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Development and validation of UHPLC-MS/MS assay for rapid determination of a carvone Schiff base of isoniazid (CSB-INH) in rat plasma: application to pharmacokinetic study

Muzaffar Iqbal[a,b]*, Mashooq A. Bhat[a] and Faiyaz Shakeel[c]

ABSTRACT: In this study, a fast UHPLC-MS/MS method was developed and validated for the determination of a novel potent carvone Schiff base of isoniazid (CSB-INH) in rat plasma using carbamazepine as an internal standard (IS). After a single-step protein precipitation by acetonitrile, CSB-INH and IS were separated on an Acquity BEH^{TM} C_{18} column (50 × 2.1 mm, 1.7 μm) under an isocratic mobile phase, consisting of acetonitrile: 10 mM ammonium acetate (95:5, v/v), at a flow rate of 0.3 mL/min. Quantification was performed on a triple quadrupole tandem mass spectrometer in multiple reactions monitoring mode by using positive electrospray ionization source. The precursor to product ion transitions were set at *m/z* 270.08 → 79.93 for CSB-INH and *m/z* 237.00 → 178.97 for IS. The proposed method was validated in compliance with US Food and Drug Administration and European Medicines Agency guidelines for bioanalytical method validation. The method was found to be linear in the range of 0.35–2500 ng/mL ($r^2 \geq 0.997$) with a lower limit of quantification of 0.35 ng/mL. The intra- and inter-day precision values were ≤12.0% whereas accuracy values ranged from 92.3 to 108.7%. In addition, other validation results were within the acceptance criteria and the method was successfully applied in a pharmacokinetic study of CSB-INH in rats. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: CSB-INH; UHPLC-MS/MS; pharmacokinetics; rat plasma

Introduction

Tuberculosis (TB) is a serious infectious bacterial disease caused by *Mycobacterium tuberculosis* (Kumar *et al*., 2014). Multidrug-resistant tuberculosis is the major issue with currently available antitubercular drugs, which limits their use in TB. Therefore, there is urgent need for the development of new potent and effective antitubercular agents. Among the available treatment option, isoniazid (INH) is still maintaining its first-line importance in the treatment of TB (Singh *et al*., 2011; Bhat and Al-Omar, 2013). Recently, Bhat and Al-Omar (2013), synthesized, characterized and evaluated the anti-*M. tuberculosis* activity of various terpenes Schiff bases of INH. Among the investigated Schiff bases, the carvone Schiff base of INH (CSB-INH) was found to be very potent and effective against TB. Compared with INH, CSB-INH was more lipophilic and more potent than isoniazid under the investigated conditions (Bhat and Al-Omar, 2013). Considering CSB-INH as a new potential antitubercular agent, a selective and sensitive bioanalytical assay was required to assess the pre-clinical pharmacokinetic studies. Among the available analytical methods, ultra-high-performance liquid chromatography tandem mass spectroscopy (UHPLC-MS/MS) has gained considerable attention and is used as the pre-eminent analytical tool for bio- and pharmaceutical analysis, as both chromatographic separation and assay performance are significantly improved using this technology (Iqbal *et al*., 2013; Kumar *et al*., 2012; Li *et al*., 2008). Additionally, the use of an Aquity BEH (Ethylene Bridged Hybrid) column (Waters Corp., Milford, MA, USA) in UHPLC increases the separation throughput and efficiency as well as reducing the retention time and volume of solvent required in chromatographic separation (Nováková *et al*., 2006; Liu *et al*., 2009). The aim of this study was to develop a selective and sensitive UHPLC-MS/MS assay that facilitates the rapid determination of CSB-INH in rat plasma and can be applied for pharmacokinetic studies in rats.

