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Simplified LC-MS/MS method for quantification of IG-105, a novel tubulin ligand, and its application to the pharmacokinetic study in rats at the anti-cancer effective dose

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IG-105, N-(2, 6-dimethoxypyridine-3-yl)-9-methylcarbazole-3-sulfonamide, a novel carbazole sulfonamide, shows a potent anticancer activity in a variety of human tumor cells *in vitro* and *in vivo*. In the present study, a rapid and convenient liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was developed and applied to the pharmacokinetic study of IG-105 in rats. Chromatographic separation was accomplished on a C_{18} column using an isocratic mobile phase of acetonitrile-water-acetic acid (56:44:0.2, v/v/v). The ion transitions of IG-105 and combretastatin A4 (internal standard) in selected reaction monitoring mode were m/z 398→154 and m/z 317→286, respectively. The assay exhibited good linearity over the range of 2–512 ng/mL. Intra- and inter-day precisions were within 8.2 %, and the accuracies ranged from -6.0 to 3.7 %. The extraction recoveries were higher than 90 %, and the matrix effects were negligible. All quality control samples were stable at different storage conditions. The validated LC-MS/MS method was successfully applied to a preclinical pharmacokinetic study of IG-105 in rats after a single oral dose of 100, 250, or 1000 mg/kg which showed tumor growth inhibition activity. The absorption of IG-105 was proved to be rapid but saturated to a certain extent into the blood circulation, from where it was distributed and eliminated gradually.

1. Introduction

Microtubules, dynamic polymers of α- and β-tubulin heterodimers, are essential in all eukaryotic cells as a key component of the cytoskeleton. Microtubules readily polymerize and depolymerize in cells to undergo profound morphological modifications to perform multiple cellular functions, ensuring cell shape maintenance, mediating intracellular trafficking and contributing to cell motility during interphase, while reorganizing to form the mitotic spindle and orchestrating chromosome segregation during mitosis (Field et al. 2014; Pasquier et al. 2008). Suppression of microtubule dynamics will block mitosis and eventually lead to cell death by apoptosis (Bhalla 2003; Jordan 2002). The dynamic behavior is crucial to cell fate and enables microtubule a major target for the discovery and development of anticancer drugs (Jordan et al. 2004; Zhou et al. 2005). The representative two classes of drugs are Vinca alkaloids (such as vincristine) that inhibit microtubule assembly and taxol analogues (such as paclitaxel) that disrupt the microtubules disassembly (Jackson et al. 2007; Liu et al. 2014; Mollinedo et al. 2003). Compounds that target the microtubule have been hugely successful in the clinic as chemotherapeutics. However, there are some limitations in use with these agents. Drug resistance caused by multidrug efflux pumps, point mutations, and overexpression of tubulin subtypes, as well as side effects such as neurologic toxicity are serious issues (Canta et al. 2009; Dumontet et al. 1999). Besides, relatively complicated molecules of the Vinca alkaloids and taxol analogues isolated from plants make the de novo synthesis difficult.

IG-105 (Fig. 1), N-(2,6-dimethoxypyridine-3-yl)-9-methylcarbazole-3-sulfonamide, a novel carbazole sulfonamides agent, inhibits microtubule assembly by binding at colchicine pocket (Hu et al. 2007). It shows a potent anticancer activity in a variety of human tumor cells *in vitro* and *in vivo*. As a tubulin-active anticancer agent with novel chemical structure, IG-105 overcomes the major limitations of the existing tubulin-active drugs. Not being a P-glycoprotein substrate, IG-105 could avoid the induction of multidrug resistance; meanwhile it shows good safety (Wang et al. 2008).

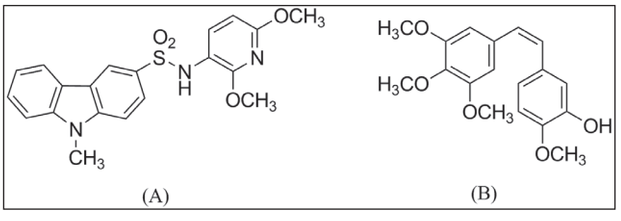

Fig 1: Chemical structures of (A) IG-105 and (B) CA4 (IS).

