Detection of sivelestat and its metabolite in small volumes of plasma from Chinese ALI/ARDS patients with SIRS via high-throughput UPLC-MS/MS: A pharmacokinetic study

Mingzhou Liu [a], Jing Zhang [b], Lingfang Dong [a], Wenhua Xue [c], Qilin He [e], Wenzhong Liang [e], Xing Liu [a], Jingying Zhang [a], Li Gu [a], Yinghua Feng [a], Jie Yang [a], Haibo Wang [b], Yaqin Wang [a], Kun Li [a], Yuanlong Li [a], Weiqin Kong [f], Xiaojian Zhang [d,*], Mengying Yao [c,*], Kai Wang [b,*], Peizhi Ma [a,*], Wei Zhang [a,*]

[a] Department of Pharmacy, Henan Provincial People's Hospital, Zhengzhou University People's Hospital, Zhengzhou, Henan, 450003, China
[b] Department of Respiratory and Critical Care Medicine, Henan Provincial People's Hospital, Zhengzhou University People's Hospital, Zhengzhou, Henan, 450003, China
[c] Department of Pharmacy, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, China
[d] Department of Respiratory and Critical Care Unit, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, China
[e] WuXi AppTec (Shanghai) Co., Ltd, Shanghai, China
[f] Umins (Wuhan) Pharmaceutical Co., Ltd, Wuhan, Hubei, 430000, China

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ABSTRACT

In this study, we developed a sensitive and efficient analytical approach combining a 96-well plate-based protein precipitation strategy with ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-MS/MS) in order to assess the pharmacokinetic (PK) properties of sivelestat and its metabolite XW-IMP-A in samples of plasma from ALI/ARDS patients with SIRS. The samples were separated via gradient elution with a C18 column (Phenomenex Kinetex, C18, 2.6 μm, 100 Å, 50 × 2.1 mm) using 0.1 % formic acid aqueous solution (A) and acetonitrile-methanol (1:1, V:V) (B) as a mobile phase at a 0.6 mL/min flow rate. UPLC-MS/MS spectra were generated in positive ion mode, and multiple reaction monitoring (MRM) was used to detect the following transitions: m/z 435.1 → 360.0 for sivelestat, m/z 469.0 → 394.0 for sivelestat-IS, m/z 351.0 → 276.0 for XW-IMP-A, and m/z 384.9 → 310.0 for XW-IMP-A-IS. This assay was run for 2.5 min in total, and achieved lowest limit of quantitation values of 2.0 ng/mL and 0.5 ng/mL for sivelestat and XW-IMP-A, respectively, while remaining highly linear from 2−500 ng/mL for sivelestat ($r^2 \geq 0.9900$) and from 0.5−125 ng/mL for XW-IMP-A ($r^2 \geq 0.9900$). These validated data were consistent with US Food and Drug Administration (FDA) and European Medicines Agency (EMA) acceptance criteria. In addition, this method was successfully applied to the steady-state PK evaluation of ALI/ARDS patients with SIRS.

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1. Introduction

Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) are primary drivers of intensive care unit (ICU) admission and patient mortality. ALI can, if not effectively treated, progress to ARDS, which is a hallmark of H1N1 influenza [1], Middle East Respiratory Syndrome (MERS) [2], and severe acute respiratory syndrome (SARS) [2] infections. Notably, ARDS is the leading cause of death among patients suffering from COVID-19 [3,4], which is a pandemic form of pneumonia first characterized in Wuhan, China in December of 2019 that is caused by the SARS-CoV-2 virus [3]. Systemic inflammatory response syndrome (SIRS) can often occur in patients who are critically ill, and these patients are also at a higher risk of ALI/ARDS. Large quantities of cytokines are generated in patients suffering from these conditions, resulting in direct or indirect tissue damage and consequent organ failure mediated in part by neutrophil activation and the secretion of cytotoxic molecules such as neutrophil elastase (NE) [5,6]. Sivelestat is a selective NE inhibitor that has been used to reduce ICU admission duration and

time of ventilator use in ALI/ARDS patients, making it the only specific treatment for ARDS caused by NE-mediated endothelial injury that is currently available [7,8]. As sivelestat is able to selectively inhibit NE while leaving the activity of other proteases unaffected, it may represent an ideal therapeutic tool for reducing lung injury severity in ALI/ARDS patients [9–14]. Both the efficacy of this compound and the frequency of adverse reactions associated with its use are likely to be concentration-dependent, and steady-state plasma concentration (Css) levels represent potent biomarkers that can be used to guide individualized treatment [15,16]. To ensure that Css values lie within a targeted therapeutic window, it is important that pharmacokinetic (PK) evaluations and therapeutic drug monitoring (TDM) be performed in treated patients [17,18]. However, owing to the large number of samples generated in clinical studies, sample preparation/processing, and detection sensitivity have become a bottleneck for high-throughput analysis efforts. A number of papers have been published describing the success of 96-well plate-based liquid-liquid extraction (LLE) for the high-throughput analysis of plasma samples [19–21]. Additionally, ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-MS/MS) systems utilize high linear velocities and columns packed with sub-3 μm shell particles coupled to a tandem mass spectrometer, yielding an extremely powerful approach that dramatically improves peak resolution, sensitivity, and speed of associated bioanalytical analyses of drug substances in biological matrices. To support clinical studies, 96-well plate-based assays were developed to handle the large number of samples generated and to match with the throughput of the UPLC-MS/MS assay. The employment of UPLC-MS/MS combined with 96-well-formatted LLE technology was a result of the optimization of sample size, preparation throughput, MS sensitivity, ease of use, and ease of automation requirements [19–21]. A promising HPLC-MS/MS method has been proposed by Wang Jing et al. [22] as a means of measuring sivelestat and its metabolite in the plasma of Chinese healthy volunteers. Plasma samples (100 μL) were manually prepared via LLE in individual test tubes to achieve a sensitivity of 10.0 and 2.50 ng/mL for sivelestat and its metabolite, with a total run time of 4.7 min. Relative to previously published methods, the present approach based upon UPLC-MS/MS combined with semi-automated 96-well-formatted LLE technology offers the advantages of higher sensitivity (2.0 and 0.5 ng/mL for sivelestat and its metabolite) and shorter total chromatographic run time (1.4 min), thereby facilitating high-throughput sample analysis. Manual extraction in discrete tubes may be sufficient, as this process was limited by overall throughput. Herein, a semi-automated 96-well plate format LLE was used and was found to significantly reduce manual labor requirements and the overall time required to conduct the associated analysis. Importantly, this approach also facilitated a reduction in the use of hazardous solvents and ensured personal safety. As such, in the present study, we describe the development of a simplified UPLC-MS/MS approach that leverages a 96-well plate-based one-step protein precipitation procedure. Using this strategy, we were able to quickly and reliably measure levels of sivelestat and its metabolite in small volume (100 μL) plasma samples, and we were able to successfully utilize this approach to conduct the steady-state PK evaluation of sivelestat and its metabolite in Chinese ALI/ARDS patients with SIRS, indicating that this strategy is amenable to other PK studies and TDM applications.
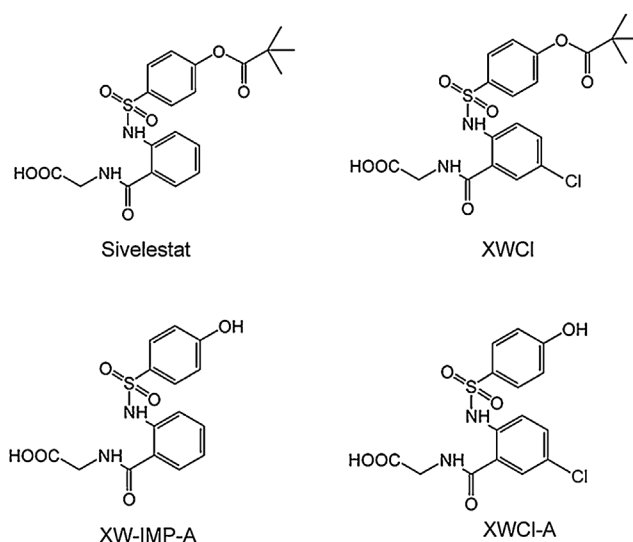