Materials and methods

Chemicals and reagents

CSB-INH (purity ≥99%; melting point 140–142 °C) was synthesized and purified by Pharmaceutical Chemistry Laboratory, College of Pharmacy, King Saud University, and further characterized by IR,

* Correspondence to: M. Iqbal, Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia. Email: muziqbal@gmail.com

[a] Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia

[b] Bioavailability Laboratory, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

[c] Center of Excellence in Biotechnology Research (CEBR), King Saud University, PO Box 2460, Riyadh 11451, Saudi Arabia

Abbreviations used: CS, calibration standard; CSB-INH, carvone Schiff base of isoniazid; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; MRM, multiple reaction monitoring; TB, tuberculosis.

NMR and LC-MS/MS (Fig. 1A). Carbamazepine (purity >98%), which was used as internal standard (IS), was obtained from Tabuk Pharmaceutical, Tabuk, Saudi Arabia (Fig. 1B). HPLC-grade methanol, dimethyl sulfoxide (DMSO) and acetonitrile were obtained from VWR International Ltd (Poole, UK), and ammonium acetate of analytical grade was obtained from Qualikems Fine Chem. Pvt. Ltd (Vadodara India). All aqueous solutions used in this study were obtained from Milli-QR Gradient A10R (Millipore, Moscheim, Cedex, France) having a pore size of 0.22 μm. Blank rat plasma was obtained from drug-free healthy rats that were received from the Animal Care and Use Centre, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Preparation of stock solutions, calibration standards and quality control solutions

Stock solutions of CSB-INH (0.5 mg/mL) in DMSO and IS (0.4 mg/mL) in methanol were prepared by dissolving the accurately weighed substance in a 10 mL volumetric flask. Two stock solutions for CSB-INH were prepared from two separate weighings. One solution was used for calibration curve samples while the other was used for quality control samples (QCs). Standard working solutions of CSB-INH and IS (4 μg/mL) were prepared by further dilution of the stock solution using 50% acetonitrile (acetonitrile–water, 50:50). All stock solutions and working solutions were stored in refrigerator at 4°C. Ten levels of calibration standard (CS) were prepared by spiking the appropriate amounts of the working standard solutions (20 μL) into 200 μL blank plasma to obtain effective concentration levels in range of 0.35–2500 ng/mL. A similar procedure was used to prepare QC samples at four different concentrations of 0.42, 1.20, 100 and 2000 ng/mL in blank plasma and was treated as lower limit of quantification for QC (LOQ QC), low QC (LQC), medium QC (MQC) and high QC (HQC), respectively. Both spiked CS and QC samples were prepared during validation and/or samples analysis.

Chromatographic separation and MS/MS conditions

The experiments were conducted on an Acquity^{tm} UHPLC system coupled to a triple-quadrupole tandem mass spectrometer (Waters Corp., Milford, MA, USA). The UHPLC system, comprising a quaternary solvent manager, a binary pump, a degasser, an autosampler with an injection loop of 10 μL and a column heater–cooler, was used in this study. The chromatographic separation was achieved on an Acquity BEH^{TM} C_{18} column (50 × 2.1 mm, 1.7 μm) maintained at 40 °C. An isocratic flow, comprising acetonitrile–10 mM ammonium acetate (95:5, v/v) at a flow rate of 0.3 mL/min, was used as mobile phase for chromatographic separation. The injection volume was 5 μL in partial loop mode and the temperature of the autosampler was set at 10 °C.

Both CSB-INH and IS were detected by triple-quadrupole tandem mass spectrometer in electrospray ionization (ESI) mode.

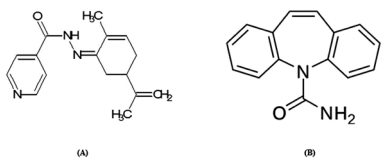

Figure 1. Chemical structure of carvone Schiff base of isoniazid (CSB-INH) (A) and carbamazepine (B).