Since IG-105 shows a promising anti-cancer activity, a quantitative determination method for IG-105 to support the pharmacokinetic study is imperative. Li et al. (2011) developed an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analytical method for IG-105 (Li et al. 2011). However, lack of the UPLC-MS/MS equipment in general laboratory limited the wide application of the method. Besides, the liquid/liquid extraction method they employed is not an ideal method for sample processing because of the extremely high protein binding rate of IG-105 (98 % based on our research). Moreover, it is more important to identify the pharmacokinetic characteristics of IG-105

at the effective dosage for anti-cancer therapy. Wang et al. (2008) reported that IG-105 monotherapy yielded 81% inhibition of tumor growth for Bel-7402 hepatoma in nude mice at 100 mg/kg. Thus, the present work was aimed to develop and validate a convenient, rapid, and sensitive LC-MS/MS method for the determination of IG-105 in rat plasma, and apply it to the preclinical pharmacokinetic study at the effective dose of 100, 250, and 1000 mg/kg.

2. Investigations, results and discussion

2.1. Method development

The selection of mobile phase was based on the consideration of better peak shape and less residue. It is proved *via* the solubility test that acetonitrile exhibited better dissolving capacity for IG-105 than methanol, and more IG-105 will be dissolved at a lower pH environment. Therefore, the mobile phase comprises water, acetonitrile, and acetic acid, and the mixture of acetonitrile and acetic acid served as the needle washing solution to avoid residue.

Owing to the high protein binding rate of IG-105, the protein precipitation is an ideal choice for the sample preparation. By employing the multi-tube vortex, the sample pretreatment process was high throughput and consistent. Although this technique provided high and reproducible recoveries for both of IG-105 and IS, the presence of high matrix effect required further optimization. Different gradient and isocratic elution programs were attempted to minimize the ion suppression or enhancement caused by matrix effect. The isocratic elution of 0.2 % acetic acid in acetonitrile and water (56:44, v/v) was proved to be an ideal mobile phase to separate the IG-105 from the matrix. Under this separation condition, the overall analysis time for each injection lasted for only 4 min, which is as rapid as the UPLC-MS/MS method developed by Li et al. (2011).

2.2. Validation of the analytical method

A full validation including specificity, linearity, precision, accuracy, extraction recovery, matrix effect, and stability was conducted in rat plasma according to the US FDA guidelines. All of the parameters were proved to meet the criteria of bioanalytical method validation.

2.2.1. Selectivity

Representative chromatograms obtained from blank plasma, blank plasma spiked with IG-105 at Lower Limit of quantification (LLOQ) level, and samples collected post-dose are shown in Fig. 2. No significant interference of endogenous substances was observed at the retention time of IG-105 and internal standard (IS) in blank plasma samples in the selected reaction monitoring (SRM) scan mode. The chromatogram of real plasma samples showed similar chromatographic behavior to spiked plasma samples.

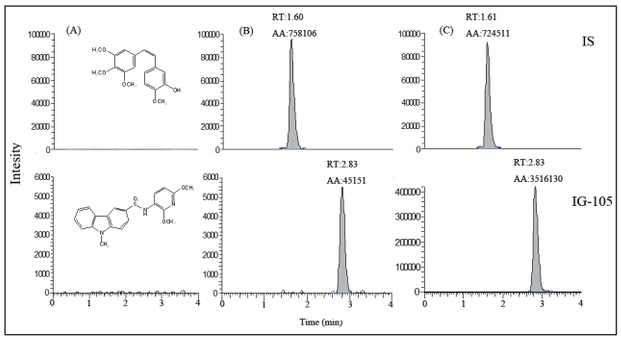

Fig. 2: Chromatograms of IG-105 and IS in SRM mode in plasma. (A) Blank rat plasma, (B) Blank plasma sample spiked with IG-105 at LLOQ, (C) Plasma sample collected 1.5h after a single 100 mg/kg oral dose of IG-105.

