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Preclinical pharmacokinetics, tissue distribution and excretion studies of a novel *anti-candidal* agent-thiosemicarbazide derivative of isoniazid (TSC-INH) by validated UPLC–MS/MS assay

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ABSTRACT

A simple and sensitive UPLC–MS/MS assay was developed and validated for rapid determination of thiosemicarbazide derivative of isoniazid (TSC-INH), a potent *anti-candidal* agent in rat plasma, tissues, urine and feces. All biological samples were prepared by protein precipitation method using celecoxib as an internal standard (IS). Chromatographic separation was achieved on Acquity BEH^{TM} C_{18} (50 × 2.1 mm, 1.7 μm) column using gradient mobile phase of acetonitrile and water (containing 0.1% formic acid) at flow rate of 0.3 mL/min. The MRM transitions were monitored at *m/z* 305.00 → 135.89 for TSC-INH and *m/z* 380.08 → 316.03 for IS in ESI negative mode. All validation parameter results were within the acceptable range described in guideline for bioanalytical method validation. The pharmacokinetic study showed that the compound TSC-INH was orally active with 66% absolute bioavailability in rats. It was rapidly absorbed with peak plasma concentration of 1985.92 ng/mL achieved within 1 h after single oral dose (10 mg/kg) administration. TSC-INH exhibited rapid distribution across the body with highest levels in liver and lungs. Penetration in brain tissues suggests that TSC-INH crossed the blood brain barrier. Only 5.23% of the orally administered drug was excreted as unconverted form in urine and feces implying that TSC-INH was metabolized extensively before excretion. With the preliminary knowledge of *in vivo* pharmacokinetics and disposition properties, this study will be beneficial for further development of compound TSC-INH in future studies.

1. Introduction

Candida is the most common fungal pathogen of humans, causing wide ranges of invasive to superficial infections known as candidiasis [1]. Over the past few decades, the incidence of candidiasis has been increasing worldwide due to increased number of immunocompromised patients and also the use of broad-spectrum antibiotics [2,3] . Invasive candidiasis is a life threatening opportunistic infection, which is associated with important cause of morbidity and mortality in immunocompromised as well as in patient hospitalized with serious underlying diseases [4]. The majority of candidiasis causing (>50%) species is *Candida albicans*. Though few classes of antifungal drugs (polyenes, azoles, echinocaniasis, allylamines and DNA analogues) are available for candidiasis treatment, but their uses are restricted due to their toxicity, side effects, drug interactions, route of administration limitation and emergence of drug resistant [5,6].

Based on docking and molecular modeling studies, the enzyme *N*- myristoyltranferase (NMT) was proposed as a novel target for the antifungal activity of 4-arylthiosemicarbazides. Therefore, in our previous study, various hydrazinecarbothioamide derivatives were synthesized and their *in vitro anti-candidal* activity have been evaluated and compared with itraconazole (as reference drug) [7]. Among the tested compounds, compound [*N*-(4-chlorophenyl)-2-(pyridin-4-ylcarbonyl)] hydrazinecarbothioamide, a thiosemicarbazide derivative of isoniazid (TSC-INH) was found to be the most potent derivative against all strains of *candida spp.* and was especially more effective against *C. albicans*

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ATCC66027 and *Candida* spp. (blood) 12,810 [MIC = 0.09 μg/mL] compared to itraconazole. Moreover, TSC-INH showed lowest cytotoxicity against three cancer cell lines and non-cancer cell lines compared to itraconazole. Further, the solubility study confirmed that TSC-INH is freely soluble in PEG 400; soluble in isopropyl alcohol, ethylene glycol, propylene glycol; slightly soluble in ethanol and insoluble in water; and results of dissolution thermodynamics indicated endothermic and non- spontaneous dissolution of TSC-INH [8,9]. Thus TSC-INH represents a good lead for the development of newer, potent and broad spectrum *anti-candidal* agents. Preclinical pharmacokinetic parameters e.g., drug exposure, distribution profile, clearance (CL), half-life ($T½$), bioavailability, and initial dosing information are critical in initial drug development especially for systemic infection because they provide useful initial *in vivo* information on drug passage and disposition specially. Of particular significance are data associated with drug exposure, [10]. In this study a sensitive and accurate UPLC–MS/MS assay was developed and validated for the determination of TSC-INH in rat plasma, urine, feces and tissue homogenates. The developed assay was successfully applied in pharmacokinetics, tissue distribution and excretion studies of TSC-INH in rats.

2. Experimental

2.1. Chemical and reagents

TSC-INH, [(*N*-(4-chlorophenyl)-2-(pyridin-4-ylcarbonyl) hydrazinecarbothioamide)] was synthesized, purified (purity >99%) and characterized in pharmaceutical chemistry laboratory at College of Pharmacy, King Saud University, KSA. The molecular structure of TSC-INH is presented in Fig. 1' [molecular formula, $C_{13}H_{11}ClN_4OS$; molecular mass, 306.77]. Celecoxib (purity >98%) was used as internal standard (IS) and was obtained from Ranbaxy Research Laboratory, Gurgaon, India. Methanol, acetonitrile and dimethyl sulphoxide (DMSO) of HPLC grade were obtained from VWR International Ltd., Leicester, UK. Formic acid (analytical grade) was obtained from BDH Laboratory, Poole, England whereas PEG-400 [IUPAC name, poly-(oxyethene)] was obtained from Fluka Chemicals (Buchs, Switzerland).

