

Development of a ultra-performance LC–MS/MS method for quantification of GW788388 and its applications in pharmacokinetic study in rats

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Aim: GW788388 is a selective and orally active TGF- β 1 receptor inhibitor that shows potent activity in renal fibrosis. We aimed to establish and validate a simple and sensitive ultra-performance LC–MS/MS method for the determination of GW788388 in plasma samples. **Methodology & results:** GW788388 in rat plasma was processed with protein precipitation method and then separated on a C₁₈ column. The calibration curve presented a good linearity in the range of 1.0–1200 ng/ml, with satisfactory accuracy (relative error, [–17.5% < relative error < 11.7%]) and precision (CV < 8.9%) for all quality control samples. After oral administration, GW788388 was absorbed quickly and reached a peak concentration of 595.3 ± 60.2 ng/ml after 20 min. **Conclusion:** The validated method provides a quantification method of GW788388 in rat plasma in detail, and can be utilized to successfully describe the pharmacokinetic profile of GW788388.

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TGF- β is considered as an important multifunctional mediator in tissue fibrosis, which upregulates matrix protein synthesis, inhibits matrix degradation and alters cell–cell interaction [1,2]. Among the three TGF- β isoforms, TGF- β 1 is expressed in hematopoietic, endothelial and connective tissue cells and observed upregulation in the lung, liver, heart and kidney fibrosis [1–3]. Thus, TGF- β 1 receptor inhibitors offer a novel approach for the treatment of fibrotic diseases such as hepatic, renal and pulmonary fibrosis [4–7].

GW788388 is a selective and orally active TGF- β 1 receptor inhibitor that shows potent activity in renal fibrosis [8,9]. It is reported to reduce the expression of collagen type I A1 in a puromycin aminonucleoside-induced renal fibrosis model [8]. Another research found that in a diabetic nephropathy mice model, GW788388 significantly reduced renal fibrosis and decreased the mRNA expression of several extracellular matrix deposition key mediators (PAI-1, COL-I and COL-III) in kidney, indicating that GW788388 attenuated TGF- β 1 signaling *in vivo* and effectively decreased fibrosis marks in mice with late-stage diabetic nephropathy [9].

GW788388 also prevents heart fibrosis in *Trypanosoma cruzi* infection-induced Chagas disease [10–12]. GW788388 reduced collagen type I and fibronectin levels, thereby slowing down the process of cardiac fibrosis. In experimental Chagas disease mouse models, GW788388 significantly decreased parasitemia, reduced mortality [10] and restored cardiac function such as increased heart rate, reversed sinus arrhythmia, reduced the prolonged PR and QTc intervals, and reversed atrial and atrioventricular conduction disorders [11,12].

Although there are many findings suggesting the clinical development of GW788388, no detailed analysis method of GW788388 has been reported, and no pharmacokinetic studies have been conducted. As LC–MS/MS method was widely used for determination of small molecule in biological matrices [13,14], we established and validated a simple and sensitive ultra-performance (UP) LC–MS/MS method for determination of GW788388 in rat plasma.

