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Robustness in bioanalysis

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Contents

Foreword

Speed, sensitivity and robustness are the three pillars of a successful bioanalytical assay, yet achieving the optimal balance between these elements remains one of the most complex challenges in bioanalysis.

In pursuit of speed, researchers often move towards simple, high-throughput sample clean up steps like protein precipitation. This 'good enough' approach often leads to large amounts of dirty matrix components entering the ion path of the mass spectrometer, resulting in deposition of these matrix components on the ion guides and quadrupoles. Along with the speed of sample extraction, short chromatographic run times to further reduce time of analysis and improve throughput contribute to a higher matrix load onto the mass spectrometer, accelerating the pace at which ions are deposited on the ion guides and quadrupoles.

Over time, this accumulation compromises instrument performance and eventually requires a deep clean of the mass spectrometer, which can result in the loss of 1-2 days of critical sample analysis, potentially compromising critical studies and delaying life-changing therapies from reaching patients who need them most.

This eBook demonstrates how intelligent analytical design and robust instrumentation can break this cycle. Through practical research articles and application notes, leading experts show how to achieve high throughput without sacrificing instrument longevity or data quality.

We hope you enjoy the eBook!

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Engineering robustness into LC–MS bioanalytical operations

Sally Hannam is the co-founder of Alderley Analytical (now part of Synexa Life Sciences; Macclesfield, UK) and a Director in Synexa's Scientific Strategies Team. With over 35 years of experience in bioanalysis using LC-MS, Sally is an expert in method development and method validation for quantitative LC-MS assays.

In this insightful interview, Sally Hannam shares her perspective on how bioanalytical workflows have evolved. From the introduction of novel technologies to practical strategies for managing instrument downtime, Sally provides a candid look at the real-world challenges and innovations shaping modern bioanalytical laboratories.

Sally Hannam
Co-founder
Alderley Analytical

How has bioanalysis evolved in the past 10 years, particularly regarding instrument reliability and performance?

LC–MS/MS instruments have continued to become more sensitive and robust over the past decade, with software that is suitable for validation in a regulated environment. For us, the triple quad mass spectrometer is the workhorse of bioanalytical study support. As long as the system has suitable sensitivity, then robustness and reproducibility are key. We are still using our first Waters TQ-S system that was installed in February 2014 at the beginning of the Alderley Analytical journey. The most significant development for us was the introduction of the Acquity Premier UPLC system coupled to the TQ-Absolute mass spectrometer. The Acquity Premier UPLC system features MaxPeak High Performance surfaces, which reduce unwanted analyte-surface interactions, resulting in consistent and reproducible results for even the most challenging analytes..

Recently, we developed an assay for a small molecule metal chelator and two metabolites. The reproducibility of reinjection was very poor on a conventional UHPLC system, and for six replicate injections of the parent compound from the same sample, a CV of greater than 20% was recorded on peak area ratios. We transferred the assay to the Waters Premier system and reinjected six replicates of the same sample. The CV achieved was less than 1% on peak area ratios. The surface also reduces non-specific binding for biological molecules, giving greater reproducibility for peptides, proteins and oligonucleotides.

Engineering robustness into LC–MS bioanalytical operations

Can you share a specific example of when you experienced unplanned instrument downtime in your lab and how it impacted ongoing studies?

Unanticipated instrument downtime is always a concern. Having to stop or delay sample analysis because the performance levels of the instrument have dropped can cause significant loss of revenue and increased workload on the team. We had an instrument that had to be decommissioned for a week. We should never have called it System 13! We had three identical LC–MS/MS systems, so we channeled the sample analysis work to the other two systems. The level of robustness we have experienced with the new Waters TQ Absolute XR where we are able to inject >30,000 injections of protein precipitated plasma without any compromise in instrument performance is unparalleled. Knowing that you can rely on the hardware for months and still achieve reliable data from the first injection to the last has been a game changer.

What processes and procedures have you implemented to minimize instrument downtime and ensure consistent analytical performance?

We have always mitigated the risk of instrument downtime affecting the delivery of data to customers by installing at least two identical systems in our LC–MS/MS laboratory. This is particularly important when supporting pivotal Phase I dose escalation studies. The pharmacokinetic data, along with the safety data, are often used in the decision-making process before proceeding to the next dose level in the clinical trial. We offer a 72-hour turnaround time from receipt of samples to delivery of quality-controlled data, so any delay from the bioanalytical laboratory can compromise the clinical study timelines.

All our LC–MS/MS systems are on service contracts with regular preventative maintenance schedules and we have weekly in-house cleaning procedures. A system suitability check is performed on each system prior to running a batch of study samples to ensure reproducibility, sensitivity and the absence of carryover from the top calibration standard.

Engineering robustness into LC–MS bioanalytical operations

When planning large bioanalytical studies that may run for weeks or months, do you worry about the change in instrument performance from the beginning to the end?

As a GLP/GCP-accredited laboratory, our typical mass spectrometry projects include method development/feasibility studies, validation in various matrices to regulatory guidelines, and subsequent sample analysis studies. Samples are typically analyzed in small batches of around 100. The maximum batch size of each method is established during the validation process. The batch size can be larger than 100 samples if it has been demonstrated that the method is suitably robust and accuracy and precision is maintained throughout the bioanalytical run, with acceptable QC samples bracketing the unknown samples.

We may change the assays on each instrument on a daily basis. We need the systems to be robust enough to change between methods without a drop in performance. This can mean changing from quantification of a small molecule in electrospray positive mode to analysis of an oligonucleotide in electrospray negative mode. This is not possible on all our systems, so we usually schedule our peptide, protein and oligonucleotide work on our Waters Premier IClass UPLC with TQ Absolute Triple Quad Mass Spectrometer. We are able to switch from a small molecule assay to an oligonucleotide with minimal system cleaning and no requirement for passivation of the system.

I can envision a scenario in which large cohort studies for translational biomarker discovery and validation can benefit from a robust mass spectrometer like the TQ Absolute XR. These studies can run for several months and can have thousands of samples in a cohort. Ensuring consistency in instrument performance throughout the lifetime of the study can be challenging and usually requires multiple QC sets bracketing the unknown samples. If the hardware is robust and reliable, the QC samples can be placed further apart, increasing the number of real revenue generating samples that can be analyzed.

Engineering robustness into LC–MS bioanalytical operations

How do you balance the need for high sample throughput with maintaining data quality and instrument longevity in regulated bioanalytical environments?

Our labs follow a robust Quality Management System. The quality and reliability of our instruments are crucial to the delivery of robust and reliable scientific data. We have a dedicated data compliance team involved in the purchase and validation of new instrumentation. The process begins with a User Requirement Specification. This document is in the form of a vendor questionnaire and outlines the “must have” and “nice to have” requirements of the entire system from sample injection to software qualification.

Systems undergo full installation, operational and performance qualification before being signed off as validated. Ongoing preventative maintenance programs are employed to ensure instruments continue to perform to the required standards. Guidance documents on computer system validation are regularly reviewed to ensure continuing compliance of systems.

If you could design the ideal mass spectrometry platform that eliminates common issues like downtime, contamination, and frequent cleaning while maintaining high throughput, what specific features would it include, and how would this transform your laboratory's capabilities?

I would like a system that features a self-cleaning procedure, eliminating the need to clean your cone. Features to prevent scientific errors would also be nice! How about an autosampler that shouts when the 96-well plate has been inserted the wrong way round? Environmental impact is also always a consideration; a compact system with a small footprint, low heat output and reduced solvent usage would be preferable. I do not think downtime can ever be eliminated - it is always best to minimise the impact of downtime with robust preventative maintenance schedules and a responsive service engineer. Having common consumables for our Waters instruments and trained scientists onsite means it is easy for us to change a needle seat and resume the analysis without the need for a callout.

Engineering robustness into LC–MS bioanalytical operations

What innovations in LC–MS technology do you believe will most significantly impact bioanalytical workflows in the next five years?

In regulated bioanalytical workflows, the current triple quad instruments are suitable for pre-clinical and clinical pharmacokinetic studies required to support the development of new drug entities. Some of the newer modalities, such as protein degraders (PROTACS and molecular glues) and macrocyclic molecules, will ideally be quantified by LC–MS/MS. With the integration of Alderley Analytical into Synexa and the move by the FDA to encourage alternatives to animal testing, there may be more focus on biomarkers and translational science. Our method development team are already looking at developing biomarkers of target engagement to measure post-translational modifications of proteins using specific signature peptides. High-resolution instruments are likely to be in common use in quantitative regulatory workflows, provided the data collected and stored is of a manageable size and compatible with the informed consent of the trial subject.

The next exciting development is likely to be Charge Detection Mass Spectrometry. Although this technology may not make an immediate impact on regulatory bioanalytical pharmacokinetic workflows, it may provide a solution for measuring very large molecules such as protein complexes, viruses, nanoparticles and vaccine components.

Research Article

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Bioanalysis

Development of an LC–MS/MS assay for quantification of intact INSL3 in rat plasma

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Background: Relatively large disulfide-linked polypeptides can serve as signaling molecules for a diverse array of biological processes and may be studied in animal models to investigate their function *in vivo*. The aim of this work was to develop an LC–MS/MS assay to measure a model peptide, INSL3, in rat plasma. **Results:** A dual enrichment strategy incorporating both protein precipitation and solid phase extraction was utilized to isolate INSL3 from rat plasma, followed by targeted LC–MS/MS detection. The method was able to measure full-length INSL3 (6.1 kDa) down to 0.2 ng/ml with acceptable accuracy and precision. **Conclusion:** The final assay was applied to support an exploratory pharmacokinetic study to evaluate steady-state concentrations of dosed INSL3 in rat plasma.

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Over the past few years, there has been increased interest in measuring intact peptides in biological matrices using LC–MS/MS. Within academic and pharmaceutical research, bioactive peptides are attractive as either therapeutic agents, biomarkers or biochemical tools [1,2]. As such, investigating peptide–protein interactions and their effect on modulating biological activities is a major priority for identifying novel therapeutic agents, understanding mechanism of action or validating targets [3]. Some notable peptide drugs include receptor agonists (e.g., GLP-1 receptor drugs – liraglutide), neurotransmitters (e.g., oxytocin) and polypeptide hormones and/or analogs (e.g., insulin) [3–5]. In general, though, native peptide ligands are not considered as drug candidates, unlike small molecules, due to their limited *in vivo* stability, short half-life and poor oral bioavailability [6,7]. Furthermore, most natural peptide ligands require structural design enhancement (e.g., cyclization, modification of amino acids, conjugation to macromolecules etc.) to improve their druglike properties while maintaining target potency [6]. Besides being utilized as biotherapeutics, peptides can also serve as endogenous biomarkers or can be synthesized and utilized as controls and/or biochemical tools for *in vitro* or *in vivo* studies. In these types of exploratory studies, peptides or peptide analogs may be evaluated for their agonist and antagonist effects (i.e., therapeutic potential or as tools) and/or to understand molecular mechanisms (i.e., biomarker) for early drug development [1,3]. Therefore, there has been an emerging interest in measuring peptides and/or their analogs in preclinical biological matrices to better explore these biological pathways or pharmacokinetic and pharmacodynamic (PK/PD) relationships within the drug discovery space.