2.2. Preparation of stock, standard and quality control samples

Two stock solutions of TSC- INH (500 μg/mL) were prepared in DMSO from separate weightings. One solution was used for the calibration standards (CS) while other for the quality controls (QC) samples. Stock solution of IS (400 μg/mL) was prepared in methanol. Stock solutions of CS and QC were further diluted with acetonitrile: water (50:50, v/v) to prepare working solution for CS and QC samples, respectively. The IS working solution (10 μg/mL) for routine use was prepared by diluting the stock solution in acetonitrile: water (50:50, v/v). All stock solutions and working solutions were stored at 4 °C and brought to room temperature before use. The final CS was prepared by spiking the appropriate amount of the working solution into 200 μL of blank biological matrices to get the effective concentration of 3.97–5000 ng/mL in plasma and 11.34–5000 ng/mL in urine, feces and tissue homogenates, respectively. Similar procedure was followed to prepare QCs samples by spiking the working QCs into blank biological substrates to achieve three different concentrations (low middle and high QC) of 12, 400, 4000 ng/mL in plasma and 35, 400, 4000 ng/mL in urine, feces and tissue homogenates.

2.3. Instrumentation and analytical conditions

The quantification of TSC-INH was performed on UPLC–MS/MS system consisting of Water Acquity UPLC connected with triple-quadrupole tandem mass spectrometer (Waters Corp., Milford, MA, USA) having a electrospray ionization (ESI) interface. The chromatographic separation was achieved on Acquity UPLC BEH™ C_{18} column (50 × 2.1 mm, 1.7 μm) maintained at 40 °C temperature at a flow rate of 0.3 mL/min. A gradient mobile phase consisting of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) was eluted as follow: 0–1 min, linear change from A to B (20:80, v/v) to A–B (80:20, v/v); 1–2 min, linear change from A to B (80:20, v/v) to A–B (50:50,v/v); 2–4 min, linear change from A to B (50:50, v/v) to A–B (20:80,v/v) and maintained to equilibrium till 5 min prior to next sample injection. The injection volume was 5 μL in partial loop mode and the temperature of the autosampler was set at 12 °C. Quantitative analysis was performed using multiple reaction monitoring (MRM) transitions of *m/z* 305.00 → 135.89 for TSC-INH and *m/z* 380.08 → 316.03 for IS, both in ESI negative mode having dwell time set at 0.106 s. The desolvating gas (nitrogen) was set at 600 L/h flow rate maintaining desolvating line and source temperature at 350 °C and 150 °C, respectively. The capillary voltage was set at 3.80 kV and argon was used as collision gas at 0.1 mL/min flow rate. The optimized compound specific parameters: cone voltage and collision energy were set at 26 V & 14 eV for TSC-INH and 54 V & 22 eV for IS, respectively. The system control and data analysis were carried out using MassLynx software (Version 4.1) and processed using TargetLynx™ program.

2.4. Sample preparation

In this study, a simple protein precipitation method was applied to extract the analyte from biological samples (plasma, urine, feces suspension and tissue homogenates). An aliquot of 200 μL plasma (blanks, CS, QC and unknown) samples were placed in 1.5 mL Eppendorf tubes. Then 20 μL of IS (10 μg/mL) was added into each tube except blank. The samples were vortex- mixed for 30 sec and were deproteinized by adding 400 μL of acetonitrile and again gently vortex-mixed for one min. The samples were cold centrifuged at 12,000 rpm for 8 min maintained at 4 ± 1 °C temperature. After centrifugation, supernatant was transferred to a fresh vial and 5 μL was injected into chromatographic system for analysis.

Same procedure was followed in other biological samples (urine, feces and tissue homogenates) except samples were 2 times diluted by deionized water and vortex-mixed before injection into chromatographic system for analysis.

2.5. Assay validation

Assay validation was performed in all biological samples (plasma, urine, feces suspension and tissue homogenates) according to US Food and Drug Administration (USFDA) and European Medical Agency (EMA) guidelines of bioanalytical method validation [11,12].

Assay selectivity was evaluated to rule out the interference from endogenous matrix compounds with analyte and/or IS. Six different blank plasma samples were spiked with TSC-INH (LLOQ level) and IS (1 μg/mL) and were processed by the proposed extraction method. The chromatograms of blank samples were compared and analyzed with chromatograms of spiked plasma samples. The response of interfering peak in blank plasma were limited to ≤20% of the response of analytes and ≤5% of the IS. The interference in other biological samples (urine, feces and tissue) were also evaluated by same procedure.

Blank plasma, tissue homogenate, urine and feces suspension was used to prepare the corresponding calibration curves to avoid different matrix effects. The calibration curves were prepared by plotting the ratio of analyte response over IS response versus the nominal concentration of analyte ranged from 3.97 to 5000 ng/mL in blank plasma and 11.34–5000 ng/mL in urine, feces suspension