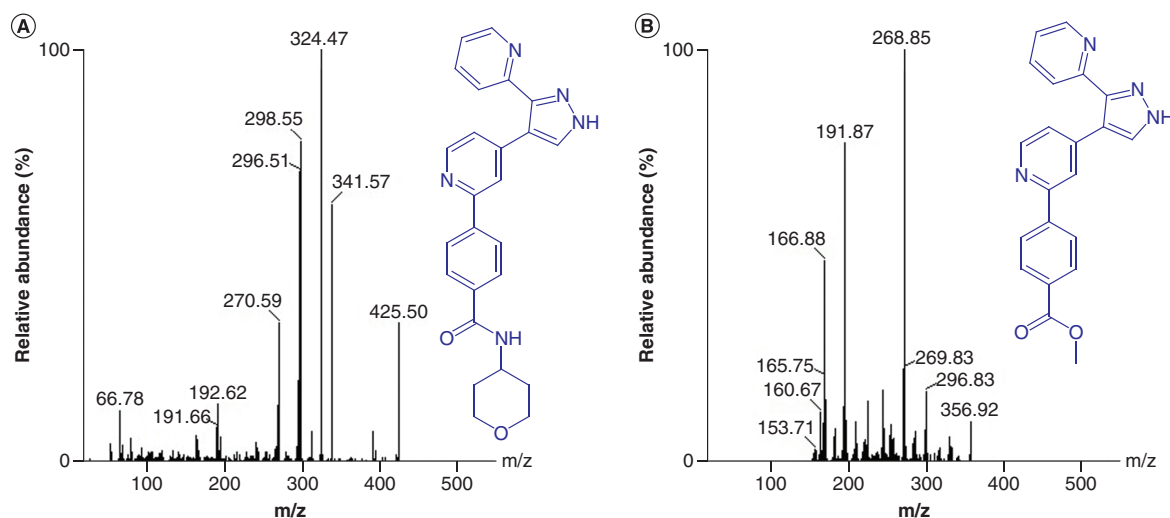


Figure 1. Chemical structures & MS² scan spectra. (A) GW788388. (B) IS.
IS: Internal standard.

The pharmacokinetic profiles after oral administration of GW788388 in adult male Sprague Dawley (SD) rats were investigated.

Experimental

Materials & reagents

Adult male SD rats were provided by Changsheng Biotechnology Co., Ltd (Benxi, China). GW788388 (>98.6%), and methyl 4-(4-(3-(pyridin-2-yl)-1H-pyrazol-4-yl)pyridin-2-yl) benzoate (>97.2%, internal standard, IS) were purchased from AZBIOCHEM Biotechnology Co., Ltd (Shanghai, China). The chemical structures of GW788388 and IS are presented in Figure 1. Acetonitrile, methanol and formic acid (HPLC-grade) were obtained from Fisher Scientific (NJ, USA). The purified water for UPLC analysis was obtained from Wahaha Co., Ltd (Hangzhou, China). Nylon filter was purchased from ANPEL Laboratory Technologies, Inc., (Shanghai, China).

Instruments & UPLC–MS/MS conditions

The analyses were carried out on an Acquity UPLC system (Waters, MA, USA). Optimum chromatographic separation was performed on an UPLC BEH C₁₈ (2.1 × 100 mm, 1.7 μm particle diameter; Waters) column, with a simple gradient elution of the mobile phase system consisting of 0.2% formic acid in water (A) and acetonitrile (B). The elution program was performed as following: 0–3.0 min, B, 20–60%; 3.01–4.0 min, B, 20%. The flow rate was 0.3 ml/min, and the column temperature was maintained at 25°C.

The mass spectrometric detection of the analytes was performed on a Waters TQ-S mass spectrometer. MS-optimized parameters were listed as follows: capillary voltage was set at 2.5 kV and the desolvation temperature was 500°C with desolvation gas flow of 800 l/h. The multiple reaction monitoring (MRM) transitions of *m/z* 425.50 → 324.47 for GW788388 and 356.92 → 268.85 for the IS were monitored in positive ionization mode. The optimized cone voltage of GW788388 and IS were 40 V, and the collision energy for GW788388 and IS were 32 and 36 V, respectively. Furthermore, the MRM transitions of *m/z* 425.50 → 298.55 for GW788388 and 356.92 → 191.87 for the IS were monitored as the qualifier ion pairs, and the corresponding collision energy for GW788388 and IS were 38 and 40 V, respectively. Dwell time for both of the analytes was 0.025 s.

Stock solutions, calibration standards & quality control samples

Stock solutions of GW788388 and IS were prepared in methanol to obtain a concentration of 1.0 mg/ml, respectively. The working solutions of GW788388 were prepared by subsequently diluting GW788388 stock solution with methanol to generate a concentration range from 10–12000 ng/ml, and then, 10 μl of corresponding working solutions were added in 100 μl of drug-free rat plasma (a mixed plasma collected from six healthy SD rats) to produce calibration standards of GW788388 (1.0, 5.0, 20, 100, 300, 600 and 1200 ng/ml). Quality control (QC) samples were prepared at 1.0 ng/ml (LLOQ), 3.0 ng/ml (low concentration of QC [LQC]), 200 ng/ml (medium

concentration of QC [MQC]) and 1000 ng/ml (high concentration of QC [HQC]) separately in the same way. The IS working solution was prepared in acetonitrile at a concentration of 15 ng/ml.