Fig. 1. Chemical structures of sivelestat, XWCl(sivelestat-IS), XW-IMP-A, and XWCl-A(XW-IMP-A-IS).

## 2. Materials and methods

2.1. Chemicals and reagents

Sivelestat (> 99.5 % pure, HPLC-grade), XW-IMP-A (> 99.5 % pure, HPLC-grade), sivelestat-IS (> 97.6 % pure, HPLC-grade), XW-IMP-A-IS (> 97.4 % pure, HPLC-grade) were from Shanghai Huilun Life Science & Technology Co,. Ltd. (Shanghai, China). Chromatographic-grade acetonitrile and formic acid were from Sigma-Aldrich (MO,USA). An ELGA water purification system (MA, USA) was used to prepare water for all analyses. Analytical grade citric acid was from the Shanghai Chemical Reagent Corporation. Chemical structures of both analytes (sivelestat and XW-IMP-A) and internal standards (sivelestat-IS and XW-IMP-A-IS) are shown in Fig. 1.

2.2. UPLC-MS/MS

For these analyses, a UPLC-NEXERA platform (LC-30AD, Shimadzu, Kyoto, Japan) with a quaternary pump, degasser, autosampler, and column oven was employed. A Phenomenex Kinetex column (2.6 μm, C18, 100 Å, 100 × 2.1 mm, Phenomenex, CA, USA) was used for chromatographic separation at a temperature of 20–25 °C. For this analysis, a mobile phase composed of 0.1 % formic acid (A) and acetonitrile/methanol (1:1, V:V) (B) was used, with gradient settings being detailed in Table 1. A constant flow rate of 0.6 mL/min was used during elution, with 3 μL and 5 μL injections being used for sivelestat and XW-IMP-A, respectively. Under these elution conditions, we found that sivelestat, sivelestat-IS, XW-IMP-A, and XW-IMP-A-IS exhibited respective retention times of approximately 1.0, 1.4, 0.9, and 1.4 min. An API 6500 triple quadrupole instrument (Applied Biosystems, Concord, Ontario, Canada) was used for MS analyses, with electrospray ionization being conducted in multiple reaction-monitoring (MRM) mode with positive ionization. Solvent evaporation was performed under nitrogen, and the following settings were utilized for compound-dependent MS analyses:

Table 1
UPLC gradient for separation of the analytes in human plasma.

