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Early Growth and Development Impairments in Patients with Ganglioside GM3 Synthase Deficiency

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Wang, H.; Wang, A.; Wang, Dan; Bright, A.; Sency, V.; Zhou, Aimin; and Xin, B., "Early Growth and Development Impairments in Patients with Ganglioside GM3 Synthase Deficiency" (2016). *Chemistry Faculty Publications*. 386.
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A rapid LC-MS/MS method for quantification of CSUOH0901, a novel antitumor agent, in rat plasma

Ramakrishna R. Voggu, Ravali Alagandula, Xiang Zhou, Bin Su, Bo Zhong and Baochuan Guo

Introduction

CSUOH0901 {benzo [1,3] dioxole-S-carboxylicacid [3-(2,5-dimethylbenzyloxy)-4-(methanesulfonylmethylamino)-phenyl] amide} (NSC751382; Fig. 1C; Zhong *et al.,* 2013) is a novel, second-generation anticancer agent derived from nimesulide, which can inhibit cyclooxygenase-2 (COX-2; Fig. 1A). In cancer therapy, nimesulide showed hepatotoxicity on long-term usage and required higher concentrations to inhibit COX-2 activity (Zhong *et al.,* 2012). This led to the development of CSUOH0901, a nimesulide derivative, which exhibited very promising anticancer activities by interacting with tubulin and Hsp27 proteins, which are important to cancer cell proliferation. CSUOH0901 inhibited the proliferation of cancer cells of lung, breast, colon, CNS, ovary, renal and prostate cancer with an IC_{50} of 0.1-0.5 µM, which is 10-fold more active than JCC76 {N-[3-(2,5-dimethylbenzyloxy)-4-(methylmethylsulfonamido) phenyl] cyclohexanecaboxamide; (Fig. 1B; Suleyman *et al.,* 2008) and 1000-fold more potent than nimesulide (Zhong *et al.,* 2013).

Recent docking studies in SKBR-3 breast cancer cell lines (Suleyman *et al.,* 2008; Yi *et al.,* 2012) revealed that CSUOH0901 interacted with both a- and /J-tubulin in the colchicine pocket and disorganized microtubules. Additionally, interaction of heat shock protein 27 (Hsp27) (Sun and MacRae, 2005) with CSUOH0901 inhibited the phosphorylation of Hsp27, leading to cell apoptosis. Hsp27 is a stress protein that is expressed when cells are stimulated by heat (Kampinga *et al.,* 1995; Stege *et al.,* 1995a, 1995b), radiation (Rau *et al.,* 1999), chemotherapeutic drugs (Ciocca *et al.,* 1992) or other agents (Wu and Welsh, 1996). A recent study showed that cancer cells with HSP27 overexpression were resistant to chemotherapeutic drugs (Huot *et al.,* 1991; Fuqua *et al.,* 1994; Hettinga *et al.,* 1996; Richards *et al.,* 1996). Antisense to inhibition of the HSP27 gene decreased cellular resistance to chemotherapy as well as to heat shock (Horman *et al.,* 1999). Other studies have suggested that HSP27 prevents cancer cells from apoptosis and dramatically enhances their tumorigenicity (Garrido *et al.,* 1998, 1999; Guenal *et al.,* 1997; Samali and Cotter, 1996). Mass spectrometric studies revealed that tubulin and Hsp27 proteins are the most prevalent targets of CSUOH0901. Recent *in vivo* studies demonstrated that CSUOH0901 significantly decreased the size of HT29 tumors in a xenograft model compared with the control group, suggesting the low toxicity and high potency *in vivo* (Zhong *et al.,* 2012).