Quantification was performed using multiple reaction monitoring (MRM) transitions of *m/z* 270.08 → 79.93 for CSB-INH and *m/z* 237.00 → 178.97 for IS, in ESI-positive mode having a dwell time of 0.106 s. Nitrogen was used as a desolvating gas at a flow rate of 600 L/h. The desolvation line temperature was 350 °C, whereas source temperature was set at 150 °C. The collision gas (argon) flow was 0.1 mL/min and capillary voltage was set at 3.5 kV. The compound specific parameters, cone voltage and collision energy, were set at 38 V and 22 eV for CSB-INH and 32 V and 34 eV for IS, respectively. The MassLynx software (version 4.1, software change note no. 714) was used to control the UHPLC-MS/MS system and data was collected and processed using TargetLynx^{TM} program.

Sample preparation

In a fresh 1.5 mL centrifuge tube, a 200 μL aliquot of plasma samples (blanks, CSs, QCs and unknown samples) was transferred and 20 μL of IS (4 μg/mL) was added in each tube except for the blank. The samples were vortex mixed for 30 s and were deproteinized by addition of 380 μL of acetonitrile and gently vortexed for 1 min. The samples were centrifuged for 10 min at 10,500 ***g*** at 8 °C. After centrifugation, supernatant was transferred to a fresh UHPLC vial, and 5 μL was injected into UHPLC–MS/MS for analysis.

Method validation

The developed method was validated in rat plasma by following guidelines for bioanalytical method validation set by the US Food and Drug Administration (2001) and the European Medicines Agency (2011). Evaluated validation parameters included selectivity, linearity, accuracy, precision, recovery, matrix effects, carryover effects and stability of analyte in both short-term sample processing and long-term storage.

Selectivity. Method selectivity was investigated by analyzing six individual blank plasma samples obtained from six different rats. Blank plasma and plasma sample spiked at lower limit of quantification (LLOQ) level (0.35 ng/mL) were processed by the proposed extraction protocol. The selectivity of the method was evaluated by comparing the MRM chromatograms of blank plasma with the corresponding spiked plasma at LLOQ level. The response of interferences in blank plasma were limited to ≤20% of the response of analytes and ≤5% of the IS (European Medicines Agency, 2011).

Linearity, calibration curve and lower limit of quantification. Assay linearity was evaluated in rat plasma by preparing three different calibration standards in concentrations ranging between 0.35 and 2500 ng/mL. The calibration curves were constructed by plotting analyte/IS peak area ratios as a function of the corresponding nominal concentration and analyzed by least squares linear regression method. The determination coefficient $R^2 > 0.995$ was desirable for all the calibration curves. For weighting factor optimization, three weighting 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. The weighting factor with the least deviation was selected for the validation procedure.

The lowest concentration of analyte on the calibration curve was recognized as the LLOQ, which could be quantified reliably, with an acceptable accuracy and precision (≤20%). In addition,

the signal intensity of the LLOQ sample should be at least five times the blank plasma signal.

Precision and accuracy. The precision was expressed as percentage coefficient of variation (CV), whereas accuracy was expressed as a percentage deviation from the nominal value. The intra-day precision and accuracy were determined at four different QC concentrations (LOQ QC, LQC, MQC and HQC) in replicate ($n = 6$) on the same day, whereas inter-day precision and accuracy were determined at four different QC concentrations in replicate ($n = 18$) over three consecutive days. The deviation in mean value of precision (CV) was limited to $<15\%$ (20% for the LOQ QC samples) and accuracy was limited to be within ±15% (20% for the LOQ QC samples).

Recovery and matrix effect. Both recovery and matrix effect for CSB-INH were determined in rat plasma at LQC, MQC and HQC levels. The blank plasma obtained from six different rats was used for this study. The recovery for CSB-INH was determined by comparing peak response ratio of plasma spiked with analyte prior to extraction (pre-extraction-spiked analyte) with those spiked with analyte after the extraction (post-extraction-spiked analyte).

The matrix effect was evaluated by post-extraction spike method. The matrix effects were determined by dividing the response of post-extraction-spiked analyte samples by that of the analyte in aqueous solution at the same concentration. The same procedure was followed to assess recovery and matrix effect for IS (400 ng/mL). The IS normalized matrix factor was also calculated by dividing the matrix factor of CSB-INH by the matrix factor of IS. The inter-subject variability of matrix effect at every concentration levels was limited to $<15\%$ as recommended in the bioanalytical guidelines of the European Medicines Agency (2011).