Run Time (min)	Flow rate (μL/min)	Sivelestat		XW-IMP-A	
		Phase A %	Phase B %	Phase A %	Phase B %
0.20	600	45	55	65	35
1.50	600	25	75	45	55
1.51	600	5	95	5	95
2.00	600	5	95	5	95
2.01	600	45	55	65	35
2.50	600	45	55	65	35
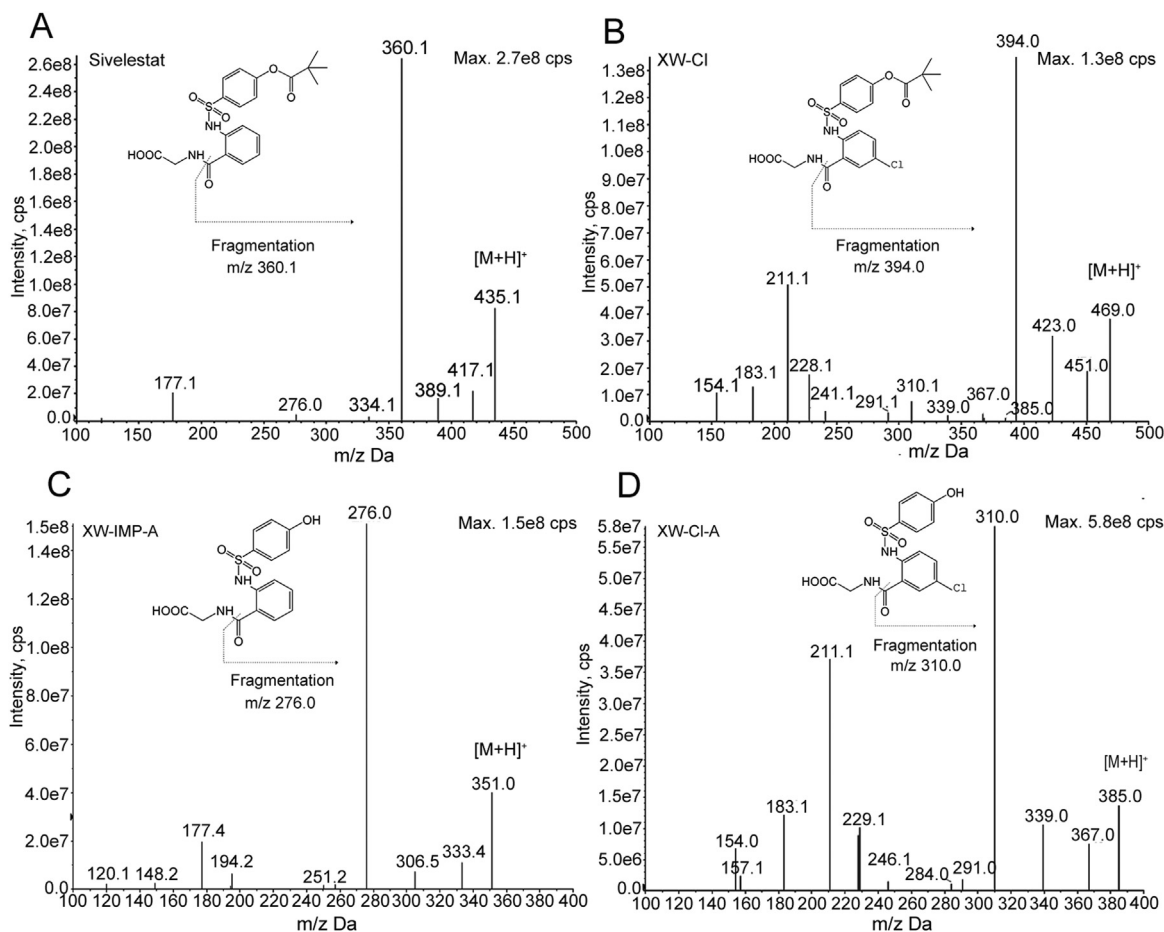
Fig. 2. Positive ion spectra for sivelestat (A), XWCl (B), XW-IMP-A (C) and XWCl-A (D).

Table 2
Optimized mass parameters for sivelestat, XW-IMP-A and the internal standards.

Analyte	MRM (*m/z*)	Dwell time (ms)	DP (V)	CE (eV)	EP (V)	CXP (V)
Sivelestat	435.1→360.0	100	50	22	10	11
XWCl	469.0→394.0	100	50	22	10	11
XW-IMP-A	351.0→276.0	100	50	22	10	11
XWCl-A	384.9→310.0	100	50	22	10	11

Note: DP: declustering potential, CE: collision energy, EP: entrance potential, CXP: collision cell exit potential, ms: millisecond.

turbo-gas temperature, 450 °C; ion-spray needle voltage, 5500 V; curtain gas, 30 psi; nebulizer gas, 50 psi; heater gas, 60 psi. For quantification purposes, the following precursor-to-product ion transitions were used: m/z 435.1→360.0 for sivelestat, m/z 469.0→394.0 for XWCl (sivelestat-IS), m/z 351.0→276.0 for XW-IMP-A, and m/z 384.9→310.0 for XWCl-A (XW-IMP-A-IS). Typical full-scan positive ion ESI mass spectra corresponding to the four compounds are shown in Fig. 2, while corresponding MS parameters are compiled in Table 2.

2.3. Sample preparation

Samples were initially thawed on ice and vortexed, after which a 96-well plate-based approach to rapid sample protein precipitation was performed. Briefly, 100 μL aliquots of fresh calibration samples (eight concentrations), QC samples (three concentrations), and prepared plasma samples from patients were added to appropriate wells, after which 50 μL of sivelestat-IS (100 ng/mL) or XW-IMP-A-IS (15 ng/mL) were added to all wells other than double-blank

wells to which an equivalent volume of 50 % isopropanol in water was instead added. Plasma protein was then precipitated by adding 400 μL of acetonitrile to each sample well, after which plates were sealed, briefly vortexed, and spun for 10 min at 6200 rpm at 4 °C. Supernatant samples (50 μL each) were then transferred to a fresh 96-well plate, and 450 μL of 50 % acetonitrile in water was added per well. Plates were then sealed, vortexed, and appropriate sample volumes (3 μL for sivelestat and 5 μL for XW-IMP-A) was directly injected into the UPLC-MS/MS system as detailed above.

2.4. Calibration and QC sample preparation

Sivelestat and XW-IMP-A reference standards were dissolved using 50 % isopropyl alcohol (IPA) at 1.00 mg/mL in order to prepare stock solutions, which were subsequently diluted serially with 50 % IPA to 40–10000 ng/mL (sivelestat) or 10–2500 ng/mL (XW-IMP-A). For IS solution preparation, 50 % IPA was used to dilute sivelestat-IS and XW-IMP-A-IS to 100 ng/mL and 15 ng/mL, respectively. Calibration curves were prepared by adding 50 μL of these IS working solutions to untreated human plasma samples to yield samples with concentrations of 2, 4, 10, 40, 100, 260, 400, and 500 ng/mL (sivelestat) or 0.5, 1, 2.5, 10, 25, 65, 100, and 125 ng/mL (XW-IMP-A). QC samples were prepared in an identical manner, with final sivelestat concentrations of 6, 240, and 380 ng/mL, and with final XW-IMP-A concentrations of 1.5, 60, and 95 ng/mL for XW-IMP-A. All prepared solutions were divided into aliquots and maintained at 4 °C prior to UPLC-MS/MS analysis.

3. Methodological validation

The assays were partially validated according to existing US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines [23,24]. The selectivity, linearity, lower limit of quantification (LLOQ), accuracy and precision, stability, extraction recovery, and matrix effect of our methodological approach were validated as detailed below.