Clearly, CSUOH0901 is a very promising anticancer drug candidate and will be further studied. However, to date, no LC-MS/MS method has been developed for the quantification of CSUOH0901. Therefore, a simple and accurate method to quantify CSUOH0901 is needed that will be essential to the future pharmacological and toxicological studies of CSUOH0901. In this work, a rapid and sensitive LC-MS/MS method was developed and validated for quantitative determination of CSUOH0901 in rat plasma. We demonstrated that the method developed was

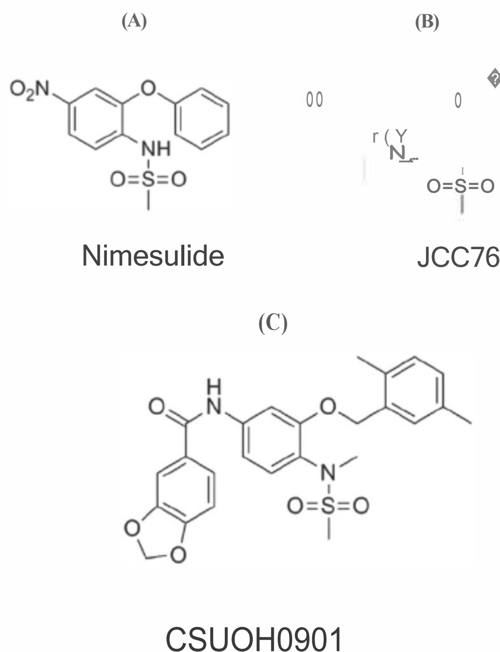

Figure 1. The chemical structures of nimesulide (A), internal standard JCC76 (B) and CSUOH0901 (C).

fast, sensitive and specific for quantifying CSU0H0901 in plasma, and can be used in pharmacological studies.

Experimental

Chemical and reagents

CSUOH0901 and JCC76 (internal standard, IS) were synthesized and purified according to the previously published procedures (Zhong *et al.,* 2013; Suleyman *et al.,* 2008). HPLC grade methanol and acetonitrile were purchased from Pharmco Apper (Philadelphia, PA, USA). Formic acid, ammonium formate and ammonium acetate were purchased from Sigma Aldrich Chemical Company (Allentown, PA, USA). Dimethyl sulfoxide was obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was generated from a Barnstead Nano Pure Water Purification System from Thermo Scientific (Waltham, MA, USA). Sprague Dawley rat plasma K2 with specific lot numbers (10577 01 06) was purchased from Innovative research (Novi, MI, USA).

Calibration standard and quality control samples

Preparation of stock and working solutions. The stock solutions of CSUOH0901 and JCC76 (IS) were prepared in dimethyl sulfoxide (DMSO) at 1 mg/ml and stored at 20°C. A set of CSOH0901 working solutions of 10, 20, 50, 150, 400, 1000 and 2000 ng/ml were prepared by serial dilution from the stock solution with DMSO. The working solution of JCC76 (IS) was obtained by diluting the stock solution with DMSO to give a concentration of 150 ng/ml.

Preparation of calibration and quality control plasma sampies. The calibration plasma solutions were prepared by spiking 10 µL of CSUOH0901 working solutions in 200 µL of blank plasma (mixture of 6 lots) to give drug concentrations of 0.5, 1.0, 2.5, 7.5, 20, 50 and 100 ng/mL. The lower limit of quantification (LLOQ) and quality control (QC) standards were prepared in a similar way at 0.5, 1.25, 10, 80 ng/ml, representing LLOQ, low QC (LQC), middle QC (MQC) and high QC (HQC) respectively. The QC and calibration samples were frozen at 20°C overnight. and then treated by the following sample preparation procedure and subjected to LC MS/MS analysis.

Sample extraction

Plasma samples were removed from the 20°C freezer and thawed to room temperature. Single and double blanks were prepared by spiking 10 µL of acetonitrile in 200 µL of rat plasma. Then 10 µL of IS working solution was spiked in all calibration, QC solutions and single blank. except in double blank and vortexed immediately for 30 s The samples were deproteinized by adding 800 µL of acetonitrile and sonicated for 15 min followed by centrifugation at 13,000g for 15 min. The supernatants were transferred into autosampler vials for LC MS/MS analysis.