Carryover effects. Owing to wide dynamic range of calibration curve (0.35–2500), it was important to eliminate any possible carryover effects with respect to the autosampler, column and tubing. Carryover effects were investigated by injecting double blank plasma extracts after injection of an upper limit of quantification (ULOQ) sample in triplicate. The percentage peak area in the double blank plasma was compared with peak area of LLOQ. Carryover in the blank plasma sample was limited to not more than 20% of the LLOQ and not more than 5% of the IS as recommended the guidelines of the European Medicines Agency (2013).

Stability. The stability of CSB-INH in rat plasma was evaluated using six replicates of LQC and HQC under different anticipated conditions. Short-term stability was assessed by processing and analyzing QC plasma samples after 8 h storage at room temperature. Freeze–thaw stability was determined after three freeze–thaw cycles by freezing the QC samples at −80 °C and thawing at 25 °C. Post-preparative stability was determined by storage of the reconstituted QC samples for 48 h in the autosampler at 10 °C before analysis. Long-term stability was evaluated by analyzing the QC plasma samples which were stored at −80 °C for 30 days. The stock solutions and working solutions of CSB-INH and the IS were also evaluated for their stability at room temperature for 12 h and at refrigerator temperature for 15 days. Analytes were considered stable if the deviation of mean calculated precision value (CV) was ≤15% and accuracy within ±15% compared with nominal concentrations.

Application to pharmacokinetic study in rats

The developed method was applied to a comparative pharmacokinetic study of CSB-INH in rats. Twelve male Wistar albino rats weighing 210 ± 15 g (mean ± SD) were randomly divided into two groups (six in each), which served as control and test groups. In a single-dose parallel group study, the control group received CSB-INH suspension (5 mg/kg, p.o), whereas the test group received CSB-INH nanoemulsion (5 mg/kg, p.o). CSB-INH suspension was prepared by dispersing an appropriate amount of CSB-INH in required quantity of water in order to obtain 0.5% w/v suspension. Blood samples (approximately 0.75 µL) were withdrawn into heparinized microfuge tubes (lithium heparin, Improvacuter®) at predose and at 0.5, 1, 2, 3, 4, 6, 8 and 24 h after administration of CSB-INH (5 mg/kg, oral). Plasma samples were harvested by centrifuging the blood at 4500 ***g*** for 8 min at 4 °C temperature and stored frozen at −80 ± 10 °C until analysis.

Results and discussion

Optimization of mass spectroscopy condition

UHPLC-MS/MS operation parameters were optimized to obtain the maximum sensitivity and selectivity for the analyte and IS. Initially the standard solution (400 ng/mL) of both CSB-INH and IS were infused into the mass spectrometer for tuning using ESI as the ionization source in both positive and negative mode. The results showed that the signal intensities of both CSB-INH and IS were much higher in positive ionization mode than in negative ionization mode. Two precursor to product ion transitions at *m/z* 270.08 → 79.93 and *m/z* 270.08 → 120.95 were produced for CSB-INH. The precursor to product ion transitions at *m/z* 270.08 → 79.93 produced maximum signal intensity and was selected as quantifier for CSB-INH determination. Source-dependent parameters such as desolvation temperature, ESI source temperature, desolvation gas and cone gas flow rate were optimized to obtain the optimum intensity of protonated molecular ions $(M+H)^+$ for CSB-INH and IS. The compound-specific parameters, that is, capillary and cone voltage, were also optimized to produce the strongest parent ion and daughter ion signals, and were set at 38 V and 22 eV for CSB-INH and 32 V and 34 eV for IS. The parent ion and daughter ion spectra of CSB-INH are shown in Fig. 2.