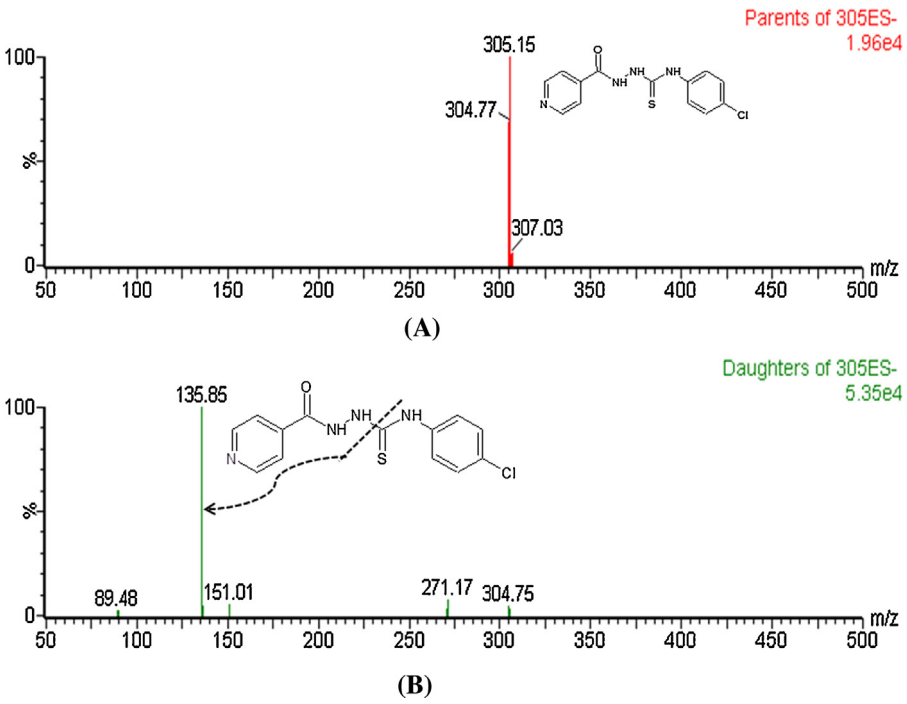


Fig. 1. Chemical structure, (A) parent ion $[M–H]^-$ and (B) product ion $[M–H]^-$ spectra of TSC-INH.

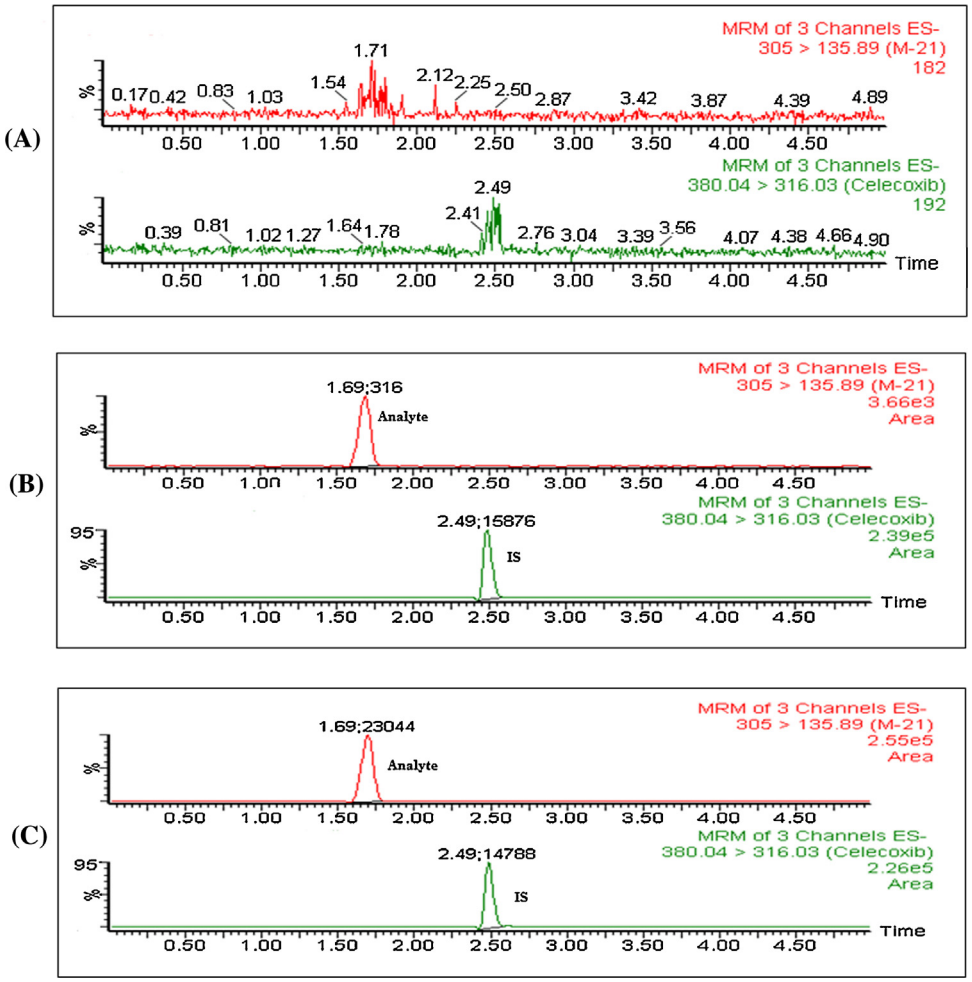


Fig. 2. Representative chromatograms of TSC-INH and IS in (A) blank plasma, (B) plasma spiked with TSC-INH and IS at LLOQ level and (C) 1 h after oral administration of TSC-INH (10 mg/kg).

Table 1
Intra- and inter-day precision and accuracy of TSC-INH in rat plasma, tissue homogenates, urine and feces ($n = 6$).