2.2.2. Linearity and sensitivity

The standard calibration curves constructed over the range of 2–512 ng/mL showed good linearity with a correlation coefficient greater than 0.99. A typical equation for the calibration curve was $y = 0.0319059\ x+0.00686265$ ($R^2 = 0.9997$, W: 1/x). With qualified accuracy (80–120%) and precision (<20%), the lowest concentration in the calibration curve of 2 ng/mL was defined as LLOQ.

2.2.3. Accuracy and precision

Intra- and inter-day precisions and accuracies at LLOQ and three quality control (QC) levels are summarized in Table 1. The intra- and inter-day accuracies were between -6.0 and 3.7 %, and precisions (RSD) were less than 8.2 % for all QC levels.

Table 1: Accuracy and precisions of IG-105 in rat plasma

Nominal concentration (ng/mL)	Intra-day accuracy (%, n=6)	Inter-day accuracy (%, n=18)	Intra-day precision (RSD%, n=6)	Inter-day precision (RSD%, n=18)
2	0.9	3.7	8.2	6.5
5	-6.0	-1.2	5.3	6.6
50	0.1	0.9	4.6	4.1
400	1.0	2.4	2.1	4.2

2.2.4. Extraction recovery and matrix effect

As presented in Table 2, the recoveries and matrix effects showed high efficiency and consistency among different concentrations and individuals. The extraction recoveries were between 95.4 and 108.6 % for IG-105 at all QC levels and for IS at the concentration used in the assay. The matrix effect was 100.3±4.8 % for IS, and ranged from 91.0 to 99.9 % for IG-105, suggesting the absence of ion-suppression or ion-enhancement of this method. Protein precipitation was proved to be a good way to extract IG-105 from rat plasma.

Table 2: The extraction recoveries and matrix effects quantitative values of IG-105 and IS in rat plasma

Compound	Nominal concentration (ng/mL)	Extraction recovery (%)		Matrix effect (%)	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)
IG-105	5	108.6	7.3	96.6	9.2
	50	96.2	4.6	99.9	9.0
	400	98.5	8.3	91.0	4.2
IS	125	95.4	8.4	100.3	4.8

2.2.5. Stability

For all of the stability investigation, mean concentrations were within ±15 % of the nominal values as summarized in Table 3. The results indicated that IG-105 was stable in untreated plasma after being placed at room temperature for 24 h, at -20 °C for 2 weeks, or after three freeze-thaw cycles. IG-105 was also stable after extraction when stored in autosampler at 4 °C for 48 h. Moreover, the respective stability tests confirmed the stability of IG-105 and the IS in stock solutions for 2 weeks at -20 °C (data not shown).

Table 3: Stability data of IG-105 in rat plasma

Storage condition	Mean of percentage remaining (%)		
	5 (ng/mL)	50 (ng/mL)	400 (ng/mL)
Bench-top	100.2	103.0	104.2
Long-term	97.3	102.5	95.8
Freeze-thaw	99.3	104.2	101.0
Autosampler	105.9	103.3	104.9

2.3. Pharmacokinetics of IG-105 in rats

In the present study, the validated HPLC-MS/MS method was successfully applied to the quantification of IG-105 in rat plasma after a single oral administration. Fig. 3 shows the mean plasma

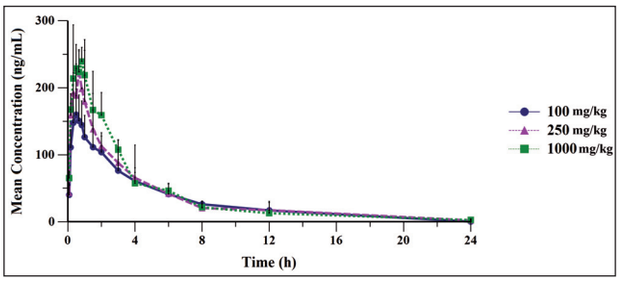

Fig. 3: Mean plasma concentration-time profiles of IG-105 following a single oral dose of 100, 250, and 1000 mg/kg in rats. Each point represents the mean±S.D. (n = 6).

concentration-time profiles of IG-105 in rats following a single oral dose of 100, 250, and 1000 mg/kg.