LC-MS/MS analysis

LC MS/MS analysis was conducted using 5500 QTRAP triple quadrupole, tandem mass spectrometer (AB Sciex, Toronto, Canada) with an electrospray ionization (ESI) source (Framingham, MA, USA) interfaced with high performance liquid chromatography (HPLC, Shimadzu, Columbia, MD, USA) with two LC 30 AD pumps, DUG 20A3R inline degasser, a SIL 30 AC autosampler, a CBM 20A controller and a CTO 10AVP column oven (Shimdazu, Tokyo, Japan). Analyst software, version 1.52 (AB Sdex) was used to control all the parameters of tandem mass spectrometer and HPLC.

A Luna C_{18} (2) HPLC column (50 x2.0 mm 5 µm) with a C_{18} security guard cartridge from Phenomenex (Torrance, CA, USA) was used for the chromatographic separation of the supernatants from the deproteinized samples. An optimized gradient flow of mobile phase A, 5 mM ammonium formate in 2% methanol, and mobile phase B, 5mM ammonium formate in 90% methanol at a flow rate of 0.2 mL/min, was developed. The column was equilibrated with the mobile phase for 10 min and the run time was 8 min for each run with 10 µL injection volume. The positive ESI mode was selected and the MRM (multiple reaction monitoring) function was used for quantification, with the transitions set at *m/z* 483.2 →404.3, m/z 483.2 →119.0 for CSUOH0901 and *m/z* 445.3 →366.3 for JCC76 (IS) (Fig. 2). The dwell time for each MRM transition was set at 120 ms. Source dependent parameters were optimized by flow infusion analysis: nebulization gas (30), heating gas (30), curtain gas (40), ion spray voltage (5000 eV) and temperature (450°C). Compound dependent parameters were manually optimized as following: declustering potential, 180; entrance potential, 10; collision energy, 20; and cell exit potential, 12.

Analytical method validation

A full method validation was performed using rat plasma according to the currently accepted FDA bioanalytical method guidelines (US Food and Drug Administration, 2001) and also other references (Liu *et al.* 2013; Ito *et al.,* 2013). The entire method was validated for precision, accuracy, linearity, selectivity, extraction recovery, LLOQ, matrix effect and stability studies.

Calibration curve, linearity and sensitivity. Seven CSUOH0901 plasma calibrators at the concentrations of 0.5, 1.0, 2.5, 7.5, 20, 50 and 100 ng/ml, double blank and single blank (only JCC76 internal standard) were selected to establish a calibration curve. The weighed linear regression, 1/x, as weighing factor was used to calculate the slope and correlation coefficient of the calibration curve. The LLOQ was defined as the concentration with precision (coefficient of variation, CV) <20%.

Accuracy and precision. Intra and inter assay precision and accuracy studies were performed using three QC standards, LQC, MQC and HQC, at 1.25, 10 and 80 ng/ml with five replicates (n =5). Intra and inter assay precisions were determined as CV, and accuracies were cakulated by comparing experimentally determined concentrations with the spiked values. Therefore, accuracy (%) = [(experimental concentration spiked concentration)/ spiked concentration) x 100.

Recovery and matrix effect. The absolute extraction recovery was determined by comparing the peak areas of CSUOH0901 in QC samples at 1.25, 10 and 80 ng/ml (CSUOH0901 added prior to deproteinization) with those of postextraction samples (CSUOH0901 added after