Sample matrix	Concentrations (ng/mL)	Intra-day		Inter-day	
		Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
Plasma	12	4.49	99.4	3.60	96.6
	400	9.91	92.9	9.71	99.2
	4000	8.64	97.8	8.12	101.0
Liver	35	10.54	103.5	7.40	104.5
	400	8.16	107.9	5.87	105.6
	4000	2.05	99.1	3.53	100.6
Heart	35	11.94	106.3	9.29	105.9
	400	3.93	102.9	6.02	102.2
	4000	8.08	95.3	6.61	94.6
Lungs	35	5.72	104.9	6.33	102.7
	400	10.51	94.6	10.33	95.3
	4000	7.49	91.6	7.56	91.7
Kidney	35	10.91	105.8	7.80	107.8
	400	4.99	103.8	8.07	100.3
	4000	3.38	101.0	4.22	99.9
Spleen	35	8.04	102.8	8.02	105.0
	400	5.56	103.4	6.99	103.2
	4000	9.61	96.4	8.03	95.7
Brain	35	8.34	107.7	6.43	109.6
	400	7.49	105.0	7.09	103.5
	4000	6.68	104.1	5.60	104.1
Urine	35	3.70	102.1	5.11	105.4
	400	8.50	92.5	8.99	96.5
	4000	9.96	99.1	8.67	101.2
Feces	35	3.66	108.6	4.46	106.8
	400	8.95	98.7	8.00	99.0
	4000	8.68	99.8	7.19	98.0

and tissue homogenates. Assay linearity was determined using least squares linear regression method. The correlation coefficient (r^2) was desired to be $\geq$0.995 for all calibration curves. Weighing factors: none, 1/X and $1/X^2$ were used to calculate the deviation in nominal values at each concentration level of the standard curve as a function of weighing factor optimization. The LLOQ was defined as the lowest concentration on the calibration curve which can be quantified reliably with acceptable accuracy (±20%), precision (RSD,%; $\leq$20) and having signal intensity of at least 5 times the signal of a blank sample.

The accuracy and precision were determined in all matrices of interests at their three different QC concentrations (low middle and high level) in six replicates using different analytical batches on the same day and during three consecutive days. The acceptance criteria for intra and inter-day precision (RSD, %) was limited to $\leq$15% and accuracy was required to be limited within ±15%.

Extraction recovery and matrix effect were evaluated in all matrices of interest at their three QC levels. The recovery was calculated by comparing the response of analyte in extracted samples with those spiked with analyte after the extraction. The matrix effect was evaluated by post-extraction spike method and was calculated by comparing the response of the analyte spiked after post extraction to the response of analyte spiked in standard solution. The matrix effects and recovery of IS was also evaluated by same procedure.

The stability of TSC-INH in all biological matrices of interest was evaluated using six replicates of two QC (low and high levels) samples against freshly spiked calibration curves. Short-term stability was assessed by analyzing the extracted plasma samples after spiking 6 h at room temperature. Freeze/thaw stability was tested by analyzing the extracted plasma samples after three times repetition of freeze thaw cycle. Sample injector stability was determined by analyzing the processed samples after storing for 12 h at 12 °C which cover the anticipated time for analysis. Long-term stability was evaluated by analyzing the spiked plasma samples after storing at −80 °C for 60 days. Analyte was deemed to stable if the deviation of mean calculated precision value (RSD, %) were $\leq$15% and accuracy within ±15% compared to nominal concentrations.

2.6. Animal experimentation

Male Wistar albino rats weighing 200 ± 20 g were obtained from the Experimental Animal Care Center, College of Pharmacy at King Saud University. Animals were housed under ideal laboratory conditions (temperature 23–25 °C, 12 h light/12 h darkness cycle, 45–55% relative humidity and maintained on standard pellet diet and water ad libitum throughout the experimental period. All the animals were kept on overnight fasting before the administration of TSC-INH.

2.7. Pharmacokinetic studies

Pharmacokinetic studies were performed in twelve rats randomly divided into two groups ($n = 6$). The 10 mg/ml solution of TSC-INH was prepared by dispersing an appropriate amount of it in PEG 400 which was further diluted with normal saline to achieve 1 mg/mL solution. Group first received a single intravenous (i.v.) administration of TSC-INH at 2 mg/kg via tail vein and blood samples were collected at 0, 0.05, 0.5, 1, 2, 3, 4 and 8 h. Group second received a single oral administration of TSC-INH at 10 mg/kg and blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h. Plasma samples were harvested by centrifuging the blood at 4500 rpm for 8 min at 4 °C temperature and stored frozen at −80 ± 10 °C until analysis.

Table 2
Matrix effects and recovery of TSC-INH in rat plasma, tissue homogenates, urine and feces ($n = 6$).