The pharmacokinetic parameters were calculated using a non-compartment model, and the major parameters are summarized in Table 4. The short time to maximum concentration (T_{max}) of 0.5± 0.2 h, 0.8±0.2 h, and 0.6±0.3 h for 100, 250, and 1000 mg/kg dosing groups, consistent with the reported data, revealed that IG-105 was absorbed rapidly. IG-105 then underwent gradual and extensive distribution with mild slops of the concentration-time curves and extraordinary large volume of distribution (*Vz/F*) of 654.7±106.1, 2072.0±902.1, and 7981.1±107.3 L/kg for 100, 250, and 1000 mg/kg, respectively. One potential explanation for the high *Vz* is the lipophilicity of IG-105 led to its intracellular accumulation. Incomplete absorption may be another factor, which can be proved by the nonlinear pharmacokinetic characteristics. When the doses were enhanced from 100 mg/kg to 250 and 1000 mg/kg, peak plasma levels (C_{max}) and areas under the plasma concentration-time curve (AUC) increased from 162.4±37.0 ng/mL to 241.8±12.5 and 270.8±27.5 ng/mL, and from 762.9±68.7 ng•h/mL to 853.9±296.8 and 922.7±28.2 ng•h/mL, respectively. Less than proportional increase of drug exposure to the administered doses suggested that the absorption is a saturable process. The disproportion of C_{max} (162.4±37.0 ng/mL vs 25.3±22.4 ng/mL) and AUC (762.9±68.7 ng•h/mL vs 63.0±31.3 ng•h/mL) between our results of 100 mg/kg and Li's study of 4 mg/kg dosage administration (Li et al. 2011) also proved that the absorption of IG-105 was saturated to some extent at 100 mg/kg. Prolonged half life (3.3±0.7, 4.6±1.6, and 5.2±1.5 h) and enlarged *Vz* (654.7±106.1, 2072.0±902.1, and 7981.1±107.3 L/kg) along with the increasing dosages demonstrated the nonlinear pharmacokinetics as well. We speculated the absorption saturation was induced by the poor water solubility of IG-105.

Table 4: Pharmacokinetic parameters of IG-105 after a single oral dose of 100, 250, and 1000 mg/kg in rats (n=6)

Pharmacokinetic parameters	Unit	Values		
		100 mg/kg	250 mg/kg	1000 mg/kg
C_{max}	ng/mL	162.4 ± 37.0	241.8±12.5	270.8±27.5
T_{max}	h	0.5± 0.2	0.8±0.2	0.6±0.3
AUC_{0-24}	ng·h/mL	762.9 ± 68.7	853.9±296.8	922.7±28.2
$AUC_{0-\infty}$	ng·h/mL	767.3 ± 69.3	865.6±308.3	945.4±90.1
$t_{1/2}$	h	3.3 ± 0.7	4.6±1.6	5.2±1.5
MRT	h	4.3 ± 1.5	4.3±1.0	4.9±0.8
Cl/F	L/h/kg	134.3 ± 13.2	312.6±101.5	1064.1±99.5
Vz/F	L/kg	654.7±106.1	2072.0±902.1	7981.1±107.3

C_{max}: the maximum concentration; T_{max}: the time to maximum concentration; AUC_{0-24}: area under the plasma concentration-time curve from time zero to 24 h; $AUC_{0-\infty}$: area under the plasma concentration-time curve from time zero to infinity; $t_{1/2}$: half life; MRT: mean residence time; *Cl*: Clearance; *Vz*: volume of distribution, *F*, bioavailability.

2.4. Conclusions

A rapid and convenient LC–MS/MS method for the determination of IG-105 in rat plasma has been developed and validated. The assay had a high recovery and proved to be highly sensitive with excellent reproducibility. Increased knowledge about the preclinical pharmacokinetic characters of IG-105 was gained based on our study. The parameters obtained at the anti-cancer effective dose will be meritorious for the further research, particularly for the dosage regimen design. However, IG-105 merits further pharmaceutical optimizing due to its limited absorption.