3.1. Selectivity

The specificity of our approach was assessed by analyzing six samples of human plasma from different sources that were not supplemented with any analytes or IS compounds. The resultant chromatograms were compared to chromatograms of pre-dosing plasma samples containing analyte concentrations close to the LLOQ, and to post-dosing plasma samples collected from an actual pharmacokinetic study of a patient following the administration of sivelestat so as to guarantee that there were no interfering peaks associated with the sivelestat, XW-IMP-A, or ISs retention times in these samples.

3.2. Linearity

The analyte-to-IS peak area ratio (y) was plotted against the concentration of that analyte (x) in calibration samples to evaluate the linearity of our methods. A weighted linear regression ($1/x^2$) model was used to generate calibration curves assessing the relationship between these two variables. All calibration curves yielded correlation values of 0.990 or higher.

3.3. Lower limit of quantification

The LLOQ was the lowest calibration curve concentration that yielded a signal-to-noise ratio (S/N) > 10. This value was determined using six independently prepared sets of standard samples, and was established by assessing precision (relative standard deviation;

%RSD) and accuracy (percentage bias against the nominal concentration; %RE). These precision and accuracy values were considered satisfactory when they were ± 20 %.

3.4. Accuracy and precision

To determine the intra-day accuracy and precision, replicate analyses of plasma samples containing sivelestat and its metabolite were performed on the same day. For these analyses, a calibration curve and six replicates of LLOQ, LQC, MQC, and HQC samples were evaluated. The inter-day accuracy and precision were assessed by analyzing six precision and accuracy batches on three consecutive validation days (n = 18 total). Accuracy was expressed as the relative error (RE) between the mean measured concentration and the nominal concentration (expressed as the percentage of nominal values), and the intra-day and inter-day precision values were evaluated based upon relative standard deviation (%RSD). The criteria used to assess the suitability of accuracy and precision were as follows: the relative error should be within ± 15 % and the RSD should not exceed 15 %.

3.5. Stability

Analyte stability was assessed by evaluating six QC sample replicates (high and low concentrations) following storage under a range of conditions. Short-term stability was assessed by evaluating samples following 24.1 h storage on ice, while long-term stability was assessed by freezing samples for 178 days at −20 °C and −80 °C. Stability following treatment was established by analyzing samples stored for 136.2 h at 6 °C for 136.2 h, and freeze-thaw stability was assessed by thawing frozen samples and refreezing them at −20 °C or −80 °C five times prior to analysis. QC sample concentrations obtained following these tests were compared to the nominal values, and the percentage concentration deviation was determined. Analytes were deemed stable when this ratio was in the 85–115 % range.

3.6. Extraction recovery and matrix effects

To investigate extraction recovery, a set of plasma samples (n = 6 at each concentration) spiked with sivelestat and XW-IMP-A at three QC concentration levels (low, medium, and high) and ISs at the working concentration was processed. A second set of samples was prepared by spiking analytes and ISs with these same concentrations into post-extracted blank plasma samples. The extraction recovery of sivelestat, XW-IMP-A, and ISs were measured by calculating the individual peak area of the pre-extraction spiked samples to the mean area of the samples spiked after extraction. The impact of matrix effects was evaluated at low and high QC levels for each analyte and each assayed IS concentration. Two sets of samples were prepared by directly spiking the analytes and IS into the reconstitution solution with or without the presence of residues extracted from six different lots of plasma. The matrix effects of each analyte and IS were evaluated by comparing the peak area of the post-extracted samples from each lot with the peak area of neat solutions at an equivalent concentration. The IS-normalized matrix effect was calculated by dividing each analyte matrix effect by the IS matrix effect. Furthermore, matrix effects in hemolysed and hyperlipidemic plasma for low and high QC sample concentrations (n = 3 at each concentration) were assessed. Precision and mean bias values were considered to be acceptable when ± 15 % of respective nominal concentrations.

3.7. Pharmacokinetic analysis

The methods described above were used to assess sivelestat and XW-IMP-A concentrations in plasma obtained from patients that had undergone continuous intravenous infusion with sivelestat sodium (0.2 mg/kg/h) for a maximum of 14 days. We analyzed 10 Chinese ALI/ARDS patients with SIRS for this PK study, which was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhengzhou University and Henan Provincial People's Hospital (*NO. CTR20182517*, http://www.chinadrugtrials.org.cn). ALI/ARDS were defined as per the American-European Consensus Conference definition [25,26]. Subjects meeting study inclusion criteria provided informed consent prior to participation, and this study protocol was consistent with the Declaration of Helsinki. During screening, patient vital signs, laboratory findings, chest radiographs, electrocardiogram, blood gas analysis, and ventilator use were assessed. In addition, human chorionic gonadotropin (HCG) levels were measured in women of childbearing age. Patients were admitted to the intensive care unit (ICU), and 4 mL blood samples were collected before dosing (0 h) and at 24, 48, 72, and 120 h post-dosing. The longest infusion period was 14 days. Patient plasma was obtained by collecting blood in heparinized tubes and spinning for 10 min at 1500 rpm at 4 °C. Plasma was then stored at −80 °C prior to analysis.

3.8. Statistical analysis

Data were analyzed using GraphPad Prism 5.0, and are given as means ± standard deviation (SD). $P < 0.05$ was this significance threshold for these analyses.

4. Results and discussion

4.1. Optimization of LC–MS/MS conditions

4.1.1. Chromatographic optimization

We initially tested a range of different reversed-phase UPLC and mobile phase columns in order to identify optimal chromatographic behaviors and ionization responses for sivelestat, XW-IMP-A, and IS compounds. This ultimately led us to utilize a Phenomenex Kinetex column (2.6 μm C18 100 Å, 100 × 2.1 mm, Phenomenex, Torrance CA) for the separation of these analytes via a gradient elution approach. Additionally, several tests were performed to optimize mobile phase composition in order to achieve symmetric chromatographic peak shapes and maximal resolution. The experimental results revealed that the addition of 0.1 % (V:V) formic acid to the aqueous portion of the mobile phase was sufficient to improve mass spectrometry ionization efficiency and to enable symmetric peak shapes. The experimental results revealed that increasing the ratio of methanol in the mobile phase may significantly improve the ionization efficiency of these analytes, and the MS response to these analytes was increased. When the methanol ratio in the organic phase exceeded 50 %, however, interference of plasma analytes was encountered, and the same was true at a similar acetonitrile ratio in the mobile phase. High sensitivity, good analyte separation, and short run times were ultimately achieved by using a 0.1 % formic acid aqueous solution (A) and acetonitrile-methanol (1:1, V:V) (B) as a mobile phase under ESI conditions. A constant flow rate of 0.6 mL/min was used during separation, and the retention times for sivelestat, sivelestat-IS, XW-IMP-A, and XW-IMP-A-IS were 1.0, 1.4, 0.9, and 1.4 min, respectively (Figs. 3 and 4).