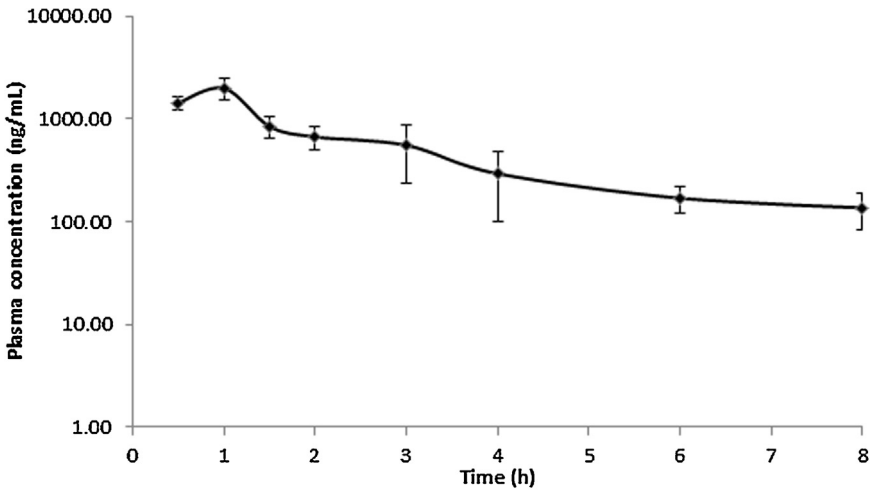
Sample matrix	Concentrations (ng/mL)	Recovery		Matrix effects	
		Mean (%)	RSD	Mean (%)	RSD
Plasma	12	80.5	9.5	90.2	4.7
	400	81.2	4.3	93.8	1.7
	4000	78.4	7.9	91.9	4.0
Liver	35	85.3	6.0	92.7	3.4
	400	78.5	7.8	94.0	3.0
	4000	83.2	8.1	95.7	2.4
Heart	35	87.2	5.9	91.8	4.6
	400	83.2	13.1	95.5	3.3
	4000	87.0	12.6	96.6	3.9
Lungs	35	83.2	7.2	94.0	4.5
	400	85.6	8.3	95.2	2.0
	4000	76.5	10.6	92.8	5.6
Kidney	35	78.2	5.3	95.7	4.2
	400	77.0	8.1	98.8	3.7
	4000	80.7	7.7	99.7	5.4
Spleen	35	74.0	7.1	87.6	5.0
	400	76.3	7.7	93.9	2.7
	4000	78.3	6.8	95.3	3.1
Brain	35	83.5	6.0	89.8	4.7
	400	86.9	6.3	92.6	4.0
	4000	84.3	8.3	89.0	7.1
Urine	35	86.2	11.3	90.6	4.6
	400	84.2	7.5	93.6	1.8
	4000	85.7	9.7	94.2	2.0
Feces	35	80.9	6.0	93.7	2.4
	400	78.5	7.8	92.3	4.0
	4000	82.4	6.5	94.9	2.3

2.8. Tissue distribution study

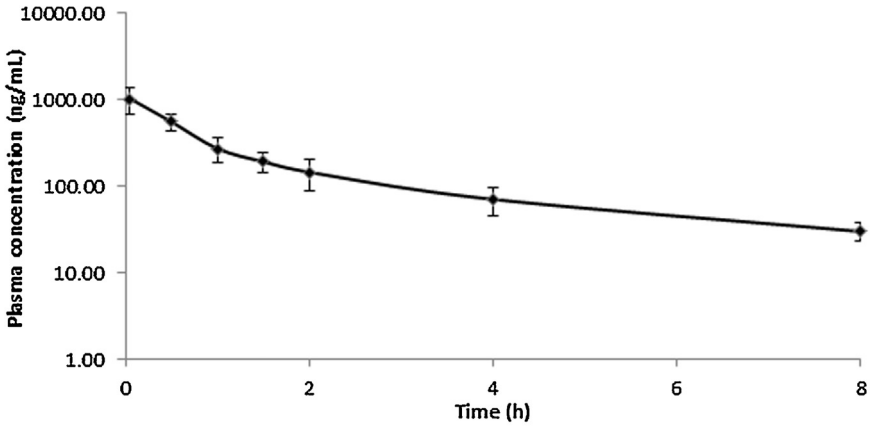
Tissue distribution study was performed in twenty four rats randomly divided in to four group ($n = 6$). All rats were orally received a single dose of 10 mg/kg TSC-INH. Tissues (heart, liver, spleen, lung, kidney and brain) were promptly harvested at 1, 2, 4 and 6 h (six rats/time point) and thoroughly rinsed in ice cold saline to eliminate blood and other content and blotted dry with filter paper. An accurately weighted amount of each tissue samples were homogenized in acetonitrile-water (50:50, v/v) solution (3 times the tissue weight, w/v) and stored at $-80 \pm 10\,^{\circ}C$ until analysis.

Table 3
Stability data of TSC-INH in rat plasma, tissue homogenates, urine and feces ($n = 6$).

Sample matrix	Nominal concen. (ng/mL)	Short-term (6 h)		Freeze/thaw (3 cycles)		Sample injector 12 h (12 °C)		Long-term 60 days	
		Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
Plasma	12	7.05	102.7	10.35	98.7	8.87	107.1	6.68	94.5
	4000	5.30	107.2	2.56	103.4	2.96	110.5	9.40	99.3
Liver	35	2.99	103.0	5.36	100.7	8.10	106.4	5.35	98.0
	4000	7.15	104.1	4.56	99.5	9.87	104.1	5.66	96.0
Heart	35	10.70	105.9	4.13	99.4	3.60	105.2	4.90	95.7
	4000	4.94	103.6	5.64	98.0	4.47	105.5	9.46	91.9
Lungs	35	6.75	97.7	7.25	105.7	7.17	107.9	5.40	96.9
	4000	7.49	100.7	8.54	99.3	8.54	103.4	7.43	93.0
Kidney	35	6.11	100.9	4.15	97.1	5.12	109.5	5.89	95.2
	4000	7.86	101.2	6.15	95.9	4.60	103.2	8.20	92.4
Spleen	35	7.55	96.1	8.92	98.0	7.80	100.1	8.27	99.6
	4000	5.17	102.0	6.65	100.7	6.02	104.0	4.27	98.2
Brain	35	6.06	97.5	7.50	105.1	10.92	100.4	6.34	103.7
	4000	8.04	101.6	6.57	99.9	4.79	105.8	8.53	95.4
Urine	35	4.52	95.7	4.38	98.0	7.50	109.5	4.22	92.3
	4000	8.97	102.4	8.19	101.6	5.74	107.1	6.78	99.9
Feces	35	9.09	106.3	6.77	103.5	9.25	106.9	5.34	102.5
	4000	4.64	105.3	2.23	101.2	4.82	108.5	6.40	97.1