3. Experimental

3.1. Standards and chemicals

IG-105 was synthesized and provided by the Medicinal Chemistry Department of Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences according to the published procedure. The purity of IG-105 was 99.5 % as determined by HPLC with UV detection at 367 nm. (Z)-2-Methoxy-5- (3,4,5-trimethoxystyryl) phenol (combretastatin A4, CA4, Fig. 1, purity >98%), which served as IS, was purchased from J&K Chemical LTD (Shanghai, China). Acetonitrile of HPLC-grade was obtained from Fisher Scientific (New Jersey, USA). Ultrapure water was obtained from Millipore system (Bedford, MA, USA). Other chemicals were of analytical grade.

3.2. Instrumentation and analytical conditions

The Thermo Fisher (San Jose, USA) LC system consisted of a Finnigan™ Surveyor® MS pump and a Finnigan™ Surveyor® autosampler. The HPLC was coupled with a Finnigan™ TSQ Quantum Discovery mass detector equipped with an electrospray ionization (ESI) source. System control and data processing were accomplished by Xcalibur software (Version 2.0.7 SP 1, Thermo Fisher, San Jose, USA).

Chromatographic separation was achieved on an Agilent Porashell 120® EC-C18 column (50 mm×2.1 mm, 2.7 µm) at column temperature of 35 °C. The isocratic mobile phase consisting of a mixture of acetonitrile, water, and acetic acid (56:44:0.2, v/v/v) was delivered at a flow rate of 0.2 mL/min. The samples were kept at 4 °C in the autosampler and the injection volume was 5 µL. The overall run time was 4 min, but the eluate was only introduced into the MS from 1 min to 4 min by using a divert valve. A needle washing and flushing program with acetonitrile containing 1% acetic acid was performed after each injection to avoid potential carryover.

Mass spectrometric analysis was acquired in positive ion mode with nitrogen as sheath and auxiliary gas and argon as the collision gas. Quantification was performed in the SRM mode with the transitions of m/z 398.0→154.0 for IG-105 and m/z 317→286 for CA4 with optimal collision energies of 22 eV and 21 eV, respectively. The main MS parameters were set as follows: spray voltage: 3800 V; sheath gas pressure: 30 Arbitrary units (Arb); auxiliary gas pressure: 10 Arb; capillary temperature: 350 °C.

3.3. Preparation of calibration standards and QC standards

Stock solutions of IG-105 and CA4 were prepared by dissolving the accurately weighed pure powder in acetonitrile to obtain the final concentration of 10 mg/mL and stored at –20 °C within 2 weeks until use. The working solutions for calibration standards and quality controls were prepared by serial diluting the stock solution with acetonitrile (to diminish the amount of organic solvent introduced to the plasma, 10-fold dilution with acetonitrile-water (20:80, v/v) was applied at the last dilution step for IG-105) to appropriate concentrations (40, 80, 160, 320, 640, 1280, 2560, 5120, and 10240 ng/mL for the calibration standards; 40, 100, 1000, and 8000 ng/mL for quality controls; and 125 ng/mL for IS). All the working solutions were stored at -20 °C for no more than two weeks.

IG-105 calibration and QC standards were prepared by spiking 5 µL of working solutions to blank rat plasma (95 µL). Nine calibration standards in the range of 2-512 ng/mL, together with LLOQ (2 ng/mL), low QC (5 ng/mL), medium QC (50 ng/mL), and high QC (400 ng/mL) were prepared from corresponding working solutions. The analytical standards and QC samples were freshly prepared each day.

3.4. Sample processing

Aliquots of frozen rat plasma samples (100 µL) were thawed at room temperature and thoroughly vortexed before analysis. Protein precipitation was employed to the sample pretreatment. Plasma (100 µL) was mixed with 100 µL IS working solution and 400 µL acetonitrile, vortexed for 1 min and centrifuged at 15000 rpm for 15 min at 4 °C. 100 µL aliquot of the supernatant and 100 µL acetonitrile-water (1:1, v/v) were transferred to a clean autosampler insert after vortex-mixing and directly injected into the LC-MS/MS system for quantitative analysis.

3.5. Method validation

The assay was fully validated for selectivity, linearity, accuracy, precision, recovery, matrix effect, and stability according to the US Food and Drug Administration (FDA) guidance for the validation of bioanalytical methods (2013).