Sample preparation

To 100 µl of plasma sample, 450 µl of IS working solution was added and vortexed for 1 min. This mixture was then centrifuged at $900 \times g$ for 5 min at 4°C. 450 µl of the supernatant was transferred into another tube, added with 450 µl purified water and then vortexed again for 30 s. After filtration with a 0.22 µm filter, 3 µl of the filtrate was introduced into the UPLC–MS/MS instrument for analysis.

Method validation

The method was validated in accordance with US FDA guidelines [15].

Selectivity was assessed by comparing blank plasma samples from six individual rats with the spiked samples at the LLOQ.

Calibration curves were obtained by plotting the peak area ratio of GW788388 and IS against GW788388 concentration. A $1/X^2$ weighted least square linear regression method was utilized, where 'X' represented the concentration of GW788388. The calibrators should be less than $\pm 15\%$ of nominal concentrations, except at LLOQ where the calibrator should be $\pm 20\%$. Linear correlation coefficients (r) >0.99 were considered acceptable for the determination of GW788388 in biological matrices.

QC samples at four concentration levels in six replicates were assessed over three independent runs to evaluate accuracy and precision. For L, M and H QCs, accuracy (relative error [RE%]) and the precision (CV%) should be less than $\pm 15\%$, while for LLOQ, the acceptance criteria were $\pm 20\%$.

Carryover was assessed by injecting blank sample after the injection of ULOQ, the peak area in blank samples should be $<20\%$ for GW788388 at LLOQ and $<5\%$ for IS [16,17].

Extraction recovery and matrix effect of GW788388 were assessed in six different samples at L, M and H QC levels. Recovery of GW788388 or IS was calculated by the ratio of the peak area of the extracted sample to that of blanks spiked with GW788388 or IS postextraction. Matrix effect was expressed by the ratio of peak area of the postextraction blanks spiked with GW788388 and IS to that of the analytes prepared according to the sample preparation with water instead of plasma.

Stability studies were evaluated under a variety of the following conditions: samples were stored at -25°C for 30 days, at room temperature for 12 h, after three freeze-thaw cycles, and processed samples were kept at 4°C for 12 h. The accuracy and precision under the above conditions should be within $\pm 15\%$. The stabilities of stock solutions were checked by comparing the peak area of the analytes in solutions stored at 4°C after 3 weeks to that in solutions freshly prepared.

Pharmacokinetic study

The animal study was approved by the animal care welfare committee of Shenyang Medical College. Male SD rats (220–240 g, SPF grade) were raised with standard diet and water in a climate-controlled room. The temperature was $23\text{--}26^\circ\text{C}$; relative humidity was between 30 and 70% with 12 h dark/light cycle. After acclimatization for 6 days, six rats were orally treated with GW788388 (10 mg/kg) to describe the pharmacokinetic profile. The dosing solution was prepared as follows: GW788388 was first dissolved in DMSO, then added with PEG-400, and further diluted with water to obtain a uniform drug solution at a concentration of 1.0 mg/ml (the mixed solvent containing 7%DMSO, 20%PEG-400 and 73% water). The dosing volume was 1 ml per 100 g bodyweight. Blood samples (about 200–300 µl) were collected at 0, 10, 20 and 30 min and 1.0, 2.0, 4.0, 6.0, 8.0 and 12.0 h from the suborbital vein after administration. Plasma was obtained after centrifuging at $900 \times g$ for 5 min, and stored at -25°C until analysis.

Results & discussion

Method optimization

To get a favorable mass response, GW788388 and IS were infused directly into the mass system with a syringe pump, respectively. Better responses were achieved in positive ionization mode, as GW788388 and IS are nitrogenous compounds. Ion m/z of 425.50 for GW788388 and 356.92 for IS were detected as the precursors $[M+H]^+$, and m/z of 324.47 for GW788388 and 268.85 for IS were chosen as the product ions for determination as these

were the most abundant product ions in the MS² scan spectra, respectively. Additionally, the ionization of m/z 425.50→324.47 for GW788388 and 356.92→268.85 for IS were stable and reproducible.