(A)



(B)

Fig. 3. Log scale plasma concentration-time profiles (mean ± SD) of TSC-INH in rats (A) after single dose oral administration of 10 mg/kg and (B) i.v. administration of 2 mg/kg ($n = 6$).

2.9. Excretion study

Excretion study was performed in six rats which were housed in separate metabolic cages after receiving a single oral dose of TSC-INH at 10 mg/kg. The urine samples were collected at 0–3, 3–6, 6–12 and 12–24 h post dosing whereas feces samples were collected at 0–6, 6–12 and 12–24 h. All animals were freely taken both food and water during housed in metabolic cages. Feces samples were weighted and homogenized in acetonitrile–water (50:50, v/v) in ratio of 1:3 (w/v) to prepare the suspension and the volume of urine samples were measured before storing at −80 ± 10 °C.

2.10. Data analysis

The plasma, tissue homogenates, urine and faces samples were subjected to UPLC–MS/MS analysis and the concentration of TSC-INH were calculated according to their respective calibration curves. The pharmacokinetic parameters including the area under

Table 4
Pharmacokinetic parameters of TSC-INH (Mean ± SD) after a single dose administration in rats ($n = 6$).

Pharmacokinetic parameters	Unit	Oral (10 mg/kg)	i.v. (2 mg/kg)
C_{max}	ng/mL	1985.92 ± 457.56	1032.81 ± 364.38
T_{max}	h	1	0.05
AUC_{0-8}	ng.h/mL	4095.27 ± 910.49	1179.35 ± 209.88
AUC_{0-inf}	ng.h/ mL	4438.40 ± 1042.09	1343.44 ± 232.35
$T^{1/2}$	h	2.20 ± 0.29	2.87 ± 0.74
MRT	h	2.96 ± 0.47	2.53 ± 0.15
Kel	h^{-1}	0.31 ± 0.04	0.25 ± 0.06
Vz	L/kg	–	6.33 ± 2.00
Vd	L/kg	–	2.21 ± 0.88
CL	L/h/kg	–	1.49 ± 0.20
F	%	66	–

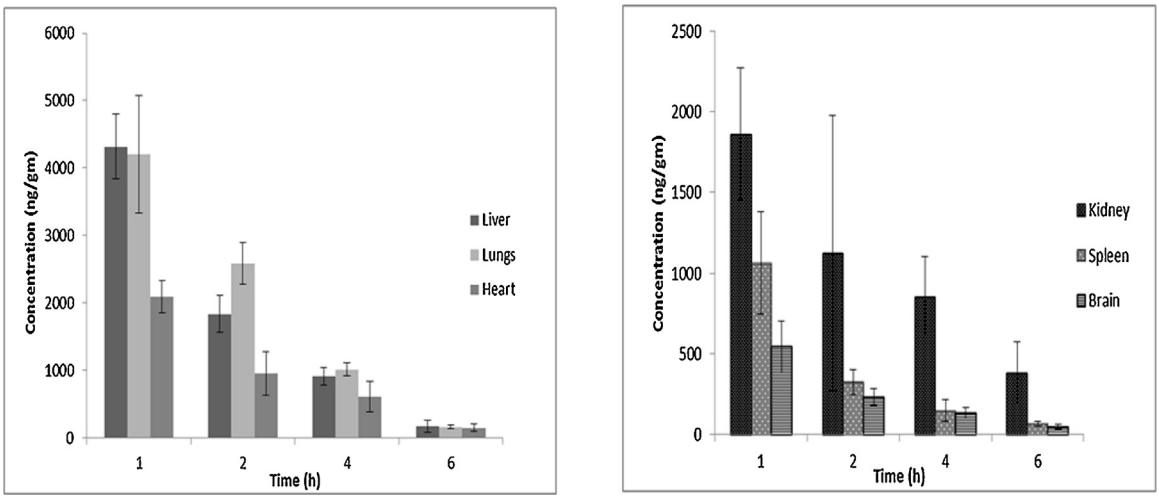


Fig. 4. Concentration (mean ± SD) levels of TSC-INH in rat tissues at 1, 2, 4 and 6 h, after single dose (10 mg/kg) administration ($n = 6$).

curve (AUC_{0-8}, AUC_{0-inf}), *T½*, initial volume of distribution (Vd), volume of distribution at terminal phase (Vz) and CL were calculated using WinNonlin software (Pharsight Co., Mountain View, CA, USA), with a non-compartment method. The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were calculated from the experimental data. The absolute oral bioavailability (F), of TSC-INH was calculated according to following equation:

$$F(\%) = (AUC_{oral} \times dose_{i.v.})/(AUC_{i.v.} \times dose_{oral}) \times 100$$

where AUC_{oral} and $AUC_{i.v}$ are the AUC values after oral and intravenous administration of TSC-INH, respectively, and $dose_{i.v.}$ and $dose_{oral}$ are the doses administered for intravenous and oral administration of TSC- INH, respectively. Data were presented as means with their standard deviation (mean ± SD).