Selectivity of the assay was assessed by analyzing the chromatograms of blank plasma from six individual rats to investigate the interference from endogenous material. Any endogenous interference at the retention time of IG-105 should be <20% of the LLOQ. Moreover, no significant interfering peaks should be present at the retention time of IS.

The calibration curve was acquired by plotting the peak area ratio of IG-105 to IS against the corresponding IG-105 nominal concentration by linear least-squares regression with a weighting index of 1/x. The deviation of 15 % for the back calculated concentrations from the nominal concentrations were considered acceptable with an

exception of ±20% for the LLOQ. The sensitivity of the method was expressed by LLOQ, which was defined as the lowest concentration of the calibration curve with qualified accuracy (within ±20 %) and precision (less than 20 %).

Determination of intra-day and inter-day accuracy and precision were carried out via the analysis of six replicates of LLOQ and QC standards within the same day or on three different days. The accuracy was assessed by relative error (RE, %) = [(mean measured concentration – nominal concentration)/nominal concentration] × 100, and the precision was expressed in terms of relative standard deviation (RSD, %) = (SD/mean) × 100. The accuracies should be within ±15 % at all QC levels except LLOQ, for which the acceptance limit is ±20 %, and the precisions should not exceed 15% with the exceptive criteria of 20 % for LLOQ.

Blank rat plasma from six different sources was used to evaluate the extraction recoveries and matrix effects of IG-105 at three QC levels (5, 50, and 400 ng/mL) and CA4 at the working concentration. Extraction recoveries were determined by comparing the peak areas of IG-105 or CA4 from extracted QC samples with those from post-extracted spiked samples at the corresponding concentration. Matrix effects were assessed by comparing the IG-105 or IS signal responses in post-extracted spiked samples with those in neat solution. A recovery of ≥70% and a matrix effect between 80% and 120% with a variation below 15% are considered ideal.

QC samples at three concentrations in six replicates subjected to the storage conditions below were evaluated for the stability of IG-105 in rat plasma. QC samples stored at ambient temperature for 24 h and at -20 °C for 2 weeks were determined for the short-term (bench-top) and long-term stability, respectively. Freeze-thaw stability was assessed after three freeze-thaw cycles. Autosampler stability was evaluated by analyzing post-preparative QC samples placed in autosampler at 4 °C for 48 h. The stock solution stability of IG-105 and IS after stored at -20 °C for 2 weeks was also evaluated by comparing with the freshly prepared stock solutions. For all of the stability investigation, the calculated percentage remaining should fall into the range of 85 to 115% to meet the acceptance criteria.

3.6. Application to pharmacokinetic study of IG-105 in rats

Eighteen Sprague-Dawley rats (three males and three females per group, weighing 200±10 g) were purchased from Chinese Academy of Military Medical Sciences (Beijing, China). All animals were housed in an environmentally controlled room with temperature of 22±2 °C, humidity of 55±6 % and dark-light cycle of 12/12 h. Rats were acclimatized for one week with free access to standard laboratory food and water, but the diet was withdrawn overnight before experiment. All animal experiments were carried out in accordance with the National Institues of Health guide for care and use of Laboratory animals, and were performed under the permission and supervision of the Ethics Committee of Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, China.

200, 500, or 2000 mg of IG-105 was moistened with 1 mL Tween 80 and prepared as a suspension by adding 1% (w/v) sodium carboxymethyl cellulose (CMC-Na) solutions to 20 mL. The freshly prepared suspension was administered to rats (1 mL/100 g) *via* gastric gavage.

250 μL blood samples were collected from the vena orbitalis into heparinized tubes at 0, 0.083, 0.167, 0.33, 0.5, 0.67, 0.83, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h post dosing. The samples were immediately centrifuged at 6000 rpm for 10 min at 4 °C. The separated plasma was stored at -20 °C until analysis.

Pharmacokinetic parameters of IG-105 were calculated by WinNonlin® (Version 6.1, Pharsight Corp., Mountain View, CA, USA) using non-compartmental model.

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