In order to obtain a suitable retention time, the isocratic and gradient elution modes were studied separately. As isocratic elution with 30% acetonitrile (flow rate 0.2 ml/min), the retention time of GW788388 was 1.55 min, while the retention time of IS was 3.31 min. In the above mobile phase proportion example, a significantly enhanced matrix effect was observed. Isocratic elution with 25% acetonitrile extended the retention time of GW788388 to 1.88 min, which slightly reduced the enhancement of matrix effect, but also increased the retention time of IS to 5.66 min, which greatly extend the total run time. Therefore, the gradient elution mode was selected. Under the optimal gradient elution condition (flow rate 0.2 ml/min), the retention times of GW788388 and IS were 2.29 and 3.34 min, respectively, and no significant matrix effect was detected. To shorten the total run time, the flow rate was set to 0.3 ml/min. Finally, the retention times of the analytes were 1.86 and 2.56 min, with a total run time of 4.0 min, including an equilibration time of 1.0 min.

To simplify the plasma preparation, protein precipitation was chosen to pretreat the plasma samples. After precipitation of methanol or acetonitrile, the chromatographic peak of GW788388 in the processed sample split severely. We found that a processed sample with a water content of <40% would lead to peak splitting. This may be due to the solvent effect. When the polarity of the sample solvent is too much different from the polarity of the mobile phase, the organic solvent in the sample will cause the local elution capacity of the mobile phase to change, which may lead to the secondary equilibrium of the solute and change the peak shape of the analyte. In this experiment, the initial ratio of organic phase in the mobile phase was 20%, and when the organic phase ratio in the sample solvent was >60%, peak splitting was observed. Thus, in the process of sample preparation, after the protein was precipitated with organic solvent, 1:1 distilled water (V/V) was added to achieve an unsplit and symmetrical chromatographic peak. As acetonitrile provided a much smoother baseline in the chromatogram than methanol, acetonitrile was selected as the precipitation solvent.

In the early stage of method development, 50 µl of rat plasma and 150 µl of acetonitrile were used to perform the sample preparation. The method showed a poor precision (CV>20%) in six replicates of QC samples. After assurance of repeatability of injection, we paid attention to the step of filtering. We prepared a large volume of QC samples at low and high concentrations and compared the peak area of the filtered sample to that of the unfiltered samples to analyze the potential adsorption of GW788388 and the IS to the Nylon filter membrane ($n = 6$). The adsorption rate of the filter was calculated as follows: the adsorption rate (%) = $(A_{\text{unfil}} - A_{\text{fil}}) / A_{\text{unfil}} \times 100\%$, here A_{unfil} denoted the peak area of the unfiltered sample, and A_{fil} denoted the peak area of the filtered sample. The results showed that at least 600 µl of the initial filtrate should be discarded to avoid the adsorption of the filter film. Therefore, 100 µl of rat plasma was used, together with the precipitant (450 µl) and water (450 µl), this method can provide at least 900 µl solution for filtration.

Method validation

Selectivity

Representative MRM chromatograms of blank plasma, plasma sample spiked with GW788388 and the IS, and rat plasma sample after administration were presented in Figure 2. Compared with LLOQ samples, no significant endogenous interference substances was observed at the retention times of GW788388 (1.86 min) and IS (2.56 min), respectively, indicating a sufficient selectivity of this method.

Linearity, LLOQ & carryover

Calibration curves were yielded with seven increasing concentration levels of GW788388, and demonstrated a good linearity in the range of 1.0–1200 ng/ml. The regression equations were $y = 0.00506x + 0.00110$ ($r = 0.9948$), $y = 0.00467x + 0.00178$ ($r = 0.9941$), $y = 0.00488x + 0.00172$ ($r = 0.9964$) for three days, and mean $\pm$ SD were 0.00487 ± 0.00020 for slope, 0.00153 ± 0.00038 for intercept and 0.9951 ± 0.0012 for correlation coefficient, respectively. The LLOQ was determined to be 1.0 ng/ml.

After injection of ULOQ, no obvious chromatographic peak were detected in blank samples at the retention times of GW788388 and the IS, thus, no carryover was found in this method.

