MS method was developed and validated for the quantification of JHL45 and its major metabolite, (+)-decursinol, from 10 μL of rat plasma. JHL45 was stable under the analysis conditions, and intra- and inter-day accuracies exceeded 90.06%, with a precision variability ≤ 13.16% for each analyte. The mean values for C_{max}, AUC_{8h}, half-life of JHL45 in rats after intravenous administration of 5 mg/kg JHL45 were 24.59 μg/mL, 10.02 μg·h/mL, and 1.88 h, respectively. The validated method herein will be useful for further pharmacokinetic studies of JHL45, as well as in preclinical and clinical phases.

Key Words: JHL45, (+)-Decursinol, LC-MS/MS, Immune modulator, Pharmacokinetics

Introduction

Atopic dermatitis (AD) is one of the most common skin diseases in children with a family history of atopy. The pathogenesis of AD is associated with the release of various cytokines and chemokines owing to an activated T-helper immune response.[1,2] However, the exact etiology of AD is unclear. Presently available treatments for AD include antihistamines, emollients, doxepin, and corticosteroids. Topical steroids are commonly used to treat AD, but their chronic use at high concentrations can cause severe adverse effects such as skin atrophy, pigmentation alterations, hypothalamic changes, and growth inhibition.[3,4] Accordingly, the safety and pathophysiological characteristics of new AD therapies must be evaluated as soon as possible.

JHL45 [3-(3,4-dihydroxy-phenyl)-acrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2*H*,8*H*-pyrano[3,2-*g*]chromen-3-yl-ester; PCT/KR2008/001017; Fig. 1] is a decursin derivative developed by Chungnam National University (Daejeon, South Korea). JHL45 was synthesized from decursin isolated from *Angelicae gigas* and was shown to act as a novel immune modulator that inhibits the release of monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-8, and total immunoglobulin E (IgE).[5,6] JHL45 is expected to be an effective agent in treating and preventing AD and allergic airway disease. It is currently being studied at the early new drug development stage.

JHL45 can be separated into its metabolites, (+)-decursinol and caffeic acid, by catalytic hydrolysis of its ester form by esterase in plasma or liver cells.[7] The presence of (+)-decursinol (Fig. 1) can be confirmed based on its molecular weight (245.8 g/mol) and mass transition (*m/z* 246.8 → 90.4), as determined with a MS/MS detector. Caffeic acid was not detected in plasma after oral administration of JHL45 using LC-MS/MS.

In order to perform pre-clinical pharmacokinetic (PK) studies of JHL45 and its metabolites in animals as well as clinical PK studies in humans, a biological plasma concentration assay method, ideally one that uses a minimal amount of biological sample, must be developed and validated. To date, no such method using high-performance liquid chromatography (HPLC) has been reported.

This study demonstrates a reliable and sensitive assay method for the quantification of JHL45 and (+)-decursinol from 10 μL of rat plasma. Using this assay, a PK model was developed based on the plasma concentrations of JHL45 and (+)-decursinol as a function of time after intravenous (IV) administration of JHL45 in rats.

Experimental

Chemicals and reagents. JHL45 and (+)-decursinol, with a purity > 98%, were supplied by the medicinal organic chemistry laboratory at Chungnam National University (Daejeon, South Korea). Acetaminophen (internal standard; IS; Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Drug-free plasma was obtained from healthy Sprague-Dawley (SD) rats weighing 200 ± 20 g (Orient Bio Inc., Seongnam, Korea). HPLC-grade organic solvents were purchased from Merck Co. (Darmstadt, Germany). All chemicals and solvents were of the highest analytical grade available.