Optimization of chromatographic conditions

A robust and high-performance assay is expected to have good peak shape, short and highly reproducible retention time, and minimum interference with high signal response. The chromatographic conditions, especially the composition of mobile phase, were optimized carefully to achieve good separation in the shortest run time. Acetonitrile and methanol are the first choice as organic modifiers owing to their miscibility, low viscosity, ability to produce good chromatographic peak shape and compatibility with MS/MS detection. Similarly, owing to their volatile nature, buffers like formic acid, acetic acid and ammonium acetate or formate are more commonly used in aqueous phase. Therefore, the feasibility of using acetonitrile or methanol with formic acid, acetic acid, ammonium acetate or formate as mobile phase was tested for separation of CSB-INH and IS in different ratios at different flow rates (range 0.2–0.4 mL/min). Finally the best results in term of peak shape, high sensitivity and low

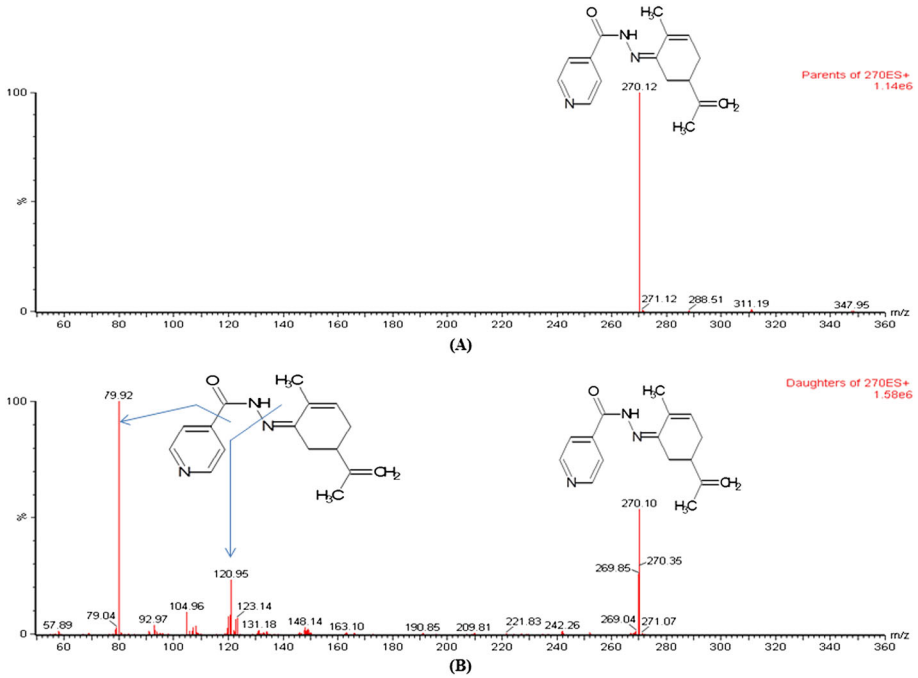

Figure 2. Typical parent ion (A) and daughter ion spectra of CSB-INH.