Accuracy & precision

As shown in Table 1, the accuracy of GW788388 in three independent runs ranged from -11.8 to 11.7 for L,

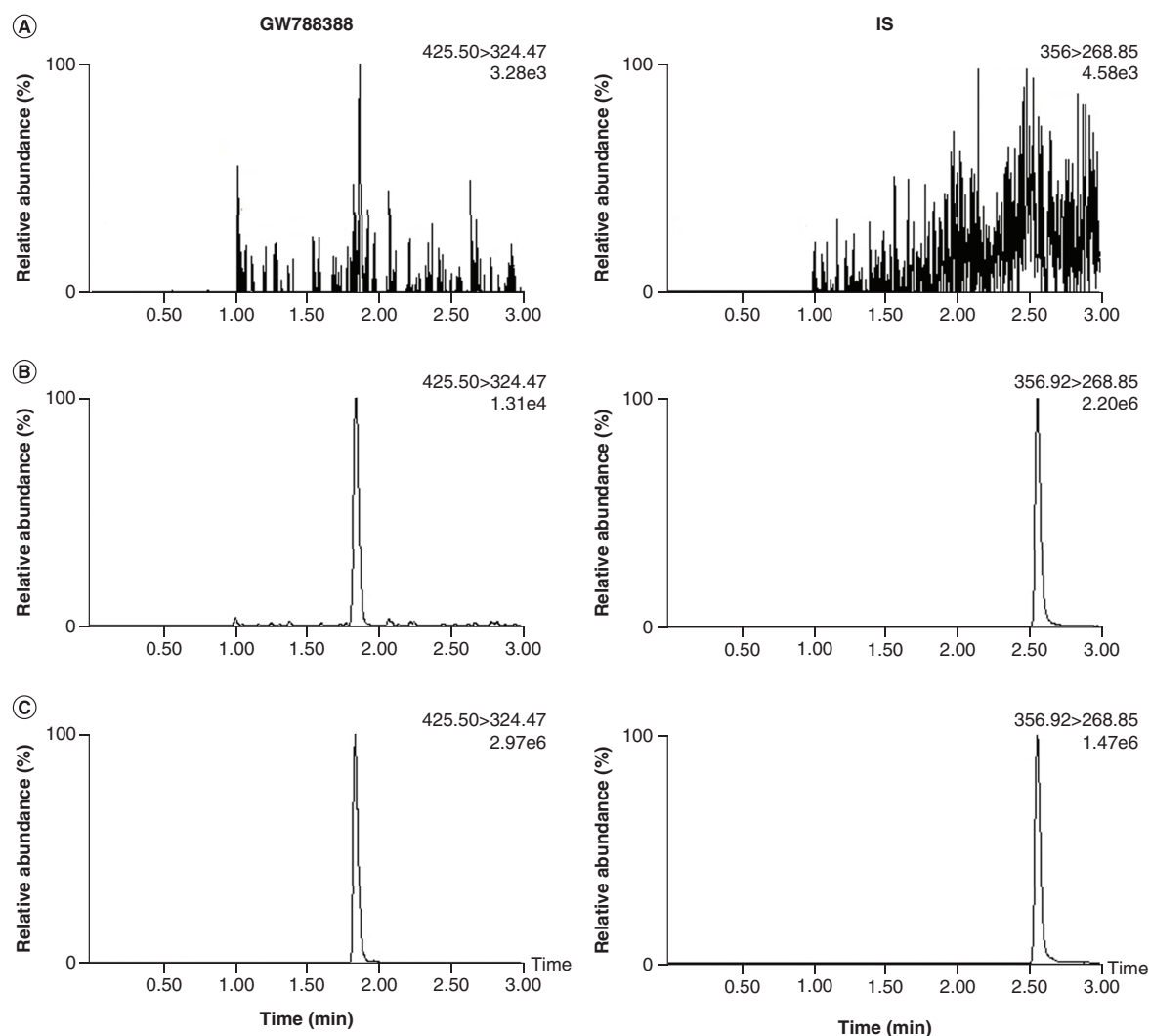


Figure 2. Representative chromatograms. (A) Blank plasma sample. **(B)** Blank plasma sample spiked with GW788388 (LLOQ) and IS. **(C)** Rat plasma sample collected at 2.0 h after dosing (the plasma concentration was 212.3 ng/ml). IS: Internal standard.