Analytical system. The plasma concentrations of JHL45 and (+)-decursinol were quantified by liquid chromatography-mass spectrometry using a PE SCIEX API 2000 (triple-quadrupole) LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface to generate positive ions $[M+H]^+$. The LC component consisted of a reversed-phase column (Xterra® C_{18}, 2.1 × 50 mm internal diameter, 3.5 μm particle size; Waters, USA) with an isocratic mobile phase of 0.1% formic acid in a mixture of MeOH and purified water (98:2, v/v). The mobile phase was eluted with an Agilent 1100 series pump (Agilent Technologies, Inc., Palo Alto, CA, USA) at 0.2 mL/min.

The turbo-ion spray interface was operated in positive ion

mode at 5500 V and 350 °C. The operating conditions, optimized with a flow injection of a mixture of all analytes, were as follows: nebulizing, auxiliary, and curtain gas flows of 1.04, 4.0, and 10.0 L/min, respectively; collision gas (nitrogen) pressure, 6×10^{-5} Torr; orifice voltage (declustering potential), 111 V; ring voltage (focusing potential), 330 V; entrance potential, 11 V; collision energy, 33 V; and collision exit potential, 6.0 V. Quantitation was performed by multiple reaction monitoring (MRM) of the protonated precursor ions and the related product ions for JHL45 and (+)-decursinol, using an internal standard method with peak area ratios. The mass transitions used for JHL45, (+)-decursinol, and the internal standard were *m/z* 408.8 → 229.0, 246.8 → 90.4, and 152.0 → 109.8, respectively (dwell time, 200 ms). Quadrupoles Q1 and Q3 were set at unit resolution. The analytical data were processed by Analyst 1.4.1 software (ABI, Inc.)

Preparation of stock and standard solutions. Stock solutions (1.0 mg/mL) of JHL45 and (+)-decursinol were prepared by dissolving 10 mg of JHL45 or (+)-decursinol in 10 mL of methanol, respectively. To prepare standard solutions of JHL45, the stock solution was serially diluted with methanol to concentrations of 0.1, 0.5, 2, 10, 50, and 100 μg/mL. Standard solutions of (+)-decursinol were similarly prepared at 0.5, 2, 8, 20, 50, and 100 μg/mL. The internal standard (IS) was prepared in methanol at 1 μg/mL. The calibration standard samples were prepared by spiking JHL45 1 μL and (+)-decursinol 1 μL into heparinized rat blank plasma 8 μL. All stock and standard solutions were kept at 4 °C throughout the study.

Sample preparation. JHL45, (+)-decursinol, and acetaminophen (IS) were extracted from rat plasma by protein precipitation. In a microcentrifuge tube, 10 μL of sample were mixed with 50 μL of IS (1 μg/mL), and the tube was vortexed for 30 s. The two phases were separated by centrifugation at 15,000 rpm for 10 min. A 20-μL aliquot of the supernatant was transferred into an injection vial, and 5 μL of this solution were injected into a LC-MS/MS for quantitative analysis. All plasma samples, including those for calibration and pharmacokinetic analysis, were processed following the same procedures.

Validation procedure. To validate the assay, the specificity, accuracy, precision, linear dynamic range, percent recovery, and stability were determined. A calibration curve was generated from the standard solutions to confirm a linear relationship between the peak area ratio and the concentration of JHL45. The slope, intercept, and coefficient of determination (r^2) were calculated as regression parameters of a weighted ($1/y^2$) linear regression. The intra- and inter-day precision and accuracy were estimated using the predicted concentration of the quality controls based on the calibration curve.[8] Accuracy was determined as the percentage difference of the measured concentration from the nominal concentration, and the coefficient of variation (CV) was used to report the precision.[9] Matrix effects and percentage recovery were investigated by analyzing three individual plasma samples at three different JHL45 concentrations (0.01 μg/mL, 1 μg/mL, and 10 μg/mL). Matrix effects were determined by comparing the JHL45 peak areas of samples spiked post-extraction with those of the same standard concentrations. The percentage recovery on ionization was determined by comparing the peak area ratio of JHL45 and the IS from extracted samples to the ratio from samples with the same standard concentration.[10] The same evaluation protocols were followed with (+)-decursinol (0.05 μg/mL, 2 μg/mL, and 10 μg/mL). For the IS, the matrix effect was evaluated only at concentrations of 1 μg/mL, 5 μg/mL, and 10 μg/mL.