Bioanalysis of bioactive peptides is generally performed using either ligand-binding assays (LBAs) or LC–MS/MS. Both techniques have advantages and limitations, and both may be used at various stages during research and development. For example, LC–MS/MS bioanalysis may be a better strategy if specific LBA reagents are not readily available (e.g., early discovery space), if there is a need to evaluate intact peptide stability or if unambiguous

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detection of a specific peptide form (e.g., endogenous vs synthetic analog or pro vs mature peptide forms) is required. While LBAs demonstrate high sensitivity, simplicity and throughput, LC–MS/MS has superior specificity (especially at the intact level), less variability due to internal standard (IS) addition and straightforward multiplexing capabilities [1,8–10]. With advancements in MS systems and sample extraction techniques, LC–MS/MS has become an excellent alternative technique to traditional LBAs for peptide and protein bioanalysis, especially in the drug discovery space [10,11].

For relatively small peptides, it is common to directly quantify them as intact peptides using conventional LC separation and targeted MS analysis in selected reaction monitoring or multiple reaction monitoring (MRM) mode on tandem quadrupole MS systems. When it comes to medium to large peptides (e.g., 5–10 kDa), though, quantification can be performed at the intact, full-length peptide level or using a surrogate peptide via reduction and/or digestion, of which the latter approach usually provides better MS sensitivity due to its smaller size [1]. Recently, there has been a trend to quantify peptides of interest at the intact level for a variety of reasons. As an example, many synthetic peptide therapeutics or tools may undergo proteolytic degradation in blood/plasma, which typically break down peptide sequences from the N- and C-termini [1,6]. In the case where a peptide may be subject to proteolytic hydrolysis, truncation or any catabolism, monitoring a surrogate peptide may result in an overestimation of the levels of the active molecule [1,11]. Furthermore, chemical reduction and/or enzymatic digestion can be time-consuming [1,12]. Measuring full-length, large peptides in biological matrices via LC–MS/MS has its caveats, though. It is generally difficult to develop sensitive LC–MS/MS assays for large peptides, especially for disulfide-bonded or cyclic peptides, which do not efficiently fragment in the MS system by collision-induced dissociation [1,9,12]. Developing LC–MS/MS workflows to quantify full-length polypeptides in biological matrices, such as plasma, is important, though, as many naturally derived disulfide-rich or cyclic peptides may be useful tools for novel therapeutic drug development [4,13,14].

In this work, a novel analytical workflow is presented for developing a sensitive LC–MS/MS assay to quantify large, disulfide bond-containing peptides from rat plasma. As a model peptide, rat INSL3 (Supplementary Figure 1A) was utilized. Rat INSL3 is a 6.1 kDa peptide hormone with three disulfide bond linkages, a member of the insulin/insulin-like growth factor/relaxin superfamily, and is produced in the testicular Leydig cells [15–18]. The exact amino acid sequence of rat INSL3 has been debated in the literature, mainly if the peptide is secreted by the testicular Leydig cells into circulation as a small heterodimer consisting of a A- and B-chain linked by disulfide bonds (mature peptide form) and/or if the C-chain of the INSL3 peptide remains uncleaved (i.e., pro-peptide form). Nevertheless, Albrethsen *et al.* [19] recently reported that the mature rat INSL3 heterodimer form was present in serum from rodents. For this work, a synthetic peptide version of mature rat INSL3 based on a predicted amino acid sequence [16] was used as a reference standard for method development and as a dosing solution in a rat PK study. Human INSL3 (Supplementary Figure 1B) was used as the IS analog to help alleviate biases during sample extraction and LC–MS/MS analysis. This work ultimately aimed to set the foundation for efficient bioanalytical LC–MS/MS method development for relatively large, bioactive peptides and/or their analogs in support of future drug or biomarker research. Therefore, the authors discuss a generic, antibody-free sample preparation strategy using protein precipitation (PPT) with trifluoroacetic acid (TFA) and solid-phase extraction (SPE) coupled with LC–MS/MS detection to quantify full-length rat INSL3 from rat plasma.

Materials & methods

Chemical & reagents

Human INSL3 (cat no.: 035-27) and rat INSL3 (custom synthesized) peptides were purchased from Phoenix Pharmaceuticals (CA, USA). Laboratory solvents and additives such as acetonitrile (ACN), isopropanol (IPA), MeOH, DMSO, formic acid (FA) and TFA were of LC–MS/MS or HPLC grade and purchased from Sigma-Aldrich (MO, USA). Pooled gender Sprague–Dawley rat plasma (K_2EDTA) was purchased from BioIVT (NY, USA). Phosphate-buffered saline (1× PBS; pH 7.4) was purchased from Thermo Fisher Scientific (NJ, USA), and β-amyloid (1–42) human peptide was purchased from GenScript® (NJ, USA). Oasis® HLB 96-well μelution plates (2 mg sorbent per well; 30 μm) were purchased from Waters Corp. (MA, USA).

Sample preparation

Lyophilized rat and human INSL3 peptides were solubilized in 1× PBS buffer to achieve a final concentration of 1 mg/ml. The rat INSL3 stock solution was then used to generate working solutions from 10 to 1000 ng/ml in rat plasma and subsequently used to generate a calibration curve over the range of 0.2–20 ng/ml. Quality control

(QC) samples were also prepared at 0.6, 2.0 and 15 ng/ml in rat plasma. Calibration standards were extracted in duplicate and QC samples were extracted in replicates of four. For the IS solution, human INSL3 peptide was diluted to 10 ng/ml in a solution of 85:5:10 (v/v/v) water:ACN:TFA containing 5 µg/ml β-amyloid peptide (added to precoat the tubes to minimize any potential nonspecific binding issues).

A 50-µl aliquot of calibration standards, study and QC samples was vortex-mixed with 200-µl 85:5:10 (v/v/v) water:ACN:TFA and 50-µl IS solution. Total blanks (control rat plasma) were prepared without IS. The samples were then vortex-mixed and centrifuged for 5 min at 3180 relative centrifugal force. Using a TomTec™ Quadra 4 (TomTec, CT, USA), the supernatant was transferred to an Oasis® HLB µelution SPE plate (Waters Corp., MA, USA) for extraction of INSL3. Prior to loading the pretreated samples, the SPE plate was conditioned with 200-µl MeOH, followed by equilibration with 200-µl water containing 1% FA. The pretreated supernatant/sample was transferred and loaded to the SPE plate. After a 10-min wait period, a low vacuum (~5 in. Hg) was applied to the SPE manifold to allow each aliquot to slowly pass through and interact with the sorbent. The SPE plate was then washed with 200-µl water with 1% FA followed by 200-µl 20% MeOH in acidified water (1% FA). The INSL3 peptides were selectively eluted off the sorbent bed using 2 × 50 µl elution solvent containing 30:40:30 (v/v/v) water:ACN:IPA containing 1% FA. The SPE eluates were then evaporated using a Savant™ Speedvac™ Integrated vacuum concentrator (Thermo Fisher Scientific™, MA, USA) for approximately 1–2 h (temperature setting: 45°C; vacuum pressure setting: 5.1 torr) and reconstituted with 100-µl 95:5 (v/v) water:ACN containing 0.1% FA.

LC–MS/MS method

A 15-µl aliquot was injected onto an Acquity UPLC® CSH C18 column (130 Å; 1.7 µm; 2.1 mm × 100 mm) using a partial loop mode on an Acquity Classic UPLC (Waters Corp., MA, USA) system equipped with a fixed loop autosampler. The sample compartment was kept cooled at 4–8°C and the column temperature was maintained at 65°C. For the weak and strong LC wash solutions, 0.1% FA in water and 20:40:40 (v/v/v) water:ACN:IPA containing 0.1% FA were used, respectively. Mobile phase A consisted of 0.1% FA in water, while mobile phase B was 1% DMSO in ACN. Rat INSL3 was eluted using a linear gradient from 5% B to 30.7% B over 3 min at a flow rate of 0.4 ml/min. Mobile phase B was then ramped to 80% over 0.1 min and held for 0.4 min to wash the column. The column was then re-equilibrated back to starting conditions for 1.8 min. The total run time was 6.5 min.

For MS analysis, a Xevo TQ-XS tandem quadrupole mass spectrometer (Waters Corp., MA, USA) with positive electrospray ionization was used. The electrospray voltage, cone voltage, source temperature, desolvation temperature and desolvation gas flow rate were 1.0 kV, 45 V, 150°C, 500°C and 950 l/h, respectively. MRM with selective transitions of m/z 766.9 (8+) → 861.0 (rat INSL3) and m/z 700.0 (9+) → 776.1 (human INSL3, IS) were used. The collision energy was set at 15 eV for rat INSL3, while 12 eV was used for human INSL3. MassLynx, version 4.2, was used for LC–MS/MS data processing. Peak area ratios of rat INSL3 to IS were determined and calibration curves were generated for rat INSL3 using a linear regression weighted by $1/x^2$. QC and study samples were determined via response ratios against the rat INSL3 calibration curve.

Rat PK study

All animal procedures were approved by the Institutional Animal Care and Use Committee of GlaxoSmithKline and were in accordance with NIH guidelines for the care and use of animals. An exploratory PK study in male Sprague–Dawley rats (n = 2 juveniles, n = 2 adults) was conducted internally at GlaxoSmithKline. Rat INSL3 lyophilized powder was suspended in PBS (pH 7.4) at 0.75 mg/ml and 2.25 mg/ml for dosing juvenile and adult rats, respectively. Alzet osmotic pumps (model 1003D) were filled with rat INSL3 solutions and implanted in the dorsal midscapular region. Briefly, animals were anesthetized with isoflurane (1–4%), the fur on the back was shaved and the area was cleaned with alcohol and an antiseptic solution. A small incision (~0.5 cm) of the skin was made in the dorsal midscapular region, a small subcutaneous pocket was made and an osmotic pump was inserted into the pocket with the delivery portal end facing away from the incision. The skin was closed with wound clips and the animals returned to a cage until awake from anesthesia. Based on the rat body weight, rats were infused continuously at 125 ng/kg/min for 3 days to produce steady-state plasma INSL3 concentrations. Serial bleed samples were collected at the 0-, 2-, 24-, 48- and 72-h time points via tail snip and transferred to K_2EDTA vacutainers. Blood samples were then centrifuged at 10,000 relative centrifugal force for 5 min and the plasma harvested and stored at -70°C until LC–MS/MS bioanalysis.

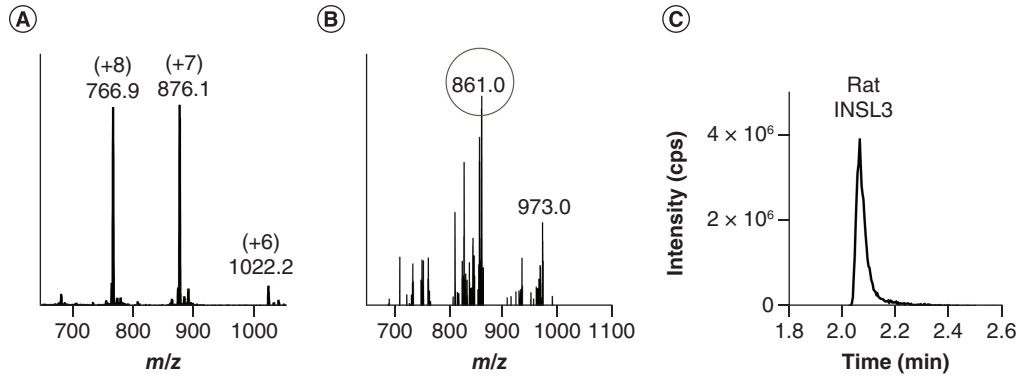

Figure 1. Development of the multiple reaction monitoring transition for rat INSL3. (A) MS spectrum (Q1 scan) in positive electrospray ionization mode. **(B)** MS/MS spectrum with the precursor fixed on *m/z* 766.9. **(C)** Representative extracted ion chromatogram (multiple reaction monitoring: 766.9 → 861.0 with collision energy setting = 15 eV).