4.1.2. MS–MS optimization

To optimize MS assay conditions, IS, sivelestat, and XW-IMP-A standard solutions (10 ng/mL) were directly introduced into the MS detector via elecrospray ionization, and MS parameters were adjusted to yield optimal ionization responses. Analyte precursor ion concentrations were detected in full scan mode, and ESI mass spectra (Fig. 2) revealed that all analytes exhibited a single dominant ion corresponding to the protonated molecule [M+H]$^+$. The [M+H]$^+$ ions at m/z 435.1, 469.0, 351.0, and 395.0 were thus identified as sivelestat, XW-IMP-A, and IS precursor ions, respectively. Product ions were identified in production scan mode, and the most appropriate collision energy values were determined based upon analyses of responses for the fragment ion peak m/z. Full-scan product-ion spectra are shown in Fig. 2, highlighting predominant fragment ion peak m/z 360.1, 394.0, 276.0, and 310.0 for sivelestat, XW-IMP-A, sivelestat-IS, and XW-IMP-A-IS, respectively. As such, precursor-to-product ion transitions were monitored at m/z 435.1→360.0; 469.0→394.0 for sivelestat and sivelestat-IS (IS), and at m/z 351.0→276.0; 384.9→310.0 for XW-IMP-A and XW-IMP-A-IS (IS). Target compounds were determined in MRM mode, such that ions unrelated to these target compounds were filtered out to yieldclean ion chromatograms.

5. Methodological validation

5.1. Selectivity

Selectivity was evaluated via the comparison of chromatograms produced from blank plasma samples to those from samples spiked with LLOQ concentrations of sivelestat, XW-IMP-A, and IS, or to those from patients at 24 h post-sivelestat administration. This analysis revealed good separation among these compounds, with retention times of 1.0, 1.4, 0.9, and 1.4 min for sivelestat, sivelestat-IS, XW-IMP-A, and XW-IMP-A-IS, respectively (Figs. 3 and 4). No interference peaks with retention times similar to those of these analytes were observed, consistent with the selectivity and specificity of this approach.

5.2. Linearity and sensitivity

A linear regression approach with a $1/x^2$ weighting factor was used to successfully generate sivelestat and XW-IMP-A calibration curves with mean correlation coefficient values > 0.99. For these two analytes, the resultant regression equations were y = 0.03742 x + 0.01854 (r^2 = 0.9959) and y = 0.1368 x + 0.01232 (r^2 = 0.9972), respectively, with x and y corresponding to analyte plasma concentration and the analyte-to-IS peak area ratio, respectively. For sivelestat and XW-IMP-A, LLOQ (S/N > 10) values of 2 ng/mL and 0.5 ng/mL were calculated, respectively, allowing for effective sample detection. Results of intra- and inter-day precision and accuracy analyses of the LLOQ are shown in Table 3, and a representative LLOQ sample chromatogram is shown in Figs. 3B and 4 B.

5.3. Accuracy and precision

Intra- and inter-day precision and accuracy analysis results for these four analytes revealed that this method was precise and accurate (Table 2), as all results were within the range proscribed by appropriate guidelines [27].