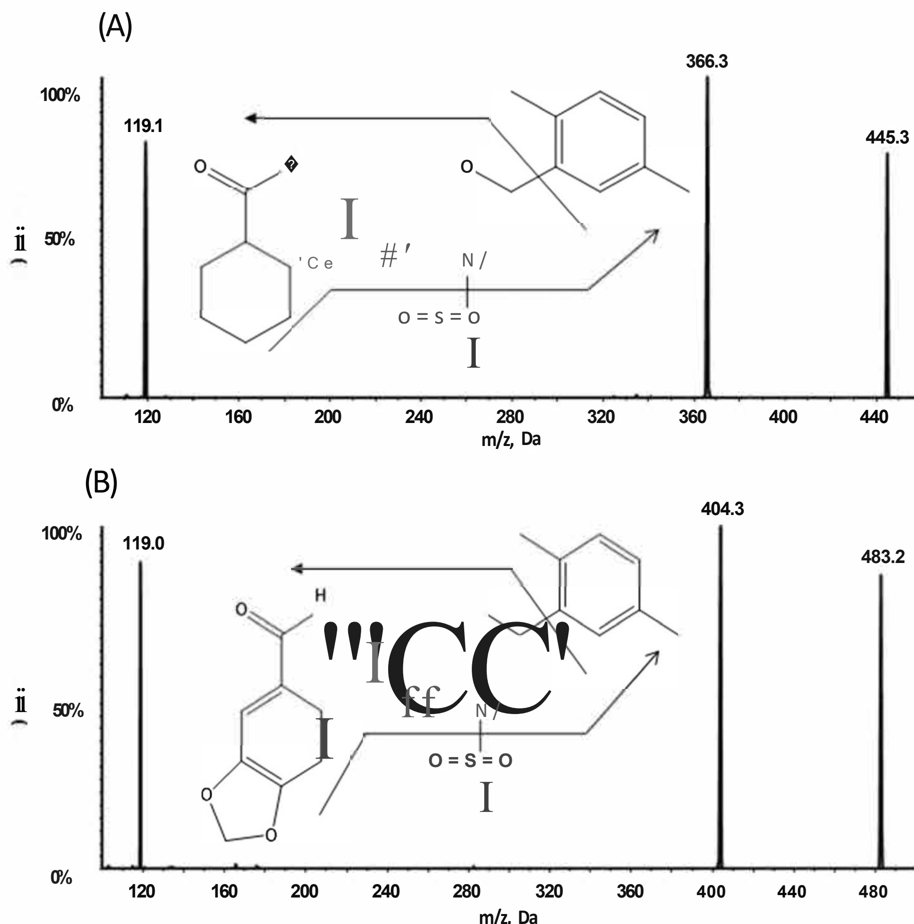

Figure 2. Precursor/product ion spectra and proposed fragmentation pathways for internal standard JCC76 (A) and analyte CSUOH0901 (B).

deproteinization) of corresponding concentrations. The relative recovery was determined by comparing peak area ratio of CSUOH0901 and IS (JCC76) spiked in plasma before extraction with that in postextraction spiked samples.

The absolute matrix effect was calculated by comparing the peak areas of postextraction blank plasma samples spiked with CSUOH0901 (1.25, 10 and 80 ng/ml) with those of corresponding standard solutions at equivalent concentrations. The relative matrix effect was calculated by comparing the peak area ratio of CSUOH0901 and IS (JCC76) spiked in the blank plasma postextraction solution with that in standard solution.

Stability studies

Effect of freeze thaw on CSUOH0901 in plasma. Two QC samples of 1.25 and 80 ng/ml concentrations were selected to verify their stability. The stability test for CSUOH0901 in plasma was studied after three freeze thaw cycles over a 3 day period.

Short- and long-term stability studies of analyte in plasma. The stability studies of CSUOH0901 in rat plasma were performed using two QC standards (1.25 and 80 ng/ml), which were kept under different storage conditions: 10 h at room temperature and 6 months at 20 °C, before and after sample extraction.

Stability of analyte in stock solutions. The stability studies of stock solutions and working solutions of CSUOH0901 and internal standard (JCC76) were also evaluated. The stock solutions of analyte were stored at 20 °C for 7 months. Two QC standards of concentrations 1.25 and 80 ng/ml were prepared from both the stored and fresh stock solutions and the experimentally determined concentrations of CSUOH0901 were compared (*n* =3 for each sample).