Results & discussion

LC–MS/MS method development

Rat INSL3 (57 amino acids; molecular weight: 6128.1 Da) consists of two peptide chains (A- and B-chains) with two inter- and one intra-chain disulfide bonds and was synthetically made based on a predicted sequence (Supplementary Figure 1A). The exact sequence of endogenous, mature rat INSL3 is debated in the literature; mainly, the B-chain amino acid sequence is not fully known; however, that was beyond the scope of this study. Herein is reported the development of a sensitive LC–MS/MS method for exogenous (dosed) rat INSL3 with a low limit of quantitation of 0.2 ng/ml as an analytical tool peptide, and method development challenges and solutions are described. Because of the concern of *in vivo* proteolytic degradation of peptides and the goal to build a workflow that is suitable for large peptide bioanalysis, the LC–MS/MS method development strategy for INSL3 was focused on measurement at the intact peptide molecule level. Human INSL3 (57 amino acids; molecular weight: 6292.3 Da) was used as an IS. Human and rat INSL3 peptides share a 62% sequence homology (Supplementary Figure 1B), and human INSL3 was commercially available, making it a good analog to be considered as an IS. Furthermore, isotopically labeled large polypeptides such as rat INSL3 with multiple disulfide bonds can be expensive and time-consuming to generate.

Rat INSL3 yielded a multiple precursor charge state profile from *m/z* 766.9 to 1022.2 (charge states 6+ to 8+) using MS detection (Figure 1A). The two most intense precursors of 766.9 (8+) and 876.1 (7+) were used as precursor ions. Collision-induced dissociation with argon of the chosen precursor was performed over the range of 200–1200 *m/z*. Both the 8+ and 7+ rat INSL3 precursor ions yielded a few fragments with relatively high intensities when the collision energy was set below 20 eV using a Waters TQ-XS system. Higher collision energies (e.g., 22 eV, 24 eV) resulted in multiple possible fragments with overall lower peak intensities, indicating that multiple INSL3 fragmentations (disulfide bond breakage and peptide backbone cleavage) occurred (Supplementary Figure 2). Choosing a fragment *m/z* higher than its precursor is generally more favorable to avoid issues such as poor MRM selectivity or high MS background [8,20]. As shown in Figure 1, 766.9 (+8) → 861.0 was chosen as the primary MRM transition using an optimized collision energy of 15 eV. Human INSL3 was also optimized for use as the IS. The addition of DMSO to the organic mobile phase was evaluated to determine its effect on INSL3 MS ionization. Interestingly, adding 1% DMSO in the mobile phase influenced the charge state distribution for human INSL3 but not for rat INSL3 (Supplementary Figure 3). For human INSL3, the most intense precursors were 899.9 (7+) in the absence of DMSO and 700.0 (9+) with 1% DMSO in the organic mobile phase, respectively. In this case, DMSO helped enhance the human INSL3 signal intensity by shifting the charge distribution of INSL3 to a more intense lower *m/z* value, indicating that DMSO can impact MS ionization based on peptide characteristics. It should be noted that DMSO adducts were observed in the MS1 scan when a cone voltage of 30 V was used. Therefore, the cone voltage was increased to 45 V to minimize DMSO adduct formation. The MRM transition for the IS was optimized using the precursor ion corresponding to the 9+ charge state. The most intense fragment was observed at *m/z* 776.1, and the collision energy for MRM transition *m/z* 700.0 → 776.1 was optimized at 12 eV. In summary, the findings included the following. First, DMSO as a mobile phase modifier helped enhance MS selectivity for human INSL3 by shifting the charge state distribution to a lower

m/z range. This can be advantageous in some cases for the analysis of large peptides because it may generate more abundant fragments with *m/z* values higher than its precursor and be within the range of the MS detector. Second, there was a clear collisional energy threshold in which INSL3 dissociates during collision-induced dissociation and undergoes cleavage of both the disulfide bonds and peptide backbones (i.e., A- and B-peptide chains) to generate multiple low-abundant fragments, thereby complicating the MS/MS spectra and hindering MS signal. As such, the MRM strategy to choose a low collision energy in which only a few large fragments are generated (leaving INSL3 interdisulfide bonds intact) was preferred. In theory, having higher *m/z* precursor/fragment ion pairs as demonstrated herein should generate a better-quality LC–MS/MS assay with high selectivity and low background issues. Finally, human INSL3 did not interfere with rat INSL3, making it a reasonable IS.

The LC column and gradient were also optimized for INSL3. Conventional reversed-phase chromatography with sub 2-µm particle size C18 columns are commonly used for peptide bioanalysis. A traditional C18, BEH, 1.7 µm, 300 Å column was initially used for INSL3 separation, but severe peak tailing was observed for the peptides of interest. Such peak tailing was also observed by Chambers *et al.* [8], and it was suggested to use a CSH stationary phase. To that end, the Acquity CSH (1.7 µm, 130 Å), CORTECS C18+ (1.6 µm, 90 Å) and Phenomenex BioZen Peptide XB-C18 LC columns were screened and initially performed better than the C18 BEH column. Ultimately, the CSH stationary phase was further utilized because it worked well for rat and human INSL3 separation. A low percentage of DMSO was also kept in the organic mobile phase, since its effect on MRM sensitivity for INSL3 was explored and did not seem to significantly increase background noise (data not shown).

Sample preparation for rat INSL3

Extraction of rat INSL3 from plasma was a critical step to achieve a sensitive, selective and reproducible LC–MS/MS method. The three most used techniques for sample extraction in peptide bioanalysis are PPT, SPE and immunoaffinity capture. PPT is generally easy, fast and cost effective; however, this approach tends to result in higher background noise and matrix effects, ultimately making it difficult to achieve highly sensitive assays [11]. Immunoaffinity capture was also ruled out for the following reasons: there were limited immunoaffinity capture reagents for rat INSL3 and capture reagents can be expensive and time-consuming to generate. SPE is a cost-effective and versatile option that can provide a relatively clean extract by selectively isolating and concentrating peptides from biomatrices. To that end, SPE was assessed and optimized during method development.

Waters Oasis® µElution SPE plates (HLB, MAX and MCX) were initially evaluated for extraction of rat INSL3, and the polymeric reversed-phase HLB sorbent was chosen for method development. To optimize assay performance, SPE conditions for loading, washing and elution were optimized. Both rat and human INSL3 were spiked into rat plasma, pretreated with 100 µl of 1% FA and washed with 200 µl of various percentages (i.e., 5–70%) of ACN or MeOH containing 1% FA, followed by elution with 2 × 100 µl of either 50:50 (v/v) water:ACN containing 1% FA or 50:30:20 (v/v/v) water:ACN:IPA with 1% FA. As shown in Figure 2A–C, the optimal wash and elution conditions were 20% MeOH with 1% FA and 50:30:20 (v/v/v) water:ACN:IPA with 1% FA, respectively. The elution condition was then further optimized by varying the total percentage of organic for the elution buffer composition containing water:ACN:IPA:FA, as demonstrated in Figure 2D. Rat and human INSL3 produced the highest MRM signals with 70% organic in the elution solution.

Fine-tuning the SPE wash and pretreatment steps is crucial to reducing background interference and improving assay sensitivity. Therefore, the sample pretreatment parameters were also evaluated for INSL3 detection in rat plasma. Specifically, 10% TFA in 5% ACN (i.e., aqueous PPT) versus 1% FA was compared for the sample pretreatment step prior to SPE. Supplementary Figure 4 shows extracted ion chromatograms of negative (plasma blanks) and positive controls (INSL3-spiked plasma) tested under both pretreatment conditions. Based on the signal-to-noise (S/N) ratio, the 10% TFA solution was selected. Incorporating the TFA-PPT step was optimal for removing highly abundant albumin, IgG and other large proteins from the plasma sample and, thus, likely helped eliminate background interference. As such, a dual enrichment step incorporating both TFA-PPT and SPE enabled a high MRM signal for the synthetic rat INSL3 peptide in rat plasma. The final assay workflow is shown in Figure 3.

Method quality assessment

The accuracy and precision of the LC–MS/MS assay were determined by analyzing QC samples against a freshly prepared calibration curve of rat INSL3 in rat plasma prepared on three different days. The dynamic range of the optimized assay was 0.2–20 ng/ml using a 50-µl rat plasma sample. Using a $1/x^2$ weighting and linear regression

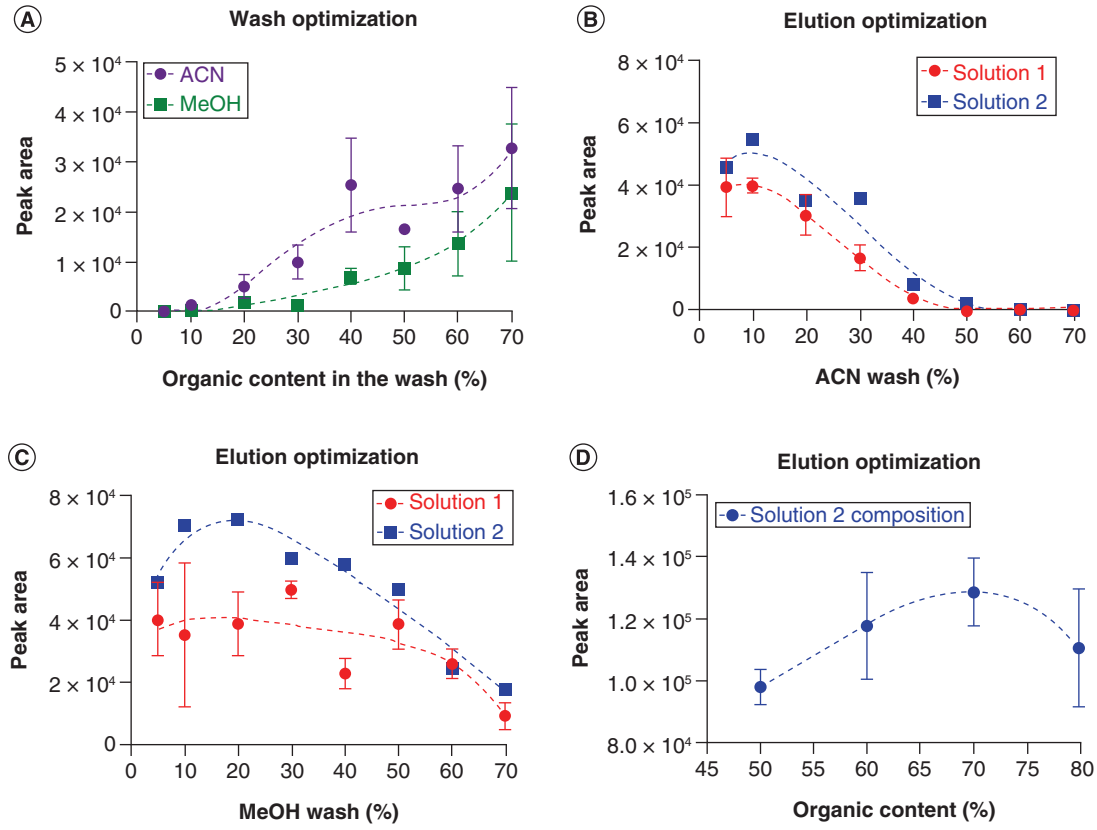

Figure 2. Peak areas of rat INSL3 during solid phase extraction optimization. (A) INSL3 levels at the wash step to monitor for analyte breakthrough. **(B & C)** Peak areas of INSL3 after elution with either elution solution 1 (50:50 H_2O:ACN with 1% formic acid [FA]) or elution solution 2 (50:30:20 water:ACN:isopropanol with 1% FA) while also varying the percentage of ACN or MeOH (containing 1% FA) in the solid phase extraction wash step. **(D)** The optimal percentage of total organic with solution 2 composition while holding the wash step at 20% MeOH containing 1% FA. Data represent mean values ± standard deviation, n = 2.
ACN: Acetonitrile.