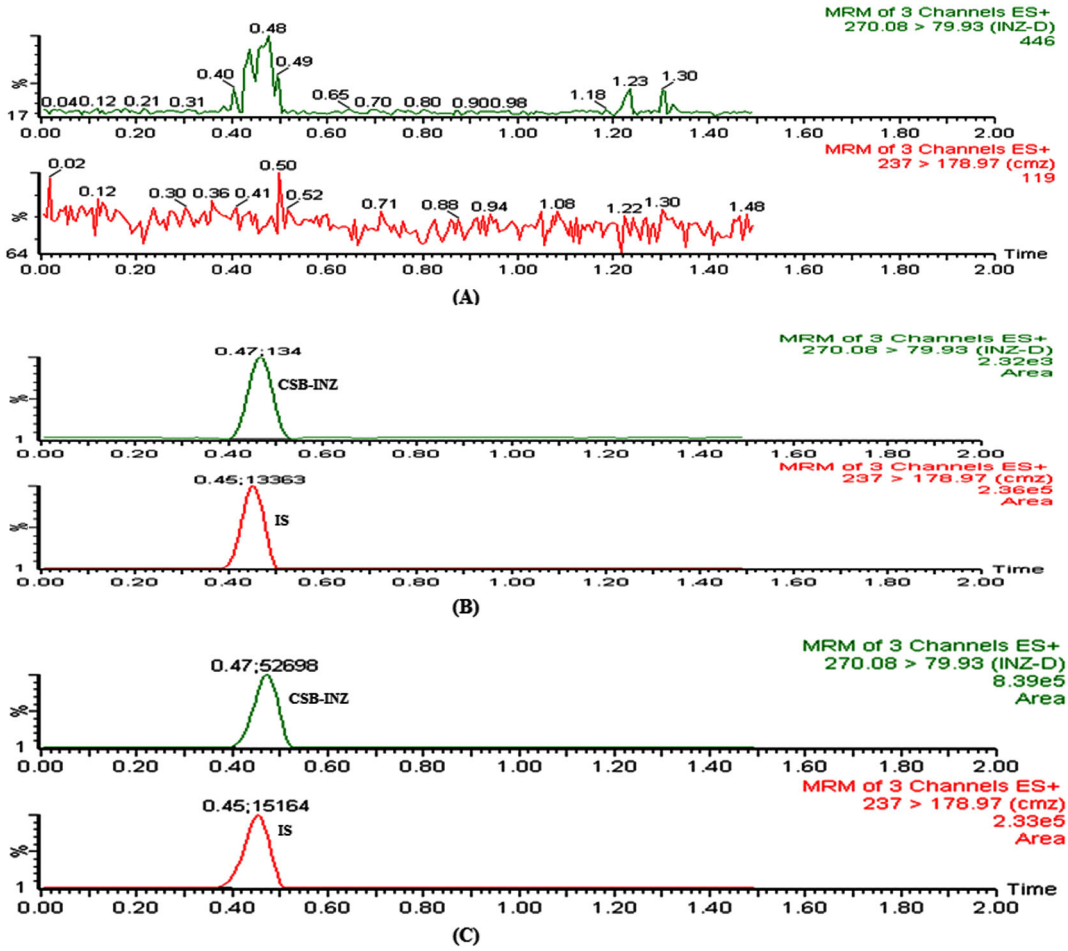

Figure 3. Representative MRM chromatograms of CSB-INH and IS in blank plasma (A); plasma spiked at LLOQ level (B) and 1.5 h after oral administration of CSB-INH suspension in rat (C).

retention time were achieved with isocratic flow of acetonitrile with 10 mM ammonium acetate in a ratio of 95:5 v/v at a flow rate of 0.3 mL/min. With respect to the column, Acquity BEH™ C_{18} columns of 100 × 2.1 mm and 50 × 2.1 mm diameters having the same particle size (i.d. of 1.7 μm) were tested. Best peak resolution with narrow peaks was obtained with 50 × 2.1 mm diameter and used for this assay having a column oven temperature of 40 °C. CSB-INH and IS were eluted at 0.47 and 0.45 min, respectively with a total run time of only 1.5 min. For IS, both INH and carbamazepine were tested. Carbamazepine produced better results with respect to co-elution with CSB-INH, and better peak symmetry, and was finally selected for IS. Other parameters such as autosampler temperature and injection volume were also optimized.

Optimization of sample processing

Optimization of sample preparation procedure is essential to produce clean sample having low matrix effect and high extraction recovery. Using UHPLC technique, together with high sensitive tandem mass spectroscopy, it is possible to quantify a very low concentration of analytes in biological fluids without a very high extraction recovery if it remains constant over time and concentration (Zheng *et al*., 2013). Owing to the ease and compatibility with mobile phase, sample extraction by protein precipitation using acetonitrile and/or methanol is often preferred over other solvents. Therefore, in this study, protein precipitation using acetonitrile and methanol was tried. Satisfactory and concentration-independent recovery and low matrix effects were achieved with acetonitrile and this was selected for the extraction procedure.