Drug-free control plasma samples were spiked with 0.2 μg/mL or 1 μg/mL JHL45, in order to study its stability in plasma. Short-term, post-extraction, freeze-thaw, and long-term stabilities were assessed.[11] To determine short-term stability, samples were analyzed after 6 h at room temperature; long-term stability was determined after 30 days of storage at −20 °C. The stability of JHL45 in extracts was examined after 24 h of storage at 4 °C. The freeze-thaw stability was evaluated by analyzing the samples after three freeze-thaw cycles (−80 °C to room temperature). The same evaluations were performed for (+)-decursinol. The stabilities of the stock solutions of JHL45 and (+)-decursinol were examined after 30 days at 4 °C.

Rat plasma dosing. Five female Sprague-Dawley (SD) rats weighing 200 ± 20 g (Orient Bio. Inc.) were housed in an animal facility at the College of Pharmacy, Chungnam National University. All animal experiments in the present study were carried out in accordance with the Standard Operating Procedures of the facility.

JHL45 was good soluble compound in both of aqueous and organic solvents. For rat dosing, JHL45 was dissolved in 10% cremophor, and the solution was clear. The analytical method described above was applied to determine the concentrations of JHL45 and (+)-decursinol in the plasma from five rats after intravenous administration of 5 mg/kg JHL45. Blood samples (50 μL) were collected from the ocular plexus venosus of each rat at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 8 h after dosing. The rats were allowed free access to water during the experiment, to maintain their normal body condition. The blood samples were placed in heparinized tubes, and the plasma was separated by centrifugation at 3000 rpm for 10 min. All plasma samples were stored at −20 °C until analysis.

Pharmacokinetic analysis. Pharmacokinetic analyses were performed using model-independent (compartmental) and model-dependent (non-compartmental) methods with WinNonlin Standard Edition 2.1 software (Pharsight Corp., Palo Alto, CA, USA). The area under the plasma concentration-versus-time curve (AUC) was calculated using trapezoidal estimation and was extrapolated to infinity. The plasma concentrations of JHL45 and (+)-decursinol as a function of time were used to determine the maximum plasma concentration (C_{max}) and the time (T_{max}) required to reach C_{max}. An elimination rate constant (k_{el}) was obtained by linear regression of the terminal phase, and the elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$.[12,13] The results are presented as the mean ± standard deviation.

Model-dependent analyses incorporated a parent-metabolite compartmental model.[8] As shown in Fig. 2, the model simultaneously describes the pharmacokinetics of JHL45 and (+)-decursinol. The pharmacokinetic parameters of JHL45 were explained by a two-compartment model, and the rate constants dictating the transport of the drug from the central to the metabolite compartment (K_{cm}) revealed a time delay

between the plasma presence of parent and metabolite. A weighting factor of $1/y$ was applied to fit the data and estimate the parameters. The criteria used to determine the fit were the AIC, SIC, ECV, SDRSS, and visual inspection.

Results and Discussion

Mass spectra. Precursor ions for JHL45, (+)-decursinol, acetaminophen (IS), and their corresponding product ions were determined from spectra obtained from the injection of standard solutions into a mass spectrometer with an electrospray ionization source. The system was operated in positive ionization mode with nitrogen collision gas in Q2 of an MS/MS system. JHL45, (−)-decursinol, and acetaminophen (IS) produced mainly protonated ions at *m/z* 408.8, 246.8, and 152.0, respectively. Both product ions were scanned in Q3 after collision with nitrogen in Q2 at *m/z* 229.0, 90.4, and 109.8 for JHL45, (+)-decursinol, and acetaminophen (IS), respectively (Fig. 1).