Results and discussion

Optimization of mass spectrometric conditions for MRM quantitation

Positive ionization mode was selected to detect and optimize the MS parameters for the detection of both CSUOH0901 and JCC76 (internal standard). It was found that the standard CSUOH0901 and JCC76 solutions prepared in methanol-water (9:1, v/v) yielded higher intensity when compared with the solutions prepared in acetonitrile-water (9:1, v/v). Fragmentation led to the formation of daughter ions in the product ion scan mode (Fig. 2). Based on the fragmentation study, the MRM transitions of *m/z* 483.2 —>404.3 for CSUOH0901 and 445.3 —>366.3 for JCC76 were selected for quantification, as these product ions yielded strong signals. The highest MS signal was obtained by fine-tuning collision energy, spray voltage and ion source temperature.

Optimization of HPLC conditions

To overcome the irreproducibility and matrix effect problems associated with the isocratic flow, a gradient flow of mobile phase A, 5mM ammonium formate in 2% methanol, and mobile phase B, 5 mM ammonium formate in 90% methanol with 0.2 ml/min flow rate, was employed. This gradient flow improved the sensitivity and signal-to-noise ratio with a total run time of 18 min. High concentration of methanol was used to elute CSUOH0901 from C_{18} column, owing to its low solubility in water with

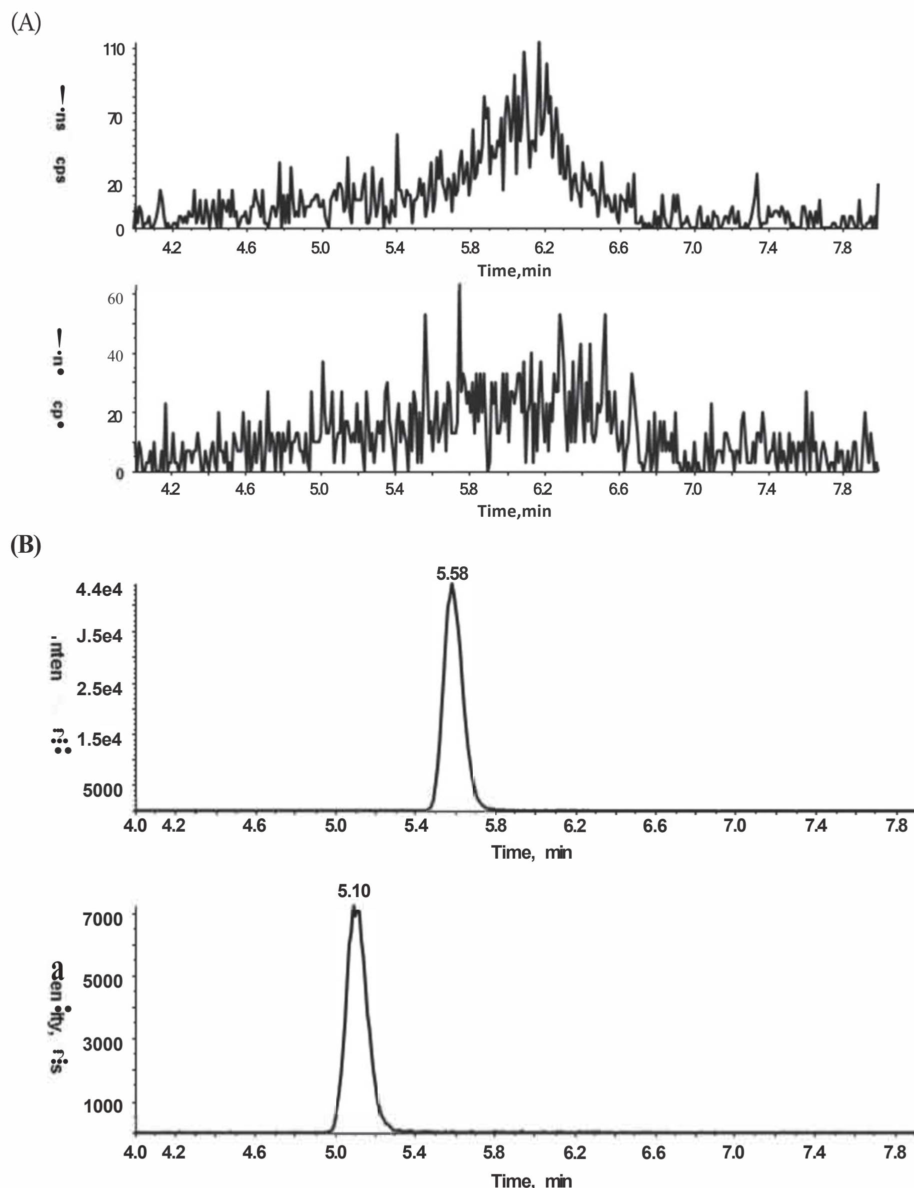

Figure 3. (A) Multiple reaction monitoring chromatograms of blank rat plasma in both IS and analyte windows. (B) IS JCC76 (10 ng/ml, S.58 min) and CSUOH0901 at LLOQ level (0.5 ng/ml, 5.10 min).

predicted logo value of 4.86. The intensity of CSUOH0901 was increased 2-fold when 5 mM ammonium formate buffer was used in the mobile phases and the retention times were around 5.09 min for CSUOH0901 and 5.58 min for JCC76 (IS) (Fig. 38).

Linearity, sensitivity, selectivity and LLOQ