Table 1. Intra- and inter-day precision and accuracy of carvone Schiff base of isoniazid (CSB-INH) in rat plasma

Nominal concentration (ng/mL)	Mean ± SD	Precision (CV, %)	Accuracy (%)
Intraday variation			
0.42	0.46 ± 0.06	12.0	108.7
1.20	1.29 ± 0.06	4.85	107.5
100	97.3 ± 9.97	10.2	97.3
2000	1889 ± 79.3	4.20	94.5
Inter-day variation			
0.42	0.44 ± 0.05	11.4	104.2
1.20	1.28 ± 0.05	3.87	106.4
100	98.5 ± 6.44	6.54	98.5
2000	1904 ± 99.38	5.22	95.2

Method validation

Selectivity. No significant endogenous interferences were observed in blank plasma (obtained from different rats) spiked CSB-INH and IS. This shows that the selectivity of this method was acceptable for quantification of CSB-INH in rat plasma. Representative MRM chromatograms of CSB-INH and IS in blank plasma (showing no significant interference) are shown in Fig. 3(A).

Linearity and lower limit of quantification. The calibration curve was constructed by plotting the peak area ratio (analyte/IS) vs concentration. The calibration curve showed good linearity in the concentration range of 0.35–2500 ng/mL in plasma samples. The linear regression of peak area ratio vs the concentration $1/x^2$ was weighted to obtain the lowest deviation, and was adopted for method validation. The determination coefficients (r^2) were consistently ≥0.997 during the course of validation. The LLOQ for this assay was 0.35 ng/mL, which was quantified with acceptable accuracy and precision (≤20%). The calculated signal to noise ratio was 6.38 compared with blank plasma. The value of LOQ QC (0.42 ng/mL) was slightly higher than the actual LLOQ (0.35 ng/mL), as it was prepared by serial dilution of stock solution of QC. Representative MRM chromatograms of CSB-INH and IS in spiked plasma at LLOQ level are shown in Fig. 3(B).

Precision and accuracy. The intra- and inter-day accuracy and precision results are summarized in Table 1. In this assay, intra-day and inter-day accuracies among the four different QCs concentrations were 94.5–108.7 and 95.2–106.4%, respectively. The intra- and inter-day precision values were ≤12.0 and ≤11.4% respectively. These results confirmed that the proposed method had acceptable precision and accuracy as set by regulatory guidelines.

Recovery and matrix effects. The extraction recoveries and matrix effects for CSB-INH at three different QC levels (1.20, 100 and 2000 ng/mL) and IS (4 μg/mL) are shown in Table 2. Results indicate that the recovery of CSB-INH by protein precipitation using acetonitrile was satisfactory, consistent and concentration independent. The matrix effects were also examined to assess the possibility of ionization suppression or enhancement. The matrix effects for CSB-INH at three QC concentrations levels (1.20, 100 and 2000 ng/mL), IS (4 μg/mL) and IS normalized were within the acceptable limits. This indicates that ion suppression or ion enhancement effect of plasma was negligible for this assay.

Carryover effects. No significant carryover effect was observed in this assay as the percentage peak area was found to be ≤20% and ≤5 % at the retention times of analyte and IS, respectively. This result ruled out any possibility of carryover effects. Addition of blank plasma extract samples after every 10–

Table 2. Extraction recovery and matrix effect for CSB-INH (three QC samples) and IS in rat plasma ($n = 6$)

Compound	Nominal concentration (ng/mL)	Extraction recovery		Relative matrix effects		IS normalized matrix effects	
		Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)
CSB-INH	1.20	69.0 ± 4.9	7.1	93.6 ± 3.3	3.5	0.98 ± 0.07	7.0
	100	66.5 ± 7.3	10.9	96.7 ± 3.7	3.9	1.01 ± 0.06	5.4
	2000	60.0 ± 6.8	11.4	92.8 ± 3.8	4.2	0.97 ± 0.04	4.5
IS	400	63.7 ± 2.3	3.7	95.6 ± 4.3	4.5		