Chromatography. Significant peak-tailing was observed for both JHL45 and (+)-decursinol with an acetonitrile mobile phase. Therefore, several combinations of methanol and water were evaluated in order to sufficiently resolve each compound while minimizing noise and peak-tailing effects. The inclusion of 0.1% formic acid in the mobile phase was improved peak shape. So and the optimal mobile phase was identified as 0.1% formic acid in a 98:2 (v/v) MeOH:H_2O. The retention times of JHL45, (+)-decursinol, and acetaminophen (IS) in rat plasma were approximately 0.9 min, and the total run time for each sample was about 3 min. Chromatograms of blank plasma, blank plasma with JHL45 and IS, blank plasma with (+)-decursinol and IS, and rat plasma after IV injection are shown in Fig. 3.

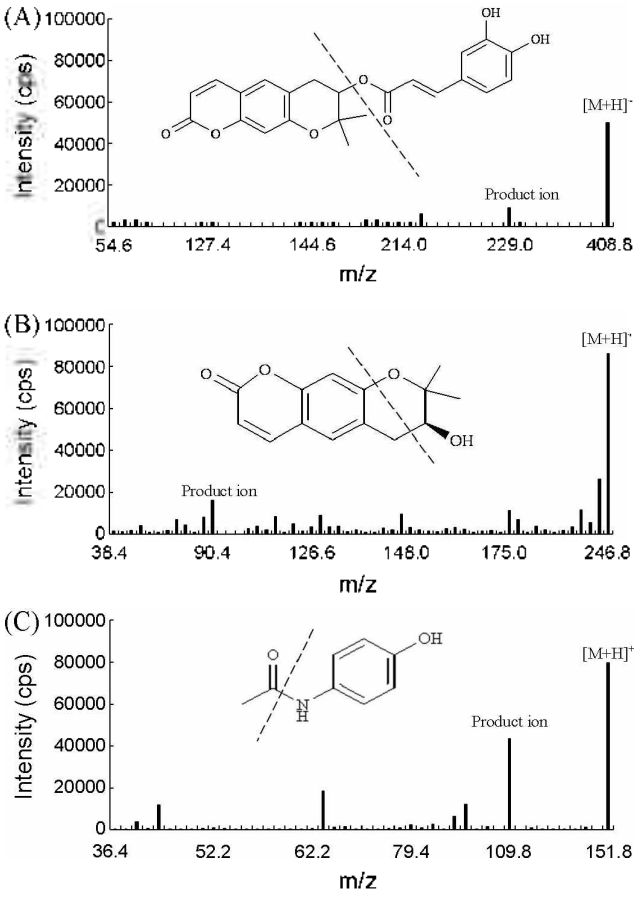

Figure 1. The molecular structures and mass spectra show the ions obtained for (A) JHL45, (B) (+)-decursinol, and (C) acetaminophen (internal standard) using electrospray ionization in positive mode.

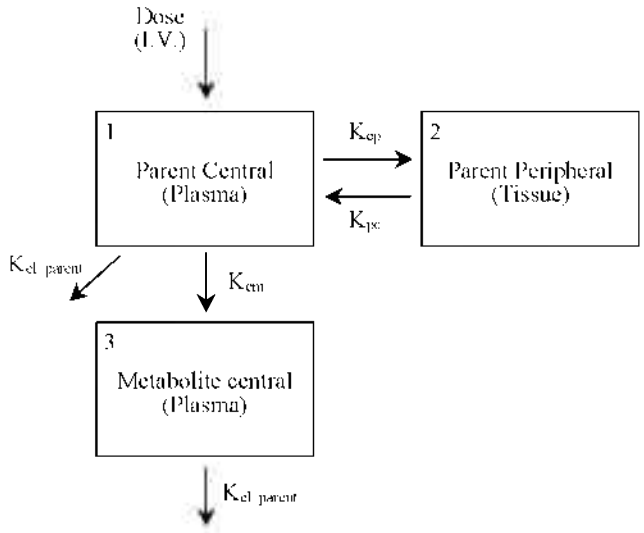

Figure 2. The parent-metabolite compartmental model used to describe the relationship between JHL45 and (+) decursinol is shown here.