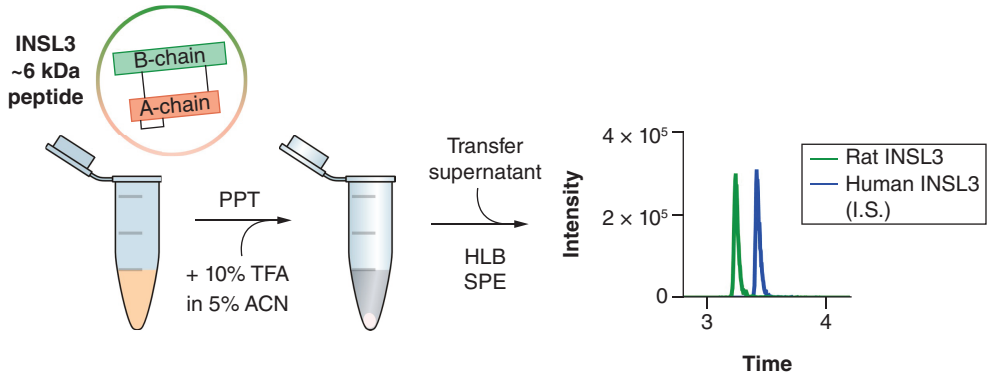

Figure 3. Overview of rat plasma sample preparation and LC–MS/MS strategy for targeted quantification of full-length rat INSL3.
ACN: Acetonitrile; IS: Internal standard; PPT: Protein precipitation; SPE: Solid-phase extraction; TFA: Trifluoroacetic acid.

fit, the coefficients of determination R^2 values for all curves were better than 0.99. The accuracy for all standard points and QC samples extracted from rat plasma was between 81.3% and 118.1% (excluding 1 point with an accuracy of 129.4%) for rat INSL3. Analysis of the frozen QC samples for runs 2 and 3 also demonstrated that

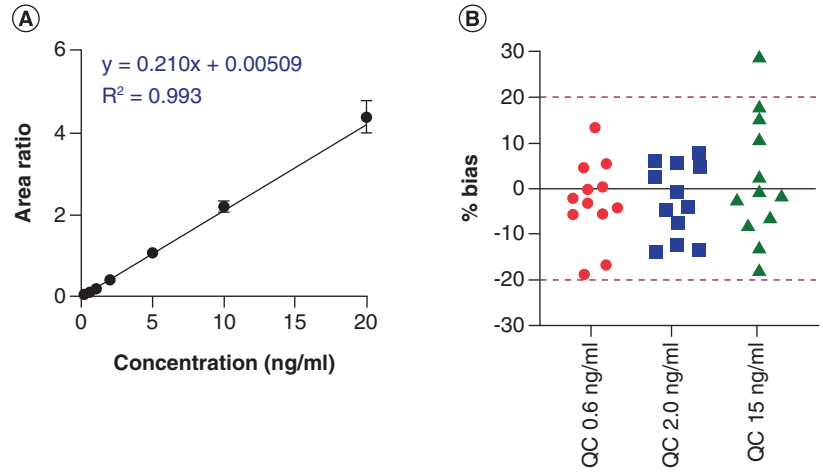

Figure 4. Assay performance of the analysis of rat INSL3 extracted from rat plasma. (A) Assay linearity using a representative calibration curve (data represent mean values +/- standard deviation, n = 2). **(B)** Quality control data (n = 12) from three consecutive analytical runs.
QC: Quality control.

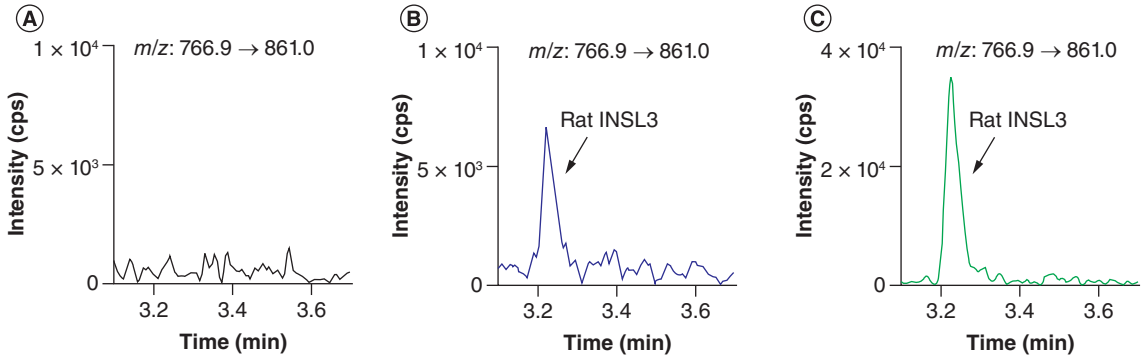

Figure 5. Representative ion chromatograms of rat INSL3 in extracted rat plasma. (A) AA control blank (rat plasma only; without spiked rat INSL3) to show background signal. **(B)** Rat INSL3 spiked at 0.05 ng/ml into rat plasma to show the limit of detection. **(C)** Rat INSL3 spiked at 0.2 ng/ml into rat plasma to show the limit of quantification.

rat INSL3 was stable for at least 35 days at -70°C. A representative plot for the standard curve and QC statistics (via % bias) are presented in Figure 4. Representative chromatograms of blank rat plasma and rat INSL3 extracted from plasma at the low limit of detection and low limit of quantitation are shown in Figure 5. The low limit of quantitation was set to 0.2 ng/ml (S/N: 15), while the low limit of detection was determined to be 0.05 ng/ml (S/N: 4.4). It should also be noted that no endogenous rat INSL3 signal was detected in the negative control plasma matrix (blank), as shown in Figure 5A.

In addition, the SPE protocol was automated on a Hamilton Microlab STAR liquid handling system (NV, USA) to allow the sample extraction (96-well plate) to be completed more efficiently and was comparable to the standard and QC statistics of the manual sample extraction (Supplementary Figure 5). Key parameters to optimize the automated extraction method included focusing on the positive pressure settings and changing from an eight-channel pipettor to a 96-well head for all SPE steps. With the final Hamilton script implemented (Supplementary Table 1), a similar MRM signal for rat INSL3 was achieved, along with similar IS plots, and enabled time savings for the analyst. Finally, the automated script could be easily transferred to other µelution SPE protocols.

Rat PK study

Like most peptides, a relatively short plasma half-life was expected for dosed rat INSL3, which likely gets cleared by proteolytic degradation and renal filtration [21]. Therefore, a rat PK study with continuous infusion of rat INSL3 was conducted to enable the accurate measurement of rat INSL3 in rat plasma. Figure 6 shows the rat INSL3 plasma

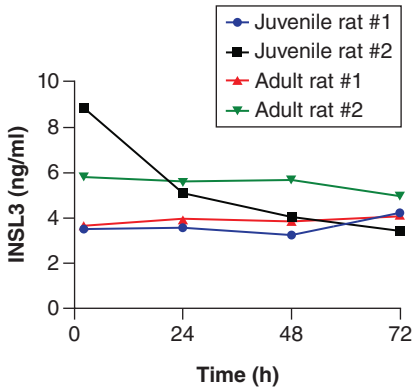

Figure 6. Rat INSL3 plasma concentration time profile during continuous subcutaneous infusion for 3 days at 125 ng/kg/min in two juvenile (~4–5 weeks of age) and two adult (~8–9 weeks of age) male Sprague–Dawley rats.

concentration–time profiles after continuous subcutaneous infusion at 125 ng/kg/min. Overall, the LC–MS/MS method was successfully employed to analyze dosed rat INSL3 in rat plasma in which levels were detected between 3.26 and 8.87 ng/ml over 3 days. These results indicate some variability in the plasma INSL3 concentrations, which is likely due to differences in drug metabolism and pharmacokinetics among rats and/or a potential variable infusion rate in the INSL3 osmotic pump. This study also confirmed that rat INSL3 in PBS solution was stable up to 3 days at 37°C. Interestingly, no endogenous rat INSL3 was detected in the control plasma matrix during method development or in predose (blank) rat plasma samples (data not shown), indicating that either endogenous levels were lower than the low limit of detection (0.05 ng/ml) or more likely the sequence of the synthetic rat INSL3 peptide reference standard was not identical to that of the endogenous peptide. Nevertheless, the focus of this work was to build an analytical capability to quantify low levels of a large polypeptide in rat plasma and was not focused on measurement of the endogenous peptide.

Conclusion

In summary, this work establishes a novel approach to quantifying very low concentrations of a relatively large disulfide-bonded di-peptide in rat plasma using a combination of aqueous PPT and SPE sample processing techniques. While the steps for SPE were systematically optimized to increase recovery of INSL3, the addition of a TFA PPT step prior to SPE proved to be critical for significantly reducing the LC–MS/MS background. Furthermore, the selection of an *m/z* precursor/fragment pair for intact rat INSL3 was important for achieving sufficient LC–MS/MS sensitivity. Using the optimized conditions, rat INSL3 could be measured down to 0.2 ng/ml within 80–120% accuracy and 20% precision. This work helped clarify the stability and concentrations of dosed rat INSL3 in preclinical matrices. Furthermore, this generic analytical strategy may be easily amenable to the development of methods for other large polypeptides or peptide analogs.

Summary points

- Quantification of full-length rat INSL3, a disulfide-bonded di-peptide, from rat plasma was achieved using a general enrichment strategy followed by LC–MS/MS analysis.
- The combination of both aqueous protein precipitation and solid-phase extraction (SPE) was critical to enrich INSL3 from rat plasma.
- SPE steps to isolate INSL3 peptide from rat plasma were systematically optimized. Adding a protein precipitation step prior to SPE significantly helped reduce the LC–MS/MS background signal. The sample preparation workflow was also automated.
- LC–MS/MS conditions to monitor INSL3 at the intact peptide level were optimized. Selection of the *m/z* precursor/fragment pair for intact rat INSL3 was vital for achieving a low quantification limit.
- The total chromatographic run time was 6.5 min.
- Using the final method, spiked/dosed rat INSL3 could be measured down to 0.2 ng/ml using 50 µl of plasma sample.
- This targeted LC–MS/MS assay was applied toward *in vivo* work.
- The concepts of this LC–MS/MS work may be broadly applied to other bioanalysis requests involving large polypeptides or peptide analogs.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2023-0120

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Financial & competing interests disclosure

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

Ethical conduct of research

All animal studies were conducted in accordance with the GSK policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the GSK Institutional Animal Care and Use Committee. All animal procedures were conducted in accordance with NIH guidelines for the care and use of animals.