5.4. Analyte stability

We confirmed acceptable stability values (± 15 %) for sivelestat and XW-IMP-A under a range of conditions (Table 4), confirming both of these compounds to be stable within QC samples. No significant sample deterioration was detected following storage on ice for 24 h, storage at −20 °C or−80 °C for 178 days, storage at 6 °C for 136.2 h, or five repeated freeze-thaw cycles at −20 °C or−80 °C.
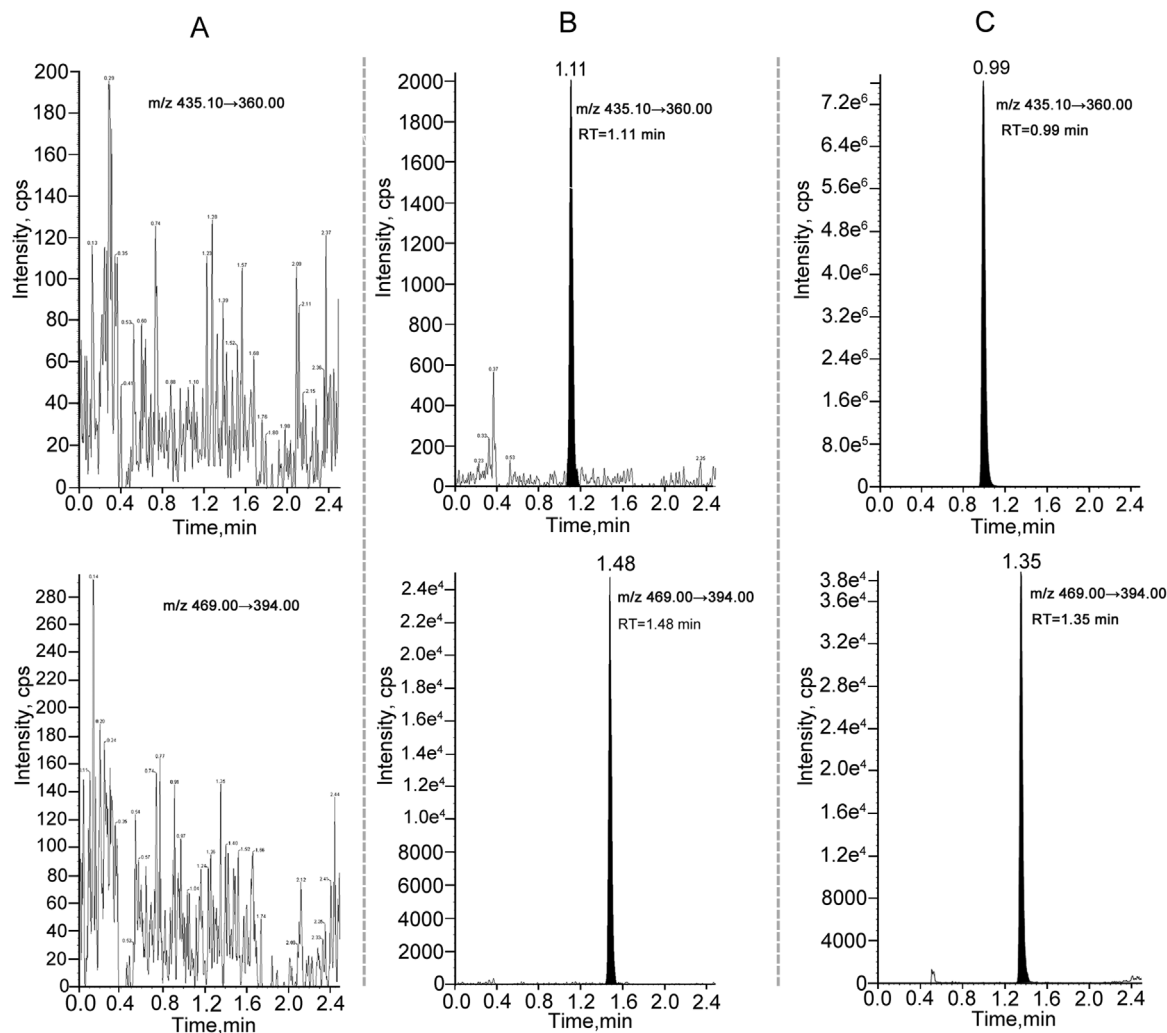
Fig. 3. Typical chromatograms for blank plasma samples (A), blank plasma spiked at the LLOQ with sivelestat (2 ng/mL) and IS (100 ng/mL) (B), and patient plasma samples at 24 h post-sivelestat administration (C).

Table 3
Intra- and inter-day precision and accuracy.

| DAY	Index	Sivelestat				XW-IMP-A			
		LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC
Intra-day (day 1)	Nominal concentration (ng/mL)	2.00	6.00	240.00	380.00	0.50	1.50	60.00	95.00
	Observed concentration (ng/mL, Mean ± SD, n = 6)	2.04 ± 0.191	6.86 ± 0.338	243 ± 3.74	408 ± 17	0.465 ± 0.0314	1.72 ± 0.0763	61.9 ± 1.58	101 ± 3.85
	Precision (RSD,%)	9.40	4.9	1.5	4.2	6.80	4.4	2.6	3.8
	Accuracy (RE, %)	2.00	14.3	1.3	7.4	−7.00	14.70	3.20	6.3
Intra-day (day 2)	Nominal concentration (ng/mL)	2.00	6.00	240.00	380.00	0.50	1.50	60.00	95.00
	Observed concentration (ng/mL, Mean ± SD, n = 6)	2.13 ± 0.124	6.46 ± 0.322	262 ± 6.74	397 ± 9.62	0.487 ± 0.0630	1.54 ± 0.0412	64.2 ± 1.90	99.30 ± 1.61
	Precision (RSD,%)	5.80	5.0	2.6	2.4	12.90	2.7	3.0	1.6
	Accuracy (RE, %)	6.50	7.7	9.2	4.5	−2.60	2.7	7.0	4.5
Intra-day (day 3)	Nominal concentration (ng/mL)	2.00	6.00	240.00	380.00	0.5	1.50	60.00	95.00
	Observed concentration (ng/mL, Mean ± SD, n = 6)	2.00 ± 0.102	6.48 ± 0.206	246 ± 4.55	387 ± 9.31	0.517 ± 0.0255	1.59 ± 0.0871	62.0 ± 1.58	96.4 ± 2.56
	Precision (RSD,%)	5.10	3.20	1.80	2.40	4.90	5.50	2.50	2.70
	Accuracy (RE, %)	0.01	8.00	2.50	1.80	3.40	6.00	3.30	1.50
Inter-day	Nominal concentration (ng/mL)	2.00	6.00	240.00	380.00	0.5	1.50	60.00	95.00
	Observed concentration (ng/mL, Mean ± SD, n = 18)	2.06 ± 0.147	6.60 ± 0.337	250 ± 9.74	397 ± 14.8	0.489 ± 0.0461	1.62 ± 0.102	62.7 ± 1.94	99.0 ± 3.39
	Precision (RSD,%)	7.1	5.1	3.9	3.7	9.4	6.3	3.1	3.4
	Accuracy (RE, %)	3	10	4.2	4.5	−2.2	8	4.5	4.2
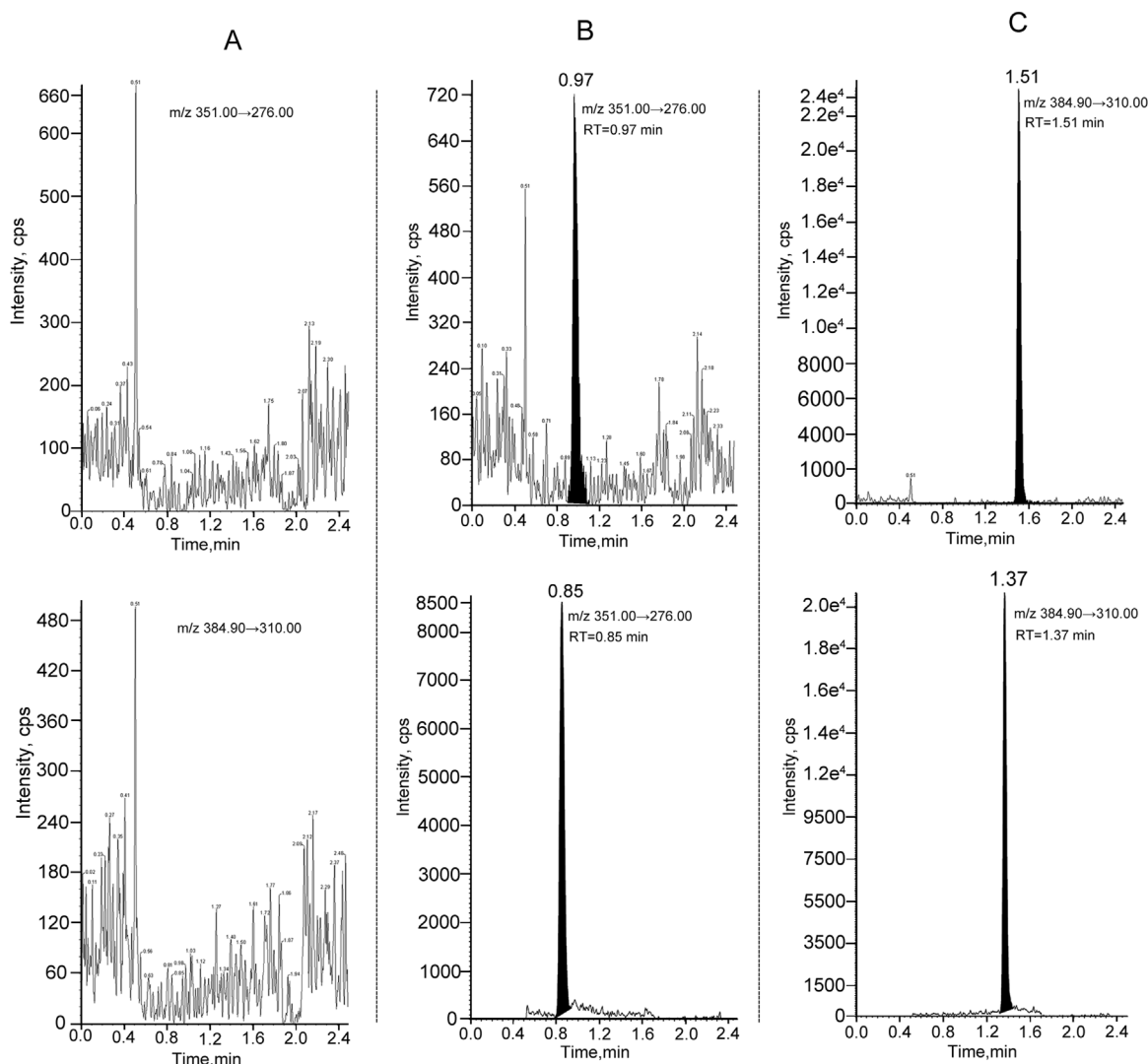
Fig. 4. Typical chromatograms for blank plasma samples (A), blank plasma spiked at the LLOQ with XW-IMP-A (0.5 ng/mL) and IS (15 ng/mL) (B), and patient plasma samples at 24 h post-sivelestat administration (C).