Table 3. Assessment of carryover effects for analyte (CSB-INH) and IS

Sample identification	Analyte response	Internal standard response	Percentage carryover observed with respect to LLOQ	Percentage carryover observed with respect to IS
ULOQ1	453,790	12,278		
Blank 1	11	5	8.2	0.04
Blank 2	8	3	5.9	0.02
ULOQ2	462,540	11,445		
Blank 1	9	4	6.7	0.03
Blank 2	7	0	5.2	0.00
ULOQ3	456,540	13,532		
Blank 1	8	6	5.9	0.04
Blank 2	9	5	6.7	0.03

Table 4. Stability data of CSB-INH in rat plasma ($n = 6$)

Stability	Nominal concentration (ng/mL) ($n = 6$)	Mean ± SD	Precision (CV, %)	Accuracy (%)
Bench-top (6 h)	1.20	1.31 ± 0.03	2.6	108.9
	2000	1918 ± 137.1	7.1	95.9
Freeze–thaw (three cycle)	1.20	1.27 ± 0.04	3.0	105.8
	2000	1852 ± 46.6	2.5	92.6
In Injector (48 h)	1.20	1.34 ± 0.04	2.9	111.6
	2000	2144 ± 91.6	4.3	107.2
30 days at −80 °C	1.20	1.25 ± 0.02	1.4	103.9
	2000	1807 ± 72.4	4.0	90.4

15 unknown samples is recommended to eliminate any possible carryover effects in routine analysis. Results of carryover effects are presented in Table 3.

Stability. The results of stability tests (freeze–thaw, post preparative, short- and long-term) in rat plasma are presented in Table 4. The results indicated that CSB-INH was stable under various routine laboratory conditions and could be used for routine analysis. The stock solutions and working standard of CSB-INH and IS were also found to be stable for 15 days at refrigerator temperature (<10 °C) and for 12 h at room temperature.

Table 5. Pharmacokinetic parameters of CSB-INH after an oral administration of nanoemulsion and suspension formulations of CSB-INH (5 mg/kg) ($n = 6$)

Parameters	CSB-INH suspension (mean ± SE)	CSB-INH nanoemulsion (mean ± SE)
C_{max} (ng/mL)	210.89 (61.28)	452.19 (55.64)***
T_{max} (h)	1.5	1.5
AUC_{0-24} (ng h/mL)	618.69 (164.90)	1313.73 (138.97)***
Relative bioavailability (%)	100	212.34

***$p < 0.001$ highly significant compared with CSB-INH suspension.
C_{max}, Peak (maximum) plasma concentration; T_{max}, time to reach C_{max}; AUC, area under the concentration–time curve.

Pharmacokinetic study in rats

The developed method was successfully applied for the quantitative determination of CSB-INH in pharmacokinetic study in rats. Representative MRM chromatogram of CSB-INH and IS at 1 h after administration of CSB-INH suspension and nanoemulsion in rat are shown in Fig. 3(C). The peak (maximum) plasma concentration (C_{max}) of 210.89 and 452.19 ng/mL was achieved at 1.5 h after administration of 5 mg/kg of CSB-INH suspension and nanoemulsion, respectively, whereas the area under the curve (AUC) was found to be 618.69 and 1313.73 ng h/mL, respectively (Table 5).

Conclusion

A simple, fast and sensitive UHPLC-MS/MS method was developed and validated for rapid determination of CSB-INH in rat plasma. The simplicity in sample preparation and isocratic chromatographic separation with shorter run time (only 1.5 min) mean that this method can be applied for high-sample-throughput analysis. The assay was successfully applied to a comparative oral pharmacokinetic study of CSB-INH in rats. The broad calibration range (0.35–2500 ng/mL) with the lowest LLOQ mean that this method can also be applied for further characterization of CSB-INH in pharmacokinetics, toxicokinetics and tissue distribution studies. Additionally, MRM transition of m/z 270.08 → 120.95 can be used as qualifier especially for qualitative analysis.

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