Table 1. Precision, accuracy, matrix effect and recovery for analysis of GW788388 in rat plasma (n = 6).

Spiked concentration (ng/ml)	Intraday precision CV (%)	Interday precision CV (%)	Accuracy (RE%)	Matrix effect (% mean $\pm$ SD)	Recovery (% mean $\pm$ SD)
1.0	7.3	8.9	-17.5	–	–
3.0	8.0	7.0	-11.8	103.5 $\pm$ 5.9	91.2 $\pm$ 8.0
200	6.3	4.8	11.7	99.3 $\pm$ 8.3	92.7 $\pm$ 9.2
1000	7.4	6.4	10.8	101.0 $\pm$ 3.6	93.5 $\pm$ 5.8
IS	–	–	–	104.2 $\pm$ 11.3	90.0 $\pm$ 3.1

IS: Internal standard; RE: Relative error.

M and H QCs, and within -17.5 for LLOQ. The intra and interday precisions for GW788388 were <8.9% for LLOQ, and 4.8–8.0% for the other QC concentration levels.

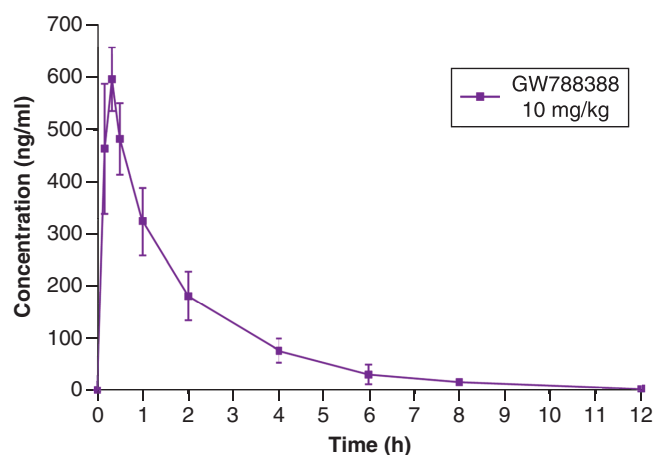
Recovery & matrix effect

Sample treatment by protein precipitation with acetonitrile produce a satisfactory extraction recovery. The percentage recoveries of GW788388 and IS spiked in rat plasma were found to be >90.0%. Table 1 shows the average

Table 2. Stability of GW788388 in rat plasma (n = 3).

Conditions	GW788388		
	Spiked (ng/ml)	RE (%)	CV (%)
30 days at -25°C	3.0	11.7	6.5
	200	-8.2	4.7
	1000	-11.8	9.4
Three freeze-thaw cycles	3.0	-7.3	6.5
	200	11.6	9.7
	1000	12.9	5.9
12 h at room temperature	3.0	-5.1	5.0
	200	12.6	9.3
	1000	9.9	9.1
4°C in autosampler for 12 h in processed samples	3.0	3.9	3.3
	200	-10.3	9.9
	1000	-8.2	5.9

RE: Relative error.

**Figure 3.** Mean plasma concentration-time curve of GW788388 after a single oral dose (10 mg/kg bodyweight) in rats (n = 6).

matrix effects obtained for three QC concentrations of GW788388. The data within 99.3–103.5% suggested that there was no measurable matrix effect interfering with the detection of GW788388 in rat plasma.

Stabilities

GW788388 and IS were stable in stock solution for 3 weeks at 4°C. The accuracy (RE%) of GW788388 and IS were ranged from -4.6 to 5.8%, with precision (CV%) of 6.7 and 4.9%, respectively. Table 2 summarized the stability data of GW788388 at L, M and H QC levels after storage. The accuracy (RE%) of the spiked samples were ranged from -11.8 to 12.9%. In rat plasma, GW788388 was stable at -25°C for 30 days, at room temperature for 12 h, and after three freeze-thaw cycles. The processed samples in autosampler remained intact at 4°C for 12 h.