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Bioanalysis

Development and validation of a high performance liquid chromatography–MS/MS method for determination of SOMCL-15-290 in a first-in-human study

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Background: SOMCL-15-290 is a novel inhibitor that targets FGF receptor, CSF1 receptor and VEGF receptor (kinase insert domain receptor). **Aim:** This study was aiming at developing a specific high performance liquid chromatography–MS/MS method for quantifying SOMCL-15-290 in human plasma and supporting the first-in-human study. **Methods:** Plasma samples were prepared using the protein precipitation method and separated on a C18 110A column with acetonitrile and 0.2% formic acid solution as mobile phases. Quantification of SOMCL-15-290 was operated on an Xevo-TQS triple quadrupole tandem mass spectrometer in electrospray ionization positive mode. **Results & conclusion:** The validated determination method of SOMCL-15-290 has proved feasible and was successfully utilized in the first-in-human study of SOMCL-15-290 in advanced solid tumor patients.

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Keywords: cancer • HPLC-MS/MS • pharmacokinetics • SOMCL-15-290

Cancer is a major disease threatening human health because of its high morbidity and mortality, and its occurrence and progression are related to a host of factors [1,2]. It is worth noting that the family of receptor tyrosine kinases (RTKs) plays a pivotal role in tumorigenesis and malignant progression in multiple cancer types [3]. The FGF receptors (FGFRs) belong to the family of RTKs and are involved in the signal transduction pathways that regulate embryonic development, differentiation, migration and angiogenesis [4–7]. Therefore, a new strategy is emerging for developing FGFR inhibitors to clearly suppress tumors [8,9]. Besides, VEGFs present pro-angiogenic activity, actively contributing to regulating the processes of normal and pathological angiogenesis. In a variety of tumor types, VEGF expression is strongly correlated with tumor microvascular density [10–12]. Given the facts, it is important to figure out the VEGF receptors (VEGFRs), including Fms-like tyrosine kinase 1 (Flt-1), kinase insert domain receptor (KDR) and Fms-like tyrosine kinase 4 (Flt-4) [10]. The CSF1 receptor (CSF1R) also belongs to the group of RTKs and is expressed on tumor-associated macrophages (TAMs) to mediate tumor occurrence in tumor-immune microenvironments. Recently, CSF1R has been regarded as a key therapeutic target for many cancers, such as colorectal cancer and prostate cancer [13–15].

SOMCL-15-290 is a novel, small-molecule, multi-targeted FGFR/KDR/CSF1R inhibitor for the clinical treatment of advanced solid tumors [16]. A first-in-human (FIH) study of SOMCL-15-290 is under way to evaluate its pharmacokinetics, safety and tolerability in Chinese patients with advanced solid tumors. Thus, to investigate the pharmacokinetic properties and evaluate the exposure–response relationship, it is critical to accurately determine drug concentrations. However, there are few publications regarding the methods for analyzing SOMCL-15-290. In the present study, a robust and specific method was established and fully validated to quantify SOMCL-15-290

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in human plasma. This method can be applied to evaluating the pharmacokinetics of SOMCL-15-290 in the FIH study and subsequent clinical research.

Materials & methods

Chemicals & reagents

The standards of SOMCL-15-290 (purity 99.8%) and D3-SOMCL-15-290 (internal standard [IS]: purity 99.0%) were supplied by the Shanghai Institute of Materia Medica (Shanghai, China). High performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Honeywell Burdick & Jackson (MI, USA). Formic acid (analytical-grade) was obtained from Sigma-Aldrich (MO, USA). Blank human plasma was supplied by Peking Union Medical College Hospital (Beijing, China) and was collected using K2-ethylenediaminetetraacetic acid (EDTA) tubes, which were purchased from Becton, Dickinson and Company (NJ, USA). The clinical registration number was NCT04058587. Deionized water was produced by a Milli-Q reagent water system (Millipore, MA, USA).

Chromatographic & mass spectrometric conditions

An ACQUITY HPLC system (Waters, MA, USA) was used for chromatographic separation. Analytical separation was performed on a Phenomenex Gemini 5u C18 110A column (2.0 × 50 mm, 5 μm) with mobile phase acetonitrile (phase A) and 0.2% formic acid in water (phase B). The total run time of a gradient program was 3 min, which was started from 20% of phase A and maintained for 1.0 min, then was elevated sharply to 80% over 0.01 min and kept for 1.0 min. Then phase A was switched back to 20% within 0.01 min and remained for 1.0 min until finishing the program. The injection volume was 5 μl for each sample. To decrease the carryover, acetonitrile-water (90:10, v/v) was prepared to wash the autosampler syringe and the injection valve. The flow rate was 0.3 ml/min. The column temperature was kept at 40°C, and the autosampler temperature was set at 10°C.

The mass spectrometric analysis was conducted on a Xevo-TQS triple quadrupole mass spectrometer (Waters, MA, USA) in the positive mode of the electrospray ionization (ESI) source. A multiple reaction monitoring (MRM) scan was applied for detection by monitoring the following transitions of SOMCL-15-290 and IS at m/z 510.3→217.2 and m/z 513.3→217.2, respectively. Other optimized parameters were set as follows: capillary voltage and cone voltage, 3.30 kV and 35 V; desolvation gas and ion source temperature, 1000 l/h and 500°C; collision gas flow, 0.015 l/h; and collision voltage, 26 V and 25 V for SOMCL-15-290 and IS, respectively.

Preparation of stock solutions, calibration standards & quality control samples

SOMCL-15-290 stock solutions (0.5 mg/ml) for calibration standards and quality controls (QCs) were prepared in duplicate using methanol-water (1:1, v/v) to dissolve the accurately weighed standards. The stock solutions were further diluted to prepare the working solutions at several concentration levels (10 μg/ml, 5 μg/ml, 1 μg/ml). IS stock solution (0.5 mg/ml) was prepared in a similar way and was diluted by methanol to obtain a working solution at a concentration of 5 ng/ml.

Calibration standards and QCs in plasma were separately prepared by serial dilution of corresponding working solutions with blank plasma. A series of calibration standards (0.2, 0.5, 2.5, 5.0, 10, 25, 50 and 100 ng/ml) were acquired, and the concentrations of the QCs were 0.2, 0.4, 8.0 and 80 ng/ml. Dilution QC (DQC) (160 ng/ml) samples were prepared and would be diluted tenfold to evaluate dilution stability. After being aliquoted, the solutions and plasma samples were frozen at a temperature of -80°C.

Sample preparation

Thawed at room temperature (RT) (average temperature: 25°C) primarily, the plasma samples were subjected to vortex mixing before analysis. A volume of 25 μl of plasma sample was transferred into 200 μl of IS working solution (5 ng/ml). The mixtures were processed by adequate vortexes for 1 min and centrifugation at 13,000 r.p.m. for 10 min. Then 100 μl of supernatant was collected and was further spiked with 100 μl of methanol.

Method validation

This method was fully validated in accordance with the updated guidelines for bioanalytical assays issued by the US FDA and the EMA [17–19], including selectivity, calibration curve linearity, lower limit of quantitation (LLOQ), stability test, precision and accuracy, matrix effect, dilution integrity and carryover.

Selectivity

Selectivity was assessed by analyzing at least six blank matrixes from different individual human plasma samples to investigate the presence of endogenous interference compared with LLOQ. One double blank (DB) sample and one LLOQ sample were prepared for each blank matrix, with a total of 12 samples. Supposing there was interference of the blank samples at the corresponding retention time of the analyte, the interference peak area had to be within 20% of the LLOQ samples and 5% of IS mean peak area in the calibration curve samples and QC samples. At least five DB samples met the above criteria.

Linearity

The calibration curve should be constructed using a DB sample, a blank sample spiked with IS and eight calibration curve samples at the concentration levels over the range from the LLOQ to the upper limit of quantitation (ULOQ). Two calibration curves were separately prepared and analyzed at the beginning and end in each analytical lot. Linear regression was performed on the peak area ratio of SOMCL-15-290 to IS (y) and analyte concentration (x), and the weighting factor was set to $1/x^2$. It was considered good linearity, while the determination coefficient R^2 of all the curves was greater than 0.980. The back-calculated concentrations should be between 80% and 120% of the nominal value of LLOQ samples and between 85% and 115% for the other calibration standards. A minimum of 75% of the standards should fulfill the above criteria.

Precision & accuracy

The intra- and inter-run precision and accuracy were performed in six replicates of QC samples (LLOQ, low concentration QC [LQC], middle concentration QC [MQC] and high concentration QC [HQC]). The intra-run precision and accuracy were evaluated in a separate batch and the inter-run over three successive batches. Independently and freshly prepared calibration curves and QC samples were used. Relative standard deviation (RSD%) and relative error (RE%) were used to evaluate the precision and denote the accuracy, respectively. The RSD% and RE% should be within ±15% of the standard concentrations for LQC, MQC and HQC samples and should be within ±20% for LLOQ samples.

Extraction recovery

The QC samples at low, medium and high concentrations were obtained and extracted. The extraction recoveries of SOMCL-15-290 were assessed by comparing the peak areas of the extracted QC samples with those of extracted blank samples spiked with an equal amount of analyte. For IS, the extraction recovery was determined in a similar way as SOMCL-15-290. The extraction recovery should be precise and reliable.

Matrix effect

Matrix effect was assessed at three levels (LQC, MQC and HQC) using blank plasma from six different sources to evaluate the influence of different sources of matrix on determination. For each batch of matrix, the matrix factor (MF) of SOMCL-15-290 or IS was acquired by calculating the ratio of the response of extracted blank matrix spiked with analyte or IS to the peak area of analyte or IS pure solution. The IS normalized MF was obtained by dividing the MF of SOMCL-15-290 by that of IS. The acceptance criterion was that the RSD% of the IS normalized MFs of six batches should not be greater than 15%.

Considering that the hemolysis and hyperlipidemia samples also occur clinically, the authors assessed the matrix effect of hemolyzed plasma (normal plasma spiked with 2% fully ruptured blood cells) and hyperlipidemic plasma (300 mg/dl). The RSD% and RE% should be kept at ≤15%.

Stability

Analyte stability was evaluated by subjecting LQC and HQC samples (N = 6, each) under various storage, processing and analysis conditions. The stock solutions' stability (25°C for 8 h and -30°C for 217 days) was evaluated by comparing the peak area of freshly prepared solutions with the stored solutions. The analyte stability in the plasma was tested under different conditions. In order to evaluate long-term and short-term stability, the samples were placed at 25°C for 15 h and at -80°C for 219 days. The extracted samples were placed in the autosampler (15°C) for 48 h and subsequently analyzed with a freshly prepared calibration curve to evaluate autosampler stability. Additionally, samples that had been analyzed were stored in the refrigerator (4°C) for 48 h, then reinjected to

evaluate the reproducibility of the repeated injection. As for freeze-thaw stability, the specimens were frozen at -80°C for over 12 h, then thawed at 25°C for at least 2 h, with five cycles.