Table 4
Stability of Sivelestat and XW-IMP-A under differrent stability conditions.

	Stability conditions	Sivelestat			XW-IMP-A			
	Nominal concentration (ng/mL)	Observed concentration (ng/mL)	RSD (%)	RE (%)	Nominal concentration (ng/mL)	Observed concentration (ng/mL)	RSD (%)	RE (%)
Bench-top stability	Wet ice bath for 24.1 h	6	6.36 ± 0.136	106.0	6.0	1.5	1.49 ± 0.109	99.3	−0.7
		380	405 ± 11.4	106.6	6.6	95	101 ± 3.07	106.3	6.3
Long-term storage stability	(−20 °C for 178 days)	6	6.51 ± 0.272	108.5	8.5	1.5	1.72 ± 0.0931	114.7	14.7
		380	408 ± 6.93	107.4	7.4	95	106 ± 1.17	111.6	11.6
	(−80 °C for 178 days)	6	6.61 ± 0.175	110.2	10.2	1.5	1.68 ± 0.0690	112.0	12.0
		380	411 ± 10.7	108.2	8.2	95	102 ± 1.83	107.4	7.4
Freeze-thaw stability	(−20 °C for 5 cycles)	6	6.40 ± 0.290	106.7	6.7	1.5	1.57 ± 0.115	104.7	4.7
		380	407 ± 20.4	107.1	7.1	95	103 ± 4.55	108.4	8.4
	(−80 °C for 5 cycles)	6	6.72 ± 0.180	112.0	12.0	1.5	1.57 ± 0.111	104.7	4.7
		380	412 ± 11.6	108.4	8.4	95	102 ± 2.06	107.4	7.4
Post-preparative stability	6 °C for 136.2 h	6	6.41 ± 0.128	106.8	6.8	1.5	1.56 ± 0.0387	104.0	4.0
		380	398 ± 13.3	104.7	4.7	95	99.2 ± 4.12	104.4	4.4

5.5. Extraction recovery and matrix effect

At low, intermediate, and high QC sample concentrations, extraction recovery rates for sivelestat were 96.2 ± 4.59 %, 98.1 ± 3.29 %, and 100.5 ± 5.89 % in human plasma, respectively, while for