Calibration curves. The calibration curve of JHL45 in rat plasma was linear from 0.01 to 10 μg/mL and was expressed by the equation $y = 0.000075x + 0.001919$ ($r^2 = 0.9996$), where y represents the peak area ratio of JHL45 to IS, and x is the analyte concentration in ng/mL. The lowest limit of quantitation (LLOQ) for JHL45 was 0.01 μg/mL. The calibration curve of (+)-decursinol was linear from 0.05 to 10 μg/mL and was expressed as $y = 0.000159x + 0.011569$ ($r^2 = 0.9996$), where x and y are analogous to those in the JHL45 calibration curve. The LLOQ for (−)-decursinol was 0.05 μg/mL. The developed analytical method of JHL45 includes protein precipitation of sample without extraction procedure, and the acetaminophen was proved to be an excellent internal standard for JHL45 assay validation.

Thus, this assay method was sufficiently sensitive, reliable, and accurate for the simultaneous determination of JHL45 and (+)-decursinol in rat plasma following the administration of a typical dosage of 5 mg/kg. In addition, the method required only 10 μL of rat plasma, which would accommodate the limited sample volume available from smaller animals such as mice.

Precision and accuracy. The intra- and inter-day accuracy and precision were validated for the simultaneous assay of JHL45 and (+)-decursinol and are presented in Tables 1 and 2, respectively. The accuracy (expressed as a percentage of nominal values) of JHL45 determination ranged from 90.06 to 106.87%, with coefficients of variation (CV%) of 0.74 - 10.51% and 3.80 - 11.85% for intra- and inter-day precision, respectively. The accuracy of (+)-decursinol determination ranged from 92.96 to 108.27%, and the intra- and inter-day