Pharmacokinetic application

The developed and validated method was successfully used for the first time to describe the pharmacokinetic profile of GW788388 in rats after an oral administration of 10 mg/kg. Plasma concentration data was processed based on noncompartmental model analysis with Phoenix WinNonlin software (version 8.0, Pharsight, CA, USA). peak concentration (C_{max}) and peak time (t_{max}) data were generated from the respective concentration-time curves. The elimination half-life equaled to $0.693/k_e$, here k_e was the constant of elimination rate. $AUC_{0 \rightarrow \infty}$ was generated by the trapezoidal rule. Apparent clearance rate (CL/F) was calculated from $D/AUC_{0 \rightarrow \infty}$, and apparent distribution volume (V_z/F) equaled to $D/(k_e \bullet AUC_{0 \rightarrow \infty})$, here D was the dose of administration.

The mean plasma concentration-time curve of GW788388 is shown in Figure 3. GW788388 was absorbed quickly and reached a peak concentration of 595.3 ± 60.2 ng/ml 20 min after dosing. The mean half-life was

Table 3. Pharmacokinetic parameters of GW788388 with noncompartmental method (n = 6).

Parameters	Units	Mean	SD	CV%
C _{max}	ng/ml	595.3	60.2	10.1
T _{max}	h	0.33	0	0
t _{1/2}	h	1.74	0.40	22.9
AUC _{0→12h}	h×ng/ml	1081.3	201.6	18.6
AUC _{0→∞}	h×ng/ml	1089.8	202.1	18.5
V _z /F	l/kg	23.86	6.93	29.0
CL/F	l/h/kg	9.46	1.84	19.5

t_{1/2}: Half-life.

found to be 1.74 ± 0.4 h. All the pharmacokinetic parameters including AUC, V_z/F and CL/F are displayed in Table 3.

Conclusion

A simple and sensitive UPLC–MS/MS method has been established and fully validated for the determination of GW788388 in rat plasma for the first time. The established analytical method was successfully applied to describe the pharmacokinetic profile of GW788388 after oral administration in rats.

Future perspective

GW788388 is a selective and orally active TGF-β1 receptor inhibitor that shows potent activity in renal and heart fibrosis. However, no detailed analytical method of GW788388 has been described and no pharmacokinetic study has been revealed so far. In the present study, a simple and sensitive UPLC–MS/MS method for quantification of GW788388 in rat plasma was established and validated and then utilized to describe the pharmacokinetic profiles after an oral administration of 10 mg/kg. This method is suitable for future preclinical and clinical studies such as pharmacokinetic studies and drug–drug interaction studies. Additionally, this method can be used as a reference for establishing a high-throughput bioassay and for therapeutic monitoring for GW788388 in human plasma.

Executive summary

Background

- As a selective TGF-β1 receptor inhibitor, GW788388 demonstrated preclinical activity for the fibrotic diseases such as hepatic and heart fibrosis.
- To describe the pharmacokinetic profile of GW788388, a simple and sensitive ultra-performance LC–MS/MS method for quantification of GW788388 in the biological matrices is urgently needed.

Methodology

- Plasma sample pretreatment was conducted by one-step protein precipitation extraction procedure with acetonitrile.
- The extracts were separated via an Acquity UPLC BEH C₁₈ column (Waters, USA), and detected with a Waters TQ-S mass spectrometer under positive ionization mode.

Results

- The bioanalytical method was fully validated over a linear concentration range of 1.0–1200 ng/ml. The extraction recoveries were 91.2, 92.7 and 93.5% for L, M and H quality control levels, respectively.
- GW788388 was absorbed quickly and reached a peak concentration of 595.3 ± 60.2 ng/ml 20 min after drug administration. The mean half-life was found to be 1.74 ± 0.4 h.

Conclusion

- The one-step protein precipitation extraction coupled with ultra-performance LC–MS/MS determination method provides a favorable character in selectivity, linearity, accuracy, precision, recovery, matrix effects and stabilities, and successfully applied for the quantification of GW788388 in real plasma samples of Sprague Dawley rats.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that the animal study was performed in accordance with the Guideline for Animal Experimentation of Shenyang Medical College, and the protocol was approved by the Animal Ethics Committee of the Institution.

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