3. Results and discussion

3.1. Optimization of mass spectrometer and chromatographic conditions

In order to achieve high sensitivity with stable fragmentation of TSC-INH, the MS/MS tuning was tested by selecting both positive and negative ESI mode. TSC-INH produced both protonated ions $[M+H]^+$ (*m/z* 307) and deprotonated (*m/z* 305) molecular ions $[M-H]^-$ in their positive and negative-ion mode, respectively. Subsequently, the protonated ions (*m/z* 307) produced two fragment ions at *m/z* 137.98 and 120.99 and deprotonated ions (*m/z* 307) at *m/z* 135.90 and 270.95. However, the fragmentation pattern of deprotonated (*m/z* 305) molecular ions was more stable with lower background noise and hence ESI in negative mode was selected for the detection of TSC-INH. The fragment ions at *m/z* 135.90 were more abundant and selected for MRM transition. Celecoxib was selected as IS, because it is more sensitive in negative mode and was compatible with TSC-INH during chromatographic separation. So the MRM transitions of *m/z* 305.00 → 135.89 for TSC-INH and *m/z* 380.08 → 316.03 for IS, both in ESI negative mode were selected

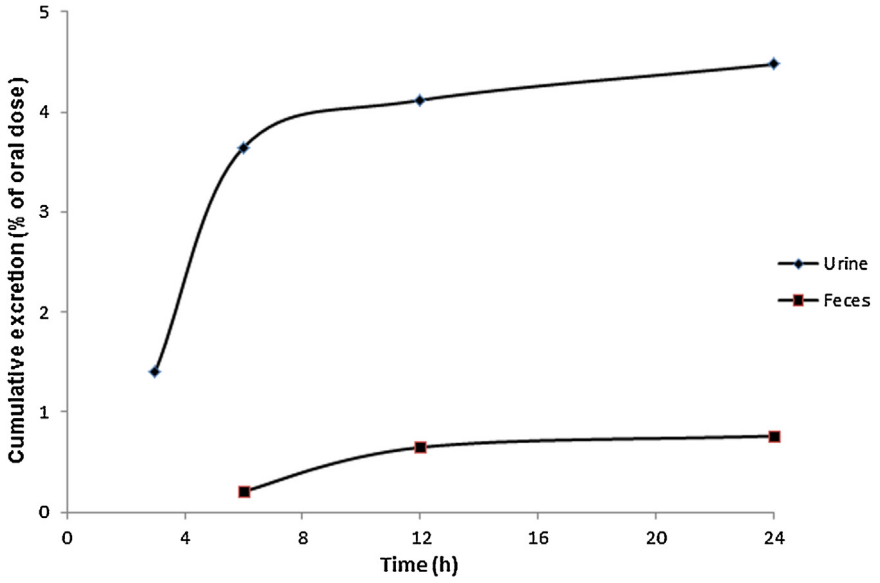


Fig. 5. Urinary and fecal cumulative excretion profile of TSC-INH in rats after single oral dose of 10 mg/kg ($n = 6$).

Table 5
Excretion profile of TSC-INH in urine and feces after oral administration of 10 mg/kg. ($n = 6$).

Matrix	Time (h)	Amount excreted (ng) Mean ± SD	Cumulative excretion (% of total amount excreted)	Cumulative excretion (% of dose)
Urine	3	14066.59 ± 4385.24	31.43	1.40
	6	2205.31 ± 3966.15	81.49	3.64
	12	4695.11 ± 1453.43	91.98	4.11
	24	3586.33 ± 815.13	100	4.47
Feces	6	2089.73 ± 270.38	27.38	0.20
	12	4395.85 ± 576.01	84.98	0.64
	24	1145.88 ± 150.15	100	0.76

for detection and quantification. The parent ion $[M–H]^-$ (A) and its product ion $[M–H]^-$ (B) spectra of TSC-INH are shown in Fig. 1.

The chromatographic condition of developed assay was also optimized for separation of analyte and IS with high resolution, to improve peak shape and to increase the signal response. Acetonitrile was preferred over methanol as organic modifiers because it produced lower background noise and good separation. Electrolyte additives such as formic acid, acetic acid and ammonium acetate were tested to achieve the maximum sensitivity. Formic acid was selected and was used in both acetonitrile and water in 0.1% composition. Gradient elution was chosen for better separation and lower interference from endogenous substances of matrices.

3.2. Method validation

3.2.1. Specificity

Fig. 2 shows the representative chromatograms of blank plasma, plasma spiked with TSC-INH at LLOQ level and 1 h after oral administration of TSC-INH (10 mg/kg). Under the optimized condition, no significant interfering peaks were observed in chromatograms of all tested matrices at the retention time of analyte and IS. All the peaks of analyte and IS were detected with high resolution and excellent peak shape. TSC-INH and IS were eluted at 1.69 and 2.49 min, respectively with total run time of 5 min.

3.2.2. Assay linearity and sensitivity

The calibration curves showed excellent linearity with correlation coefficients (r^2) ≥ 0.996 in all matrices. The weighing factor $1/X^2$ was selected for back calculation of nominal value because it produced best linear fit with minimum bias. Developed assay offered LLOQ of 3.97 ng/mL in plasma; 11.34 ng/mL in urine, feces and tissues and were quantified with acceptable accuracy and precision (≤20%). In addition, the signal intensity of the LLOQ sample was □5 times compared with the blank plasma signal. The sensitivity in urine, feces and tissues were lower than plasma because their samples were diluted with deionized water before analysis. However it was sufficient for tissue distribution and excretion studies after oral administration of TSC-INH.