the Calibration curve for CSUOH0901 in plasma was linear in the range of 0.5-100 ng/ml. Linearity results showed the quadratic fit for CSUOH0901 with a seven-point calibration curve of concentrations 0.5, 1.0, 2.5, 7.5, 20, 50 and 100 ng/ml including double-blank and single-blank (only JCC76 internal standard) plasma samples. An excellent linearity was obtained with the correlation coefficient of 0.9996 and the linear regression equation was y=0.073x 0.0085. This method exhibited high selectivity with no interfering peak in six different blank plasma samples from different sources. The LLOQ was found to be 0.5 ng/ml, where the signal intensity was 20-fold higher than the blank signal (Fig. 3). The lowest concentration in a calibration curve (LLOQ) was quantified with the accuracy and precision within 15% (Table 1).

Table 1. Accuracy and precision of CSUOH0901 calibration standards (n =5) over 0.5-100 ng/ml

Nominal concentration	Determined concentration	Accuracy	Precision
(ng/ml)	(ng/ml)	(RE)	(CV)
0.5	0.49±0.01	2.0%	4.5%
1	0.90±0.09	10.0%	6.0%
2.5	2.58±0.08	3.2%	6.7%
7.5	7.23 ±0.27	3.6%	11.0%
20	20.42±0.42	2.1%	1.7%
50	49.14±0.86	1.7%	1.6%
100	97.30 ± 2.70	2.7%	2.2%

Table 2. Inter- and intra-assay accuracy and precision of CSUOH0901 in rat plasma

	Intra- assay				Inter- assay			
Spiked (ng/ml)	Determined (ng/ml)	Accuracy (%RE)	SD	Precision (CV)	Determined (ng/ml)	Accuracy (RE)	SD	Precision (CV)
1.25	1.39	11.2%	0.04	2.9%	1.39	11.2%	0.07	5.0%
10	10.26	2.6%	0.18	1.8%	10.35	3.5%	0.46	4.5%
80	78.52	1.9%	1.44	1.8%	79.58	0.5%	4.36	5.5%

Accuracy and precision

Intra- and inter -assay accuracies of the validated method ranged from 1.9 to 11.2% and from 0.5 to 11.2%, respectively. The intra- and inter-assay precision values ranged from 1.8 to 2.9% and from 4.5 to 5.5%, respectively. The inter- and intra-assay accuracy and precision of the QC samples are depicted in Table 2.