For whole-blood stability assessment, the concentrations of LQC and 1/4 HQC samples were acquired using freshly collected whole blood and then divided into two groups (group A and group B). Plasma samples were obtained by centrifuging the QC samples immediately for group A and centrifugation after being placed at 25°C for 2 h for group B. For the sample of each concentration in group A, the average value of the ratio of analyte peak area to IS peak area was considered the nominal value. The mean value in group B had to be within ±15% of the value in group A, and the RSD% could not exceed 15%.

Dilution integrity

Dilution integrity was assessed to ascertain if the samples with concentrations above calibration range could be diluted and accurately measured. Six replicates of DQC samples (160 ng/ml) were diluted tenfold with blank matrix before analysis. The RE% and RSD% for the six replicates should be within ±15%.

Carryover

The carryover was evaluated after the ULOQ sample injection followed by a DB sample. The peak response of SOMCL-15-290 in the DB sample had to be less than 20% of that in the LLOQ sample, and the peak response of the IS in the DB sample had to be less than 5% of the average of all the IS peak responses.

Pharmacokinetic application

This method was applied to illuminate the plasma pharmacokinetics of SOMCL-15-290 in a multi-center, dose-escalation clinical phase I study, which was conducted in Chinese patients with advanced solid tumors (NCT04058587). It was performed according to the Declaration of Helsinki and Good Clinical Practice and approved by the Ethics Committee of Cancer Hospital Chinese Academy of Medical Sciences and Peking Union Medical College Hospital. During the study, patients were initially administered a single dose; 7 days later, once a week multiple doses were administered. Blood samples were harvested at the following time points. For the single dose period, samples were collected within 30 min before dosing and at 1, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h after dosing. For the period of multiple doses, samples were collected within 30 min before dosing on days 1, 7, 14, 21 and 28 and at 1, 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h after dosing on day 28.

Blood samples were collected from each subject at designated time points after dosing, and plasma samples were obtained by centrifuging harvested blood samples (anticoagulated in K2-EDTA) at 1800× *g* for 10 min with a temperature of 4°C and were kept at -80°C until further assay.

Data acquisition & analysis

Masslynx software (version 4.1, Waters) was utilized to acquire data. Watson LIMS software (version 7.3, Thermo Fisher Scientific) was used to perform calibration regression and statistical analysis. A weighted least square linear regression model ($1/x^2$) was used to calculate the concentrations of QC samples and the unknown clinical samples by equation interpolation. Pharmacokinetic parameters were obtained by non-compartmental analysis employing Phoenix WinNonlin (version 6.3, Pharsight, NJ, USA).

Results & discussion

Method development

It was the first time that a rapid, robust and sensitive HPLC-MS/MS method was established for the determination of SOMCL-15-290 in human plasma.

Optimization of mass spectrometric parameters

The nitrogen and oxygen atoms are presented in the chemical structure of SOMCL-15-290 and increased the electronegativity of this compound, which indicated that positive ionization mode with an ESI source was applicable with a better ionization performance, and a robust and stable response was observed. The representative chromatograms of product ion spectra of SOMCL-15-290 and IS are presented in Figure 1. It turned out that the most intense responses were at m/z 217.2 for both the analyte and IS. Therefore, MRM scan mode was chosen with the precursor ions for SOMCL-15-290 and IS at m/z 510.3→217.2 and m/z 513.3→217.2. Moreover, source/gas

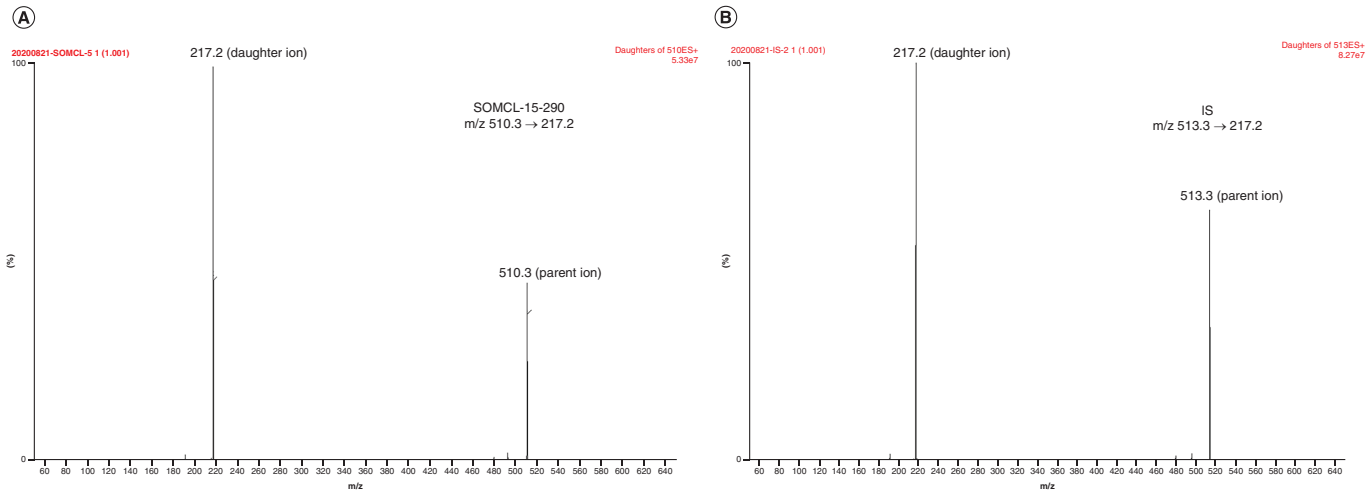

Figure 1. MS spectra of SOMCL-15-290 and internal standard. (A) SOMCL-15-290. **(B)** Internal standard.

Table 1. Ionization conditions for SOMCL-15-290 and internal standard.

Analyte	Multiple reaction monitoring transitions (*m*/*z*)	Capillary voltage (kV)	Cone potential (V)	Collision energy (V)	Dwell time (s)
SOMCL-15-290	510.30→217.20	3.30	35.00	26.00	0.16
Internal standard	513.30→217.20	3.30	35.00	25.00	0.16

and compound parameters, including capillary voltage, cone voltage and collision energy, were optimized to achieve the most appropriate ionization conditions (Table 1).

Optimization of chromatographic conditions

Based on the presence of the benzene rings of the SOMCL-15-290 chemical structure, reversed-phase chromatography was preferred. A variety of analytical columns were explored, such as BEH C18 column, XBridge Pheyl C18 column and Phenomenex Gemini 5u C18 column. And The Phenomenex Gemini 5u C18 110A column (2.0 × 50 mm, 5 µm) was finally selected for separation due to the adequate retention and satisfactory peak shape. After investigation of different mobile phase components, acetonitrile was preferred because of its better elution effect and negligible background noise; 0.2% formic acid was added in the aqueous phase to increase the response of the protonated molecules. Better chromatograms and higher sensitivity were achieved by gradient elution compared with isocratic elution. Eventually, gradient elution was performed within 3.0 min.

Optimization of sample preparation

To prepare plasma samples efficiently, at first, liquid–liquid phase extraction was investigated, but it led to chemical contamination and low sensitivity. The solid phase extraction was also taken into consideration, but this method would take up a lot of time due to complicated procedures. For the protein precipitation method, although there may be matrix effect affecting the accuracy of the determination, the validation showed a negligible matrix effect and clear plasma samples. Therefore, protein precipitation was used and a sharp chromatographic peak and satisfactory reproducibility were obtained while using methanol as an extraction reagent.

Method validation

Selectivity

For SOMCL-15-290, the LLOQ was established at 0.2 ng/ml, and the typical chromatograms that depict DB samples, DB samples with IS, LLOQ samples and an unknown clinical sample are shown in Figure 2. The retention time of SOMCL-15-290 was 0.98 min, the endogenous interference of one DB sample was greater than 20% of the average response of LLOQ at the corresponding retention time of the SOMCL-15-290 but other samples were not beyond 12.0%.

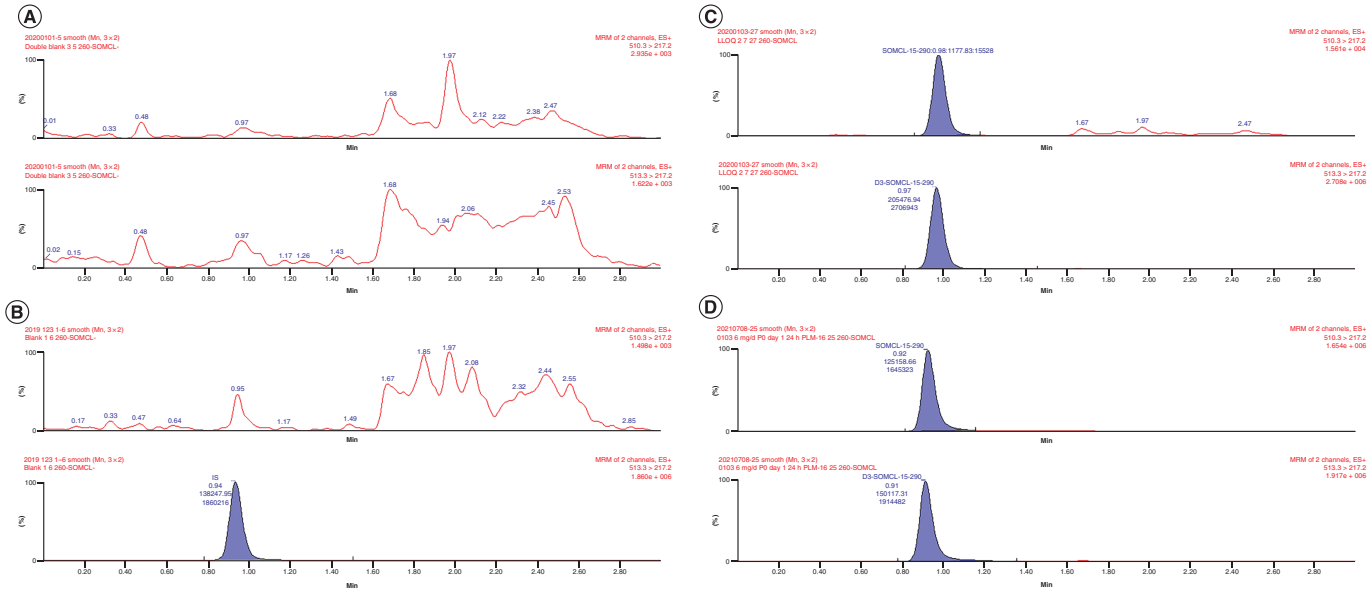

Figure 2. Typical multiple reaction monitoring chromatograms of analyte and internal standard. (A) Blank plasma. **(B)** Blank plasma spiked with internal standard. **(C)** Lower limit of quantitation sample (0.2 ng/ml). **(D)** Clinical plasma sample from a subject receiving 6 mg of SOMCL-15-290.

Table 2. Back-calculated calibration standards of SOMCL-15-290 in plasma.

Matrix	Concentration (ng/ml)	0.20	0.50	2.50	5.00	10.00	25.00	50.00	100.00
Plasma	Mean	0.20	0.49	2.49	4.99	10.20	24.70	49.90	101.00
	SD	0.02	0.02	0.07	0.10	0.29	0.70	0.99	3.11
	RSD%	8.50	3.90	2.90	2.00	2.80	2.80	2.00	3.10
	RE%	0.50	-1.00	-0.40	-0.20	2.00	-1.20	-0.20	1.00
	n	32	36	36	35	36	36	36	36

n: Number of concentrations participating in standard curve fitting; RE%: Relative error; RSD%: Relative standard deviation; SD: Standard deviation.