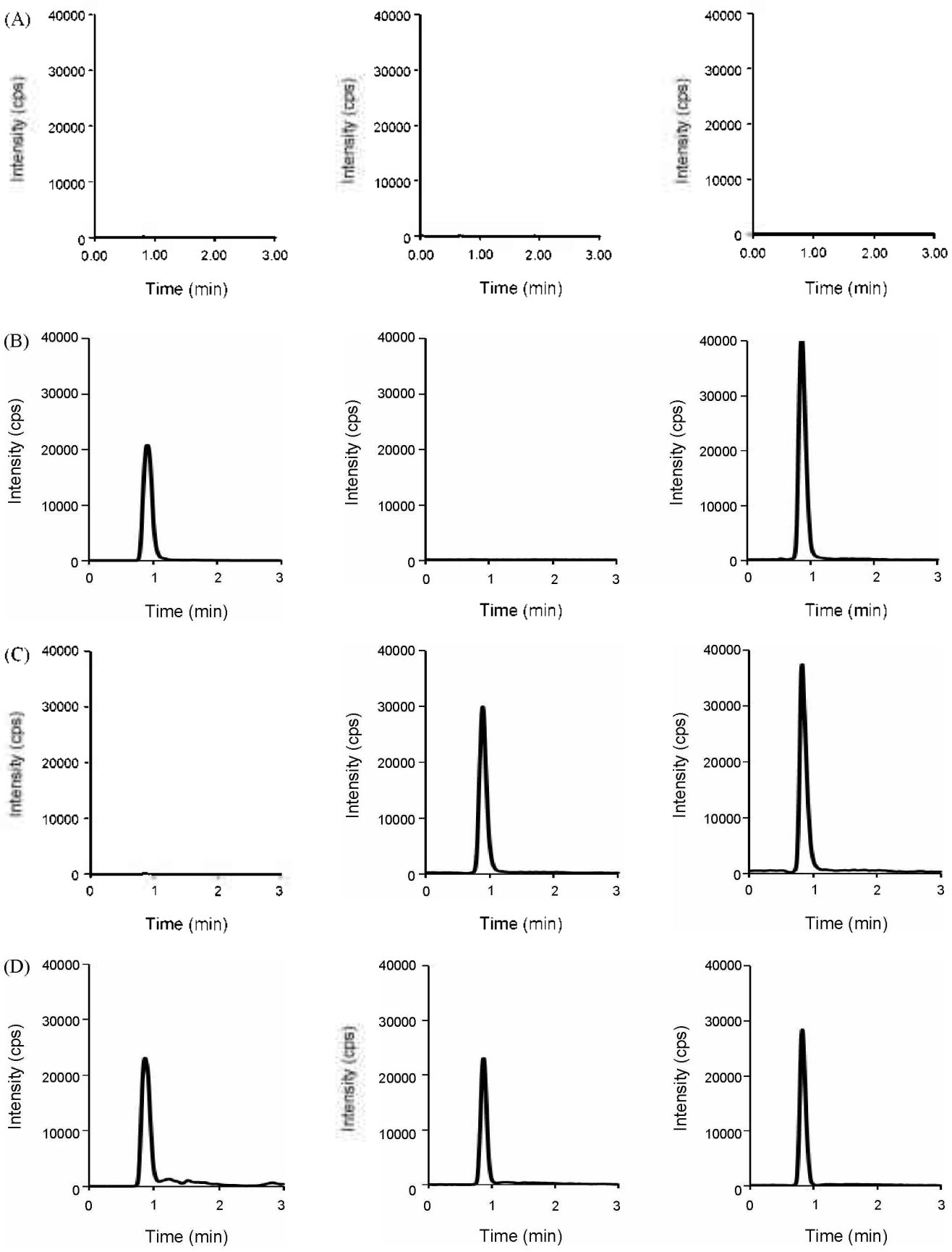

Figure 3. Chromatograms show the peaks obtained for JHL45 (left), (+)-decursinol (center), and acetaminophen (IS) (right) in (A) blank plasma, (B) plasma spiked with 1 μg/mL JHL45 with IS, (C) plasma spiked with 5 μg/mL (+)-decursinol with IS, and (D) rat plasma 5 minutes after IV administration of 5 mg/kg JHL45.

Table 1. Validation of the intra-day precision and accuracy of JHL45 and (+)-decursinol assays (n = 5)

Added (μg/mL)	Measured (μg/mL) (mean ± S.D.)	CV (%)	Accuracy (mean% ± S.D.)
JHL45			
0.01	0.0094 ± 0.00021	0.74	94.18 ± 2.51
0.05	0.052 ± 0.0032	4.02	106.87 ± 6.55
5	4.50 ± 0.48	10.51	90.06 ± 9.52
10	9.97 ± 0.72	7.98	99.74 ± 7.19
(+)-decursinol			
0.05	0.0049 ± 0.00022	10.95	98.45 ± 5.27
0.2	0.21 ± 0.014	8.214	104.14 ± 7.49
5	5.12 ± 0.28	5.47	102.40 ± 5.57
10	9.30 ± 0.30	3.24	92.96 ± 3.00

Table 2. Validation of the inter-day precision and accuracy of JHL45 and (+)-decursinol assays (n = 5)

Added (μg/mL)	Measured (μg/mL) (mean ± S.D.)	CV (%)	Accuracy (mean% ± S.D.)
JHL45			
0.01	0.0098 ± 0.0013	3.80	98.29 ± 13.45
0.05	49.86 ± 5.27	11.09	99.73 ± 10.55
5	4.84 ± 0.69	11.85	96.86 ± 13.81
10	10.04 ± 0.47	4.62	100.38 ± 4.65
(+)-decursinol			
0.05	0.058 ± 0.0042	13.16	108.27 ± 8.47
0.2	0.20 ± 0.0024	1.64	100.61 ± 1.44
5	5.04 ± 0.42	8.41	100.77 ± 8.43
10	10.53 ± 0.44	4.18	105.33 ± 4.39

precision ranged from 3.24 to 10.95% and 1.64 to 13.16%, respectively. These results demonstrated that the assay method was both reliable and reproducible.