3.2.3. Precision and accuracy

The intra-day and inter-day precision and accuracy data of TSC-INH in biological matrices are presented in Table 1. The intra-day and inter-day precision (RSD, %) values were ≤11.94% and ≤10.33%, respectively while the intra-day and inter-day accuracy (%) were in the range of 91.6–108.6% and 91.6–109.6%, respectively. All these results were within the criteria set by regulatory guidelines and so the assay was suitable for accurate quantification of TSC-INH in rat biological samples.

3.2.4. Recovery and matrix effects

The extraction recovery and matrix effects for TSC-INH (at three different QCs levels) in biological matrices are shown in Table 2. The % recovery of TSC-INH in all biological matrices were consistent, reproducible and concentration independent. The matrix effects for TSC-INH in all biological matrices were within ±15% with %RSD of ≤7.2% which was considered as negligible or insignificant matrix effects.

3.2.5. Stability

The stability of TSC-INH in different conditions (short-term, freeze/thaw, sample injector and long term stability) were within the acceptable levels i.e precision values (RSD, %) were ≤15% and accuracies were within ±15% range (Table 3). The results confirmed that the TSC-INH is stable under all anticipated sampling and storage condition. The stock solutions and working standard of TSC-INH and IS were also stable at least for 15 days at refrigerator temperature (below 10 °C) and for 12 h at room temperature.

3.3. Plasma pharmacokinetic study

The mean plasma concentration time profiles and summary of the pharmacokinetic parameters of TSC-INH after oral (10 mg/kg) and i.v. (2 mg/kg) administration are presented in Fig. 3 and Table 4, respectively. The C_{max} of 1985.92 ng/mL was achieved after 1 h of oral (10 mg/kg) administration of TSC-INH in rats, indicating that it is orally active and could be quickly absorbed into blood circulatory system after administration. The $AUC_{0–8}$ and $AUC_{0–inf}$ was found to be 4095.27 and 4438.40 ng.h/mL, respectively after oral administration, whereas, it was 1179.35 and 1343.44, respectively, after i.v. (2 mg/kg) administration. The percentage $AUC_{0–8}/AUC_{0–inf}$ value for both oral and i.v. administration was found to be >80%, suggesting that the samples collection duration was appropriate, covering >80% of the complete drug profile. The oral plasma concentrations profiles showed a biexponential decay (with a rapid decline) followed by a slow decay with a $T½$ of 2.20 h. After i.v. administration, the calculated Vd and Vz were 2.21 and 6.33 L/kg, respectively whereas CL was 1.49 L/h/kg. The absolute oral bioavailability was 66% which may be due to its adequate absorption (freely soluble in PEG 400) and high lipophilicity (c Log $P = 2.36$) [7].

3.4. Tissue distribution study

The distribution of TSC-INH in the various rat tissues, namely heart, lungs, liver, kidney, spleen and brain is presented in Fig. 4. The results demonstrated that TSC-INH underwent rapid and wide distribution to tissues within the time course examined. The highest concentration level was achieved at 1 h in all tissues which is equivalent to T_{max} of TSC-INH in plasma after oral administration in rats. The results showed that TSC-INH was well distributed into all abundant blood supply tissues and blood perfusion rate is the key factor affecting the distribution of TSC-INH in rats. The highest tissues concentrations were found in liver and lungs, followed by heart, kidney and lowest levels in spleen and brain. Detection of TSC-INH in brain suggests that it was able to cross the blood brain barrier (BBB). TSC-INH was more lipophilic with low molecular weight of 306 Da, which might be resulted to its penetration in brain tissues (the upper limit of molecule weight for efficient permeability through the BBB is about 300–400 Da) [13].

3.5. Excretion study

The excretion results of TSC-INH in urine and feces of rats after oral administration of 10 mg/kg are presented in Fig. 5. The amount of TSC-INH excreted, cumulative excretion (% of total amount excreted) and cumulative excretion (% of dose) are presented in Table 5. The results of TSC- INH excretion data indicate that merely 5.23% (4.47% and 0.76% for urine and feces, respectively) of TSC-INH excreted was unconverted after oral administration, which indicate that it mainly eliminated through metabolite (s) formation. The recovery from feces was less than 1%, which implied that TSC-INH may be adequately absorbed in rats. Therefore further excretion study including bile excretion with exploration of metabolite characterization is required to fully ascertain TSC-INH excretion profile.

4. Conclusion

In this study, a simple and sensitive UPLC–MS/MS assay was developed and validated for the determination of TSC-INH in rat biological matrices. The developed assay was successfully applied in the pharmacokinetics, tissue distribution and excretion studies of TSC-INH in rats. TSC-INH was characterised as orally active compound with 66% bioavailability. It was well distributed in all tissues (liver, kidney, lungs, heart, spleen and brain). Distribution in brain tissues suggested that TSC- INH cross the BBB and will play important role in the treatment of central nervous system infection. Only 5.23% of the orally administered TSC-INH was excreted as unconverted form in urine and feces implying that it was metabolized extensively before excretion. With the preliminary knowledge of *in vivo* pharmacokinetics, tissues distribution and excretions profiles, this study will provide helpful information for further development of compound TSC-INH in future studies.

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