Linearity & carryover

The calibration curve over the range of 0.2–100 ng/ml in all the batches showed good linearity. The mean regression coefficients (R^2) were beyond 0.994. The results of back-calculated concentrations of calibration standards are presented in Table 2.

In terms of the assessment of carryover, the peak areas of SOMCL-15-290 and IS in the DB plasma sample following the ULOQ sample were not greater than 17.6% and 0.1% of the peak area of LLOQ, which indicated that there was no obvious carryover effect for either SOMCL-15-290 or IS.

Precision & accuracy

The within- and between-run precision and accuracy are summed up in Table 3. The average bias of QC samples was less than 11.0% and the batch precision of each concentration level was less than 8.4%. It turned out that the accuracy and precision values met the specified acceptance criteria, which manifested that this method was precise and accurate for the quantification of SOMCL-15-290.

Extraction recovery & matrix effect

The average extraction recoveries of SOMCL-15-290 obtained from plasma samples of LQC, MQC and HQC levels were 104.0%, 100.7% and 109.3%, respectively, with RSD% less than 4.6%. That of IS was 103.6%. The IS-normalized matrix effect of SOMCL-15-290 ranged from 151.3% to 223.3%, with RSD% less than 2.6%. These results (Table 4) showed an applicable extraction efficiency and an uninfluential matrix effect in validating the method.

Table 3. Intra- and inter-day accuracy and precision of plasma quality control samples of SOMCL-15-290.

Run	Item	LLOQ	LQC	MQC	HQC
Run 1	Mean ± SD	0.18 ± 0.10	0.39 ± 0.01	8.42 ± 0.16	85.10 ± 0.49
	RSD%	5.30	2.10	1.90	0.60
	RE%	-11.00	-3.80	5.30	6.40
Run 2	Mean ± SD	0.18 ± 0.02	0.39 ± 0.01	8.40 ± 0.09	85.00 ± 0.72
	RSD%	8.40	1.40	1.10	0.80
	RE%	-10.00	-3.80	5.10	6.30
Run 3	Mean ± SD	0.19 ± 0.01	0.39 ± 0.01	8.31 ± 0.10	83.90 ± 0.75
	RSD%	7.00	1.50	1.30	0.90
	RE%	-4.50	-3.30	3.90	4.90
Inter-run	Mean ± SD	0.18 ± 0.01	0.39 ± 0.06	8.38 ± 0.13	84.70 ± 0.83
	RSD%	7.30	1.60	1.50	1.00
	RE%	-8.50	-3.50	4.80	5.90

HQC: High concentration quality control; LLOQ: Lower limit of quantitation; LQC: Low concentration quality control; MQC: Middle concentration quality control; RE%: Relative error; RSD%: Relative standard deviation; SD: Standard deviation.

Table 4. Matrix effect and extraction recovery of SOMCL-15-290 in plasma.

Nominal concentration (ng/ml)	Extraction recovery		Matrix effect	
	Mean	RSD%	Mean	RSD%
LQC	104.00	4.60	223.30	1.80
MQC	100.70	1.70	176.70	2.50
HQC	109.30	1.90	151.30	2.60
IS	103.60	2.70		

HQC: High concentration quality control; IS: Internal standard; LQC: Low concentration quality control; MQC: Middle concentration quality control; RSD%: Relative standard deviation.

Table 5. Intra- and inter-day accuracy and precision of quality control samples of SOMCL-15-290 in hemolytic and hyperlipidemic plasma.

Run	Item	LQC	MQC	HQC
Hemolytic plasma	Mean ± SD	0.39 ± 0.02	8.11 ± 0.27	81.60 ± 1.90
	RSD%	5.20	3.30	2.40
	RE%	-2.30	1.40	1.90
Hyperlipidemic plasma	Mean ± SD	0.39 ± 0.02	8.52 ± 0.52	83.10 ± 4.00
	RSD%	4.10	6.10	4.80
	RE%	-1.50	6.50	3.90

HQC: High concentration quality control; LQC: Low concentration quality control; MQC: Middle concentration quality control; RE%: Relative error; RSD%: Relative standard deviation; SD: Standard deviation.

In addition, the evaluation of the matrix effect of hemolytic (containing 2% lysed blood) and hyperlipidemic plasma (300 mg/dl) is shown in Table 5. The accuracy of SOMCL-15-290 in hemolytic and hyperlipidemic plasma ranged from 97.8% to 101.4%, and the RSD% was less than 5.2%. It was clearly demonstrated that the special matrixes had a negligible influence on the determination of SOMCL-15-290.

Stability

The SOMCL-15-290 stability in solution and plasma was evaluated under diverse storage and operating conditions during the routine analysis. Table 6 presents the stability test data, which revealed that plasma samples maintained good stability at 25°C for 15 h and -80°C for 378 days, and they were still stable after five freeze-thaw cycles from -80°C to 25°C. Briefly, whole-blood samples prior to centrifugation showed good stability at 25°C for 2 h, and repeated injection reproducibility was still feasible after 48 h in the 4°C refrigerator; the samples remained stable at 15°C for 48 h. In addition, stock solution samples did not influence the stability results of SOMCL-15-290 at 25°C for 8 h and -30°C for 217 days.

Table 6. Stability of SOMCL-15-290 in plasma samples and processed samples (n = 6).

Matrix	Storage conditions	Theoretical value	Found value	RSD%	RE%
Solution	SOMCL-15-290-short-term (25°C, 8 h)	83,557.00	84,980.00	1.90	
	IS-short-term (25°C, 8 h)	77,570.00	79,060.00	1.50	
	SOMCL-15-290-long-term (-30°C, 217 days)	105,433.00	107,988.00	0.70	
	IS-long-term (-30°C, 217 days)	98,760.00	100,256.00	0.90	
Plasma	Short-term (25°C, 15 h)	0.40	0.40	3.20	-1.30
		80.00	83.20	1.90	4.00
	Long-term (-20°C, 32 days)	0.40	0.41	6.60	1.40
		80.00	85.60	0.80	7.00
	Long-term (-80°C, 378 days)	0.40	0.40	2.20	-0.80
		80.00	77.10	0.30	-3.70
	Autosampler (15°C, 48 h)	0.40	0.40	2.30	-0.40
		80.00	81.90	0.70	2.40
	Reinjection (4°C, 48 h)	0.40	0.41	2.20	1.60
		80.00	84.80	0.70	6.00
	Freeze-thaw (five cycles)	0.40	0.40	2.60	-0.80
		80.00	87.20	4.00	8.90
	Whole blood (25°C, 2 h)	0.40	89.50	7.40	
		80.00	98.60	3.10	

IS: Internal standard; RE%: Relative error; RSD%: Relative standard deviation.

Dilution integrity

After dilution of the plasma samples by tenfold to 160 ng/ml, the RE% and RSD% of the six replicate samples were 5.3% and 3.5%. These results showed that samples with higher concentrations than ULOQ could be reliably diluted by tenfold.

Application to the FIH study

The method was commendably applied to evaluate the pharmacokinetic profiles of SOMCL-15-290 in human plasma in a randomized, open-label, single and multiple dose-escalation study. This study is still ongoing and currently a total of 180 plasma samples in four analytical runs have been analyzed successfully from 15 enrolled subjects, who were randomized into four groups to receive a single oral dose of 1, 2, 4 and 6 mg of SOMCL-15-290. The mean concentration–time curves of the analyte are depicted in Figure 3.

Conclusion

It was the first time that a rapid, reliable and highly sensitive HPLC-MS/MS method was developed and validated for the quantification of SOMCL-15-290 in human plasma. This method guaranteed an economic and rapid sample preparation procedure with a broad linearity and a low LLOQ. The results of methodological validation showed that the stability was satisfactory and the extraction recovery was high, which proved that the HPLC-MS/MS method was reliable for the determination of the concentration of SOMCL-15-290 in plasma samples. Particularly, the LC-MS/MS method presented in this paper might be applied to support the further clinical trial of SOMCL-15-290.

Future perspective

SOMCL-15-290 is a novel, small-molecule, multi-targeted FGFR/KDR/CSF1R inhibitor for the clinical treatment of advanced solid tumors. An FIH study was initiated to evaluate the pharmacokinetics, safety and efficacy of SOMCL-15-290 in Chinese patients. To support the FIH study, a novel analytical method was urgently needed for the determination of SOMCL-15-290 in human plasma. In the present study, a reliable and robust HPLC-MS/MS method was developed and validated. This method can be applied to subsequent clinical research.

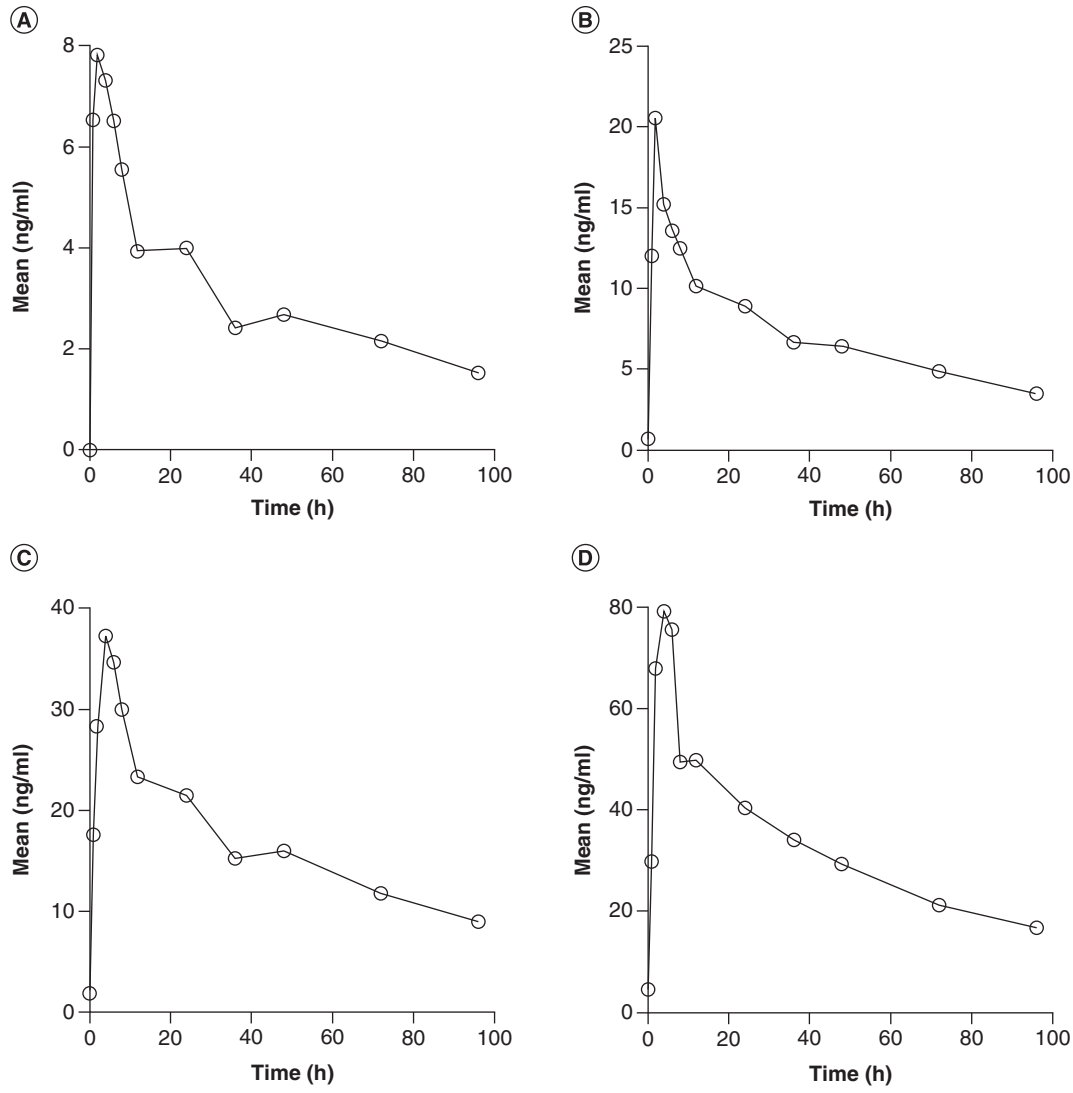

Figure 3. The mean concentration–time profiles of SOMCL-15-290 in plasma after a single oral dose in patients. (A) 1 mg (n = 3). **(B)** 2 mg (n = 4). **(C)** 4 mg (n = 4). **(D)** 6 mg (n = 4).