Matrix effects and percentage recovery. When analyzing supernatants from protein-precipitated plasma samples, salt and endogenous materials can cause ion suppression or enhancement, which may result in greater variation than that in solid-phase extracts or liquid-liquid extracts.[14] Prudent assessment of these matrix effects constitutes an important and necessary part of validation for quantitative LC-MS/MS methods that support pharmacokinetic studies in biological matrices.[15]

Table 3. Matrix effects and percentage recovery of JHL45 and (+)-decursinol in rat plasma (n = 3)

Concentration (μg/mL)	Matrix effect (mean% ± S.D.)	Recovery (mean% ± S.D.)
JHL45		
0.01	87.63 ± 2.57	94.21 ± 1.01
1	96.27 ± 1.54	101.11 ± 1.02
10	94.63 ± 1.78	97.54 ± 0.84
(+)-decursinol		
0.05	91.21 ± 2.47	95.44 ± 1.04
2	89.63 ± 1.33	98.78 ± 0.27
10	97.64 ± 1.41	97.64 ± 1.27
IS		
1	96.31 ± 1.12	-
10	94.11 ± 1.40	-
100	97.66 ± 1.54	-

Matrix effects and percentage recoveries of JHL45 and (+)-decursinol are shown in Table 3. For all samples, including JHL45, (+)-decursinol, and IS, neither matrix effects nor the percentage loss exceeded ± 20%. Therefore, no significant matrix effects or interference from endogenous compounds were present in rat plasma.

Stability. A summary of the assay stability under various conditions is presented in Table 4. The mean integrated peak areas of the 0.2 and 1 μg/mL samples were compared before and after the stability testing procedures described in the experimental section. Stock solutions were stable for 30 days at 4 °C. JHL45 and (+)-decursinol were both stable in plasma after 6 h at room temperature, and no significant decrease of either compound was detected after three freeze-thaw cycles. Both JHL45 and (+)-decursinol were stable in methanol-extracted samples for 24 h at 4 °C, and both were stable in plasma for 30 days at −20 °C. Therefore, the short-term, long-term, freeze-thaw, and extraction stabilities were acceptable.

Table 4. Stability of JHL45 and (+)-decursinol

Stability experiment	Storage condition	Stability (%)	Stability (%)
JHL45		0.2 μg/mL	1 μg/mL
Short-term in plasma	Room temperature, for 6 h	96.05	95.67
Process (extracted sample)	4 °C, for 24 h	94.76	103.22
Freeze-thaw cycle in plasma	−80 °C, after third cycle	103.29	89.27
Long term in plasma	−20 °C, for 30 days	107.61	103.63
Stock solution	4 °C, for 30 days	101.13	97.64
(+)-decursinol		0.5 μg/mL	1 μg/mL
Short-term in plasma	Room temperature, for 6 h	94.75	99.15
Process(extracted sample)	4 °C, for 24 h	102.98	106.04
Freeze-thaw cycle in plasma	−80 °C, after third cycle	88.79	89.11
Long-term in plasma	−20 °C, for 30 days	107.61	103.64
Stock solution	4 °C, for 30 days	99.78	98.47

Table 5. Pharmacokinetic parameters of JHL45 in SD rats after IV administration of 5 mg/kg JHL45 (n = 5)