Extraction recovery and matrix effect

The absolute recoveries of the extraction method were 104.0, 105.0 and 104.0% for the QC standards at 1.25, 10, and 80 ng/ml, and the relative recoveries of the extracted method were 99.9, 96.1 and 97.7% for the QC standards at 1.25, 10, and 80 ng/ml, respectively, as indicated in Table 3. Absolute matrix effects for each of three plasma samples at 1.25, 10, and 80 ng/ml were 5.5, 8.1 and 9.9%, and relative matrix effects were 2.6, 5.1 and 12.4% indicating the minimal matrix effect. Hence, the protein precipitation technique for sample preparation was found to be effective, as it not only extracted the analyte and internal standard well but also removed impurities causing interferences from the sample matrix.

Performing MRM confirmed the absence of significant matrix effect by comparing the peak areas ratio of CSUOH0901 MRM transitions (MRM_1, m/z 483.2 → 404.3; and MRM_2, m/z 483.2 → 119.0) for the spiked rat plasma samples with the average peak area ratio for seven calibrators:

$$\text{MRM ratio} = \text{peak area } MRM_1/\text{peak area } MRM_2 \quad (1)$$

The average MRM ratio of the seven calibrators was 1.5 ±1.9 (±SD). This confirmed the absence of matrix effect in the plasma samples and that they are in the acceptable range.

Stability

CSUOH0901 was stable for at least 8 h at room temperature (bench top) and for 10 h when postextracted at room temperature and the results were summarized in Table 4. The recovery of CSUOH0901 was 112.0% at LQC and 104.5% at HQC levels after three freeze-thaw cycles. Stability studies of stock solutions and working solutions of CSUOH0901 and internal standard (JCC76) were performed by storing them at 20˚(for at least 6 months. The analyte and the internal standard were found to be stable in stock solutions and the results are summarized in Table 5.

Table 3. Absolute and relative matrix effect and recovery of CSUOH0901 in rat plasma

Concentration of QC samples (ng/ml)	Matrix effect		Recovery	
	Absolute	Relative	Absolute	Relative
1.25	5.5%	2.6%	104.0%	99.9%
10	8.1%	5.1%	105.0%	96.1%
80	9.9%	12.4%	104.0%	97.7%

Table 4. Stabilities of CSUOH0901 under various conditions

Stability	Concentration (ng/ml)	Recovery (%)
Bench-top (8 h)	1.25	112.0%
At room temperature	80.00	99.1%
Freeze-thaw (three cycles)	1.25	112.0%
	80.00	104.5%
Post-extraction (10 h) at room	1.25	106.4%
temperature	80.00	97.8%

Table 5. Stabilities of CSUOH0901 and JCC76 Stock solutions after storage at 20˚(for 6 months

Type of solutions	Concentration	Recovery (%)
CSUOH0901 stock solution	1 mg/ml	111.8%
JCC76 stock solution	1 mg/ml	106.0%
CSUOH0901 working solution	25 ng/ml	99.6%
	1600 ng/ml	105.0%
JCC76 working solution	150 ng/ml	124.6%

Conclusion

In conclusion, a highly sensitive LC-MS/MS method for the quantitation of CSUOH0901 in rat plasma was developed and validated for the first time. The method developed has a short run time of 18 min employing a simple one-step sample preparation. The accuracy and precision were <10% and the LLOQ was as low as 0.5 ng/ml. The results from the validation studies illustrated that this method can be used to determine the pharmacological and toxicological profiles of CSUOH0901 in rats.

Acknowledgments

We thank Cleveland State University for providing the financial support of this research. We also acknowledge The National Science Foundation Major Research Instrumentation Grant (CHE-0923398), which supported the use of the AB Sciex QTrap 5500 mass spectrometer instrument in the current project.

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