XW-IMP-A these respective values were 94.6 ± 7.73 %, 95.2 ± 1.82 %, and 95.4 ± 0.79 %. For the sivelestat-IS and XW-IMP-A-IS samples (*n* = 6), mean IS-normalized matrix factors were 1.03 ± 0.05, 0.98 ± 0.03 for sivelestat at low and high QC sample concentrations, with respective percent coefficient of variation (%CV) values of 5.0
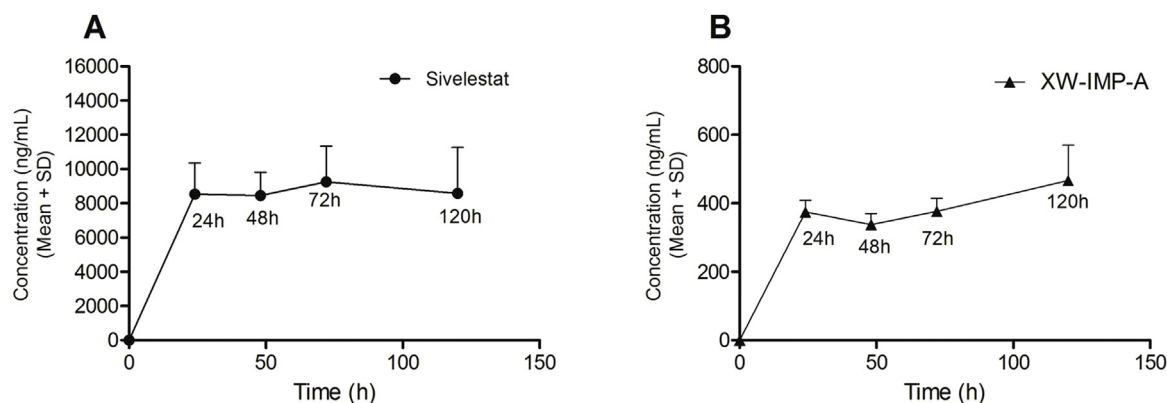
Fig. 5. The Mean plasma concentration-time profiles of analytes in human plasma following the continuous i.v. infusion of sivelestat sodium (0.2 mg/kg/h) for a maximum of 14 days. The data were from 10 Chinese ALI/ARDS patients with SIRS. Error bars represent the standard deviation ($n = 10$).

% and 2.5 %, while for XW-IMP-A these values were 0.98 ± 0.03, 1.01 ± 0.01 at low and high QC sample concentrations, with CV% values of 5.0 % and 2.5 %, respectively. These IS-Normalized matrix effect %CV values were within the acceptable range, and as such, no endogenous substances were found to significantly impact analyte ionization. As per FDA and EMA guidance, we also assessed matrix effects in hemolysed and hyperlipidemic plasma, and found that in all cases the calculated percent bias values in QC samples were < 5%, consistent with an absence of any significant matrix effects.

6. Pharmacokinetic study

Lastly, we successfully utilized this UPLC-MS/MS approach to assess sivelestat and XW-IMP-A steady-state PK dynamics in Chinese ALI/ARDS patients with SIRS following the continuous i.v. infusion of sivelestat sodium (0.2 mg/kg/h) for a maximum of 14 days (Fig. 5). We found that sivelestat and XW-IMP-A reached steady-state levels within the first day and that these levels were maintained through the 120 h study period. Mean Css values at 24, 48, 72, and 120 h were 8585.56 ± 1918.37, 8456.67 ± 1368.09, 9264.44 ± 2072.81, and 8577.78 ± 2692.27 ng/mL for sivelestat, and 374.22 ± 115.69, 338.67 ± 93.86, 376.56 ± 112.76, and 466.89 ± 308.25 ng/mL for XW-IMP-A in these patient plasma samples, with no evidence of clinically relevant drug accumulation.

7. Discussion

Steady-state PK analyses are a fundamental component of TDM efforts, which are commonly employed in clinical settings in order to ensure that patients receive an optimal drug dose that is efficacious but that does not rise to toxic levels or induce undesirable side effects [28,29]. Herein, we developed a straightforward, sensitive, and efficient UPLC-MS/MS protocol that was utilized to detect sivelestat and XW-IMP-A levels in samples of patient plasma. This approach yielded good linearity across concentration ranges of 1−500 ng/mL and 0.5−50 ng/mL for sivelestat and XW-IMP-A, respectively, and both of these compounds were detectable in the plasma of patients that had been continuously infused (i.v.) with sivelestat (0.2 mg/kg/h) for up to 14 days. No endogenous compound-related matrix effects were detected in this assay. This study is the first to describe the Css of sivelestat and its metabolite in Chinese ALI/ARDS patients with SIRS undergoing constant infusion via this dosing strategy. Our data suggest that this strategy may be deployed more broadly for steady-state PK analyses of sivelestat, and they may also serve as a useful reference for future safety and efficacy studies in ALI/ARDS patients. As we utilized a 96-well plate-based approach for sample preparation, our meth-

ods can be used to rapidly prepare many samples in parallel while reducing the risk of exposure to potentially harmful or infectious samples. The application of this strategy has the potential to guide future high-throughput clinical analyses of real-time drug exposureso as to facilitate the individualized treatment of hospitalized patients.

CRediT authorship contribution statement

Mingzhou Liu: Investigation, Formal analysis, Visualization, Formal analysis, Validation, Methodology, Writing - original draft. **Jing Zhang:** Investigation, Resources. **Lingfang Dong:** Investigation, Resources. **Wenhua Xue:** Investigation, Resources, Project administration. **Qilin He:** Investigation, Formal analysis, Resources, Data curation, Methodology. **Wenzhong Liang:** Investigation, Formal analysis, Resources, Data curation, Methodology. **Xing Liu:** Investigation, Resources. **Jingying Zhang:** Investigation, Resources. **Li Gu:** Investigation, Resources. **Yinghua Feng:** Investigation, Resources. **Jie Yang:** Investigation, Resources. **Haibo Wang:** Investigation, Resources. **Yaqin Wang:** Investigation, Resources. **Kun Li:** Investigation, Resources. **Yuanlong Li:** Investigation, Resources. **Weiqin Kong:** Data curation, Resources. **Xiaojian Zhang:** Supervision, Conceptualization, Writing - review & editing. **Mengying Yao:** Data curation, Resources. **Kai Wang:** Data curation, Resources. **Peizhi Ma:** Supervision, Conceptualization, Writing - review & editing. **Wei Zhang:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

Appropriate Ethics Committee approval has been obtained for the data reported. This work has not been submitted for publication elsewhere either in print or electronically, including on a web site, nor has it been accepted for publication elsewhere or is under consideration by another journal. All of the authors listed on the title page have read and agreed to its submission to *Journal of Pharmaceutical and Biomedical Analysis*, have met the criteria for authorship as established by the International Committee of Medical Journal Editors, believe that the paper represents honest work, and are able to verify the validity of the results reported. The authors declare no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and that there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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