Model-independent analysis		
	JHL45	(+)-decursinol
AUC_{8h} (μg·h/mL)	10.02 ± 3.09	44.31 ± 17.20
AUC_{inf} (μg·h/mL)	10.43 ± 3.35	58.03 ± 23.41
C_{max} (μg/mL)	24.59 ± 7.23	12.00 ± 1.98
T_{max} (h)	0.10	0.55 ± 0.27
$t_{1/2}$ (h)	1.88 ± 0.63	3.02 ± 1.05
Model-dependent analysis		
K_{el_parent} (h^{-1})	1.67 ± 1.63	
K_{cp} (h^{-1})	2.73 ± 0.77	
K_{pc} (h^{-1})	0.36 ± 0.23	
K_{cm} (h^{-1})	0.22 ± 0.03	
$K_{el\_metabolite}$ (h^{-1})	0.76 ± 0.49	
V_{c_parent} (L)	253.83 ± 77.61	
$V_{c\_metabolite}$ (L)	29.81 ± 3.17	

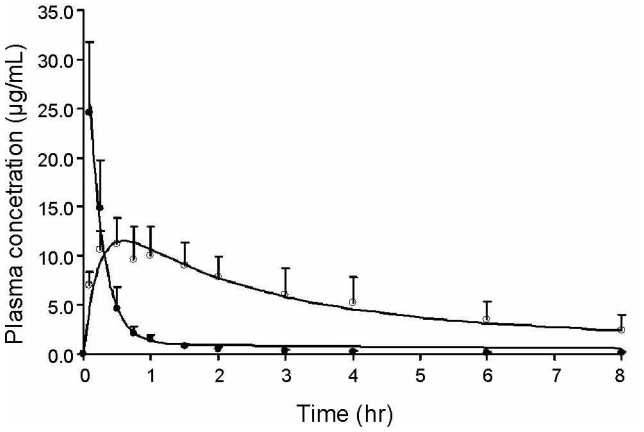

Figure 4. Observed plasma concentrations of JHL45 (●) and (+)-decursinol (○) are shown as a function of time following IV administration of 5 mg/kg JHL45 in rats. Each point represents the mean of five measurements. Error bars represent the standard deviation. The solid line is a fit generated from the parent-metabolite compartmental model.

Pharmacokinetic studies. The analytical procedures described above were employed to quantify all analytes in plasma samples obtained from five SD rats that had been administered a single IV dose of 5 mg/kg JHL45. The plasma concentrations of JHL45 and (+)-decursinol are presented as a function of time in Fig. 4; the solid lines represent the best fit of the parent-metabolite compartmental model. The model-independent and model-dependent PK parameters are shown in Table 5.

After dosing, the mean value of C_{max} was 24.59 ± 7.23 μg/mL at 0.10 h. The AUC_{8h} and AUC_{inf} of JHL45 were 10.02 ± 3.09 μg·h/mL and 10.43 ± 3.35 μg·h/mL, respectively. The half-life calculated from the terminal phase was 1.88 ± 0.63 h. The C_{max} of (+)-decursinol was 12.00 ± 1.98 μg/mL at 0.55 ± 0.27 h. The AUC_{8h} and AUC_{inf} were 44.31 ± 17.20 μg·h/mL and 58.03 ± 23.41 μg·h/mL, respectively. The half-life of (+)decursinol, determined from the terminal phase, was 3.02 ± 1.05 h.

The parent-metabolite compartment model was successful in performing model-dependent PK analyses of JHL45 and (+)-decursinol; the parameters for this model are shown in Table 5. K_{cm}, which was 0.22 ± 0.03 h^{-1} in this study, is an empirical parameter that describes the time delay between the presence of the parent compound and the appearance of the metabolite.

Conclusions

This paper describes the validation of a simple, rapid, and sensitive LC-MS/MS method for simultaneous quantification of JHL45 and its major metabolite, (+)-decursinol, in only 10 μL of rat plasma. A parent-metabolite compartment model was used to describe the pharmacokinetics of JHL45 and (+)-decursinol in rats. This analysis method and pharmacokinetic model will be useful for further pharmacokinetic studies of JHL45 during preclinical and clinical trials.

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