Summary points

- SOMCL-15-290 is a novel inhibitor that targets FGF receptor, CSF receptor and VEGF receptor (kinase insert domain receptor) for the clinical treatment of advanced solid tumors.
- A high performance liquid chromatography–MS/MS method was developed for the determination of SOMCL-15-290 in human plasma to support the first-in-human study.
- Plasma samples were prepared using the protein precipitation method and were separated on a Phenomenex Gemini 5u C18 110A column (2.0 × 50 mm, 5 μm) with acetonitrile and 0.2% formic acid solution as mobile phases. Quantification of SOMCL-15-290 (m/z 510.3→217.2) was performed on a Xevo-TQS triple quadrupole tandem mass spectrometer in electrospray ionization positive mode using multiple reaction monitoring mode.
- The validated quantitative determination method of SOMCL-15-290 has proved feasible and was successfully applied to support the first-in-human study of SOMCL-15-290 in Chinese patients.

Financial & competing interests disclosure

This study was supported by the National Key R&D Program of China (2020YFC2008303) and Shanghai Runshi Pharmaceutical Technology Co. Ltd (Shanghai, China). The authors have no other 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 apart from those disclosed.

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

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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RESEARCH ARTICLE

Development of a sensitive LC-MS/MS assay to support human microdose study for an oral agonist of the GLP-1 receptor

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ABSTRACT

Aim: The purpose of this work was to determine the feasibility of supporting a clinical microdose study for PF-06882961 (danuglipron), an oral small molecule agonist of the GLP-1 receptor, by LC-MS/MS. **Methodology:** Statistical instrument parameter optimization using response surface methodology was employed to develop a LC-MS/MS method for the analyte, PF-06882961. **Results:** An LC-MS/MS method was developed and validated to support a proof of concept microdose pharmacokinetics preclinical study in monkeys, administered PF-06882961 (0.005 mg total, average dose = 0.0007 mg/kg) via intravenous bolus injection. **Conclusion:** The present study demonstrated the feasibility of analyzing human microdose plasma samples for PF-06882961 by LC-MS/MS, instead of accelerator mass spectrometry, thereby reducing cost and eliminating synthesis and exposure to ^{14}C labeled material.

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accelerator mass spectrometry; bioanalysis; chromatography; danuglipron; LC-MS/MS; microdose; microflow; pharmacokinetics; statistical tuning; validation

1. Background

Microdosing is an approved method that is often utilized to obtain exploratory clinical pharmacokinetics data for investigational drug candidates at sub-therapeutic doses [1–3]. The low dose administered also minimizes potential safety concerns. Microdosing is applied to clinical programs as a Phase 0 study, usually occurring between regulatory preclinical toxicological assessments and Phase I first-in-human clinical trials [1]. Phase 0 studies can provide valuable insights into the pharmacokinetics parameters, analyte concentration over time at the site of action, as well as provide insights into pharmacokinetics variability due to metabolic elimination via polymorphic enzyme(s) [1–3]. Phase 0 studies can also prove potentially useful in providing insights into the clinical disposition of investigational drug candidates with uncertainty around preclinical pharmacokinetics projections. Microdose studies typically rely on accelerator mass spectrometry (AMS) to measure radiolabeled (e.g., ^{14}C) drugs to achieve the required low limits of analyte quantitation [1–6]. This type of mass spectrometer can achieve detection limits of fg/ml [4–6]. AMS sample preparation is typically performed by the conversion of organic samples to thermally and electrically conductive graphite. The graphite sample is exposed to a cesium sputter ion source that produces $^{14}C/^{12}C$ ratios, thereby allowing the total amount of ^{14}C to be determined [4–6]. AMS has limitations such as ready instrumentation access, time consuming sample preparation, training and cost associated with the analysis.

The purpose of the present work was to determine the feasibility of conducting a clinical microdose pharmacokinetics study utilizing liquid chromatography coupled with tandem quadrupole mass spectrometry (LC-MS/MS). PF-06882961 (danuglipron) (Figure 1) is a novel orally active small molecule agonist of the human glucagon-like peptide-1 receptor (GLP-1R), which is currently in clinical development for the treatment of Type 2 diabetes mellitus and obesity [7,8]. Pharmacokinetic modeling of a 0.1 mg IV bolus of PF-06882961 based on clinical single ascending oral dose data indicated that a LC-MS/MS method with a lower limit of quantitation (LLOQ) of approximately 0.200 pg/ml would be required to precisely capture the pharmacokinetic profile. Use of LC-MS/MS would eliminate the need for synthesis of [^{14}C]-PF-06882961 and preclude the conduct of a radiolabeled microdose clinical study. To demonstrate the feasibility of a clinical microdose study with unlabeled PF-06882961, bioanalytical method development was initiated in K_3EDTA cynomolgus monkey plasma utilizing the Waters iKey microflow mass spectrometry

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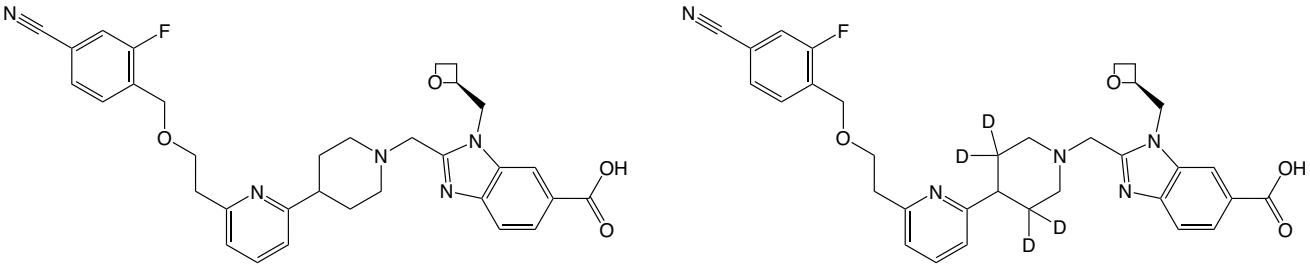

Figure 1. Structures of analyte (PF-06882961) and deuterated internal standard (IS) (PF-06974801).

source coupled to a tandem quadrupole mass spectrometer (microflowLC-MS/MS) operated in multiple reaction monitoring (MRM) mode [9,10]. MicroflowLC-MS/MS was chosen because of the enhanced ionization efficiency afforded by the relatively low flow rates (0.3–10 μl/min). The enhanced ionization efficiency in conjunction with low internal analytical column diameters (0.2–0.3 mm) also provide a significant increase in sensitivity [11].

Over the past few decades, statistical optimization of electrospray ionization (ESI) mass spectrometer sources has been utilized to improve the sensitivity of various analyte moieties, such as trace degradants of pharmaceutical compounds, metabolites in human urine, and tripeptides [12–15]. We decided to employ statistical optimization of the microflowLC-MS/MS, considering the relatively low quantitation limit of 0.200 pg/ml that is required to successfully conduct a microdose study.

2. Experimental section

2.1. Chemicals & reagents

PF-06882961 (2-[[4-[6-[(4-cyano-2-fluorophenyl)methoxy]pyridin-2-yl]piperidin-1-yl]methyl]-3-[[(2*S*)-oxetan-2-yl]methyl]benzimidazole-5-carboxylic acid) and PF-06974801 (PF-06882961 D_4) were synthesized at Pfizer, Inc. (CT, USA). Optima™, LC/MS grade water, water containing 0.1% (v:v) formic acid, acetonitrile containing 0.1% (v:v) formic acid, pH 4.00 and pH 7.00 calibration buffers, methyl tertiary-butyl ether (MTBE) and HPLC grade methanol were purchased from Fisher Scientific, Inc. (NH, USA). Reagent grade ammonium acetate was acquired from Sigma Aldrich (MO, USA). Reagent grade glacial acetic acid was obtained from J.T. Baker (NJ, USA). Cynomolgus monkey control K_3EDTA plasma was purchased from BioIVT Inc. (NY, USA).

2.2. Analyte & IS stock solution preparation

PF-06882961 and PF-06974801 stock solutions were prepared in 1:1 (v:v) water: acetonitrile containing 0.1% (v:v) formic acid, at a concentration of 0.100 mg/ml, sonicated for approximately 1 min and stored at 4°C.

2.3. IS preparation

An intermediate IS solution was prepared containing 40,000 pg/ml of PF-06974801 by diluting the stock solution in aqueous 100 mM ammonium acetate buffer, pH 5.5. The ammonium acetate buffer pH was adjusted with glacial acetic acid. A working IS solution was prepared by diluting the intermediate IS solution with 100 mM ammonium acetate, pH 5.5 to provide a concentration of 5.00 pg/ml of PF-06974801.

2.4. Plasma calibration standards & validation sample preparation

Separate analyte spiking solutions were used to prepare plasma calibration standards and validation samples in control cynomolgus monkey K_3EDTA plasma. Calibration standards were prepared in polypropylene vials by serial dilution to produce concentrations of 0.200, 0.500, 2.00, 5.00, 10.0, 14.0, 18.0 and 20.0 pg/ml. Validation samples were prepared in polypropylene vials by serial dilution to generate concentrations (pg/ml) of 0.200 (quality control lower limit of quantitation [QC LLOQ]), 0.600 (quality control low [QC low]), 3.00 (quality control middle [QC middle]), 16.0 (quality control high [QC high]) and 100 (5x upper limit of quantitation). The plasma calibration standards were prepared fresh for each validation batch run and stability assessment. The validations samples were stored at -70°C until time of use.

2.5. LC-MS/MS conditions

A Waters Corporation Aquity M Class chromatographic system using a Waters Corporation iKey, 150 μm x 50 mm peptide BEH C18 130 Å 1.7 μM, Waters P/N 186006764 was employed for the chromatographic separation. The iKey temperature was operated at 50.0°C. Mobile phases A and B consisted of Optima™ LC/MS grade water containing 0.1% (v:v) formic acid and Optima LC/MS grade

acetonitrile containing 0.1% (v:v) formic acid, respectively. A flow rate of 4.000 µl/min was used. The chromatographic gradient used is shown in Supplementary Table S1. The high organic solvent autosampler needle wash solution was composed of Optima LC/MS grade 10:90 (v:v) water: acetonitrile containing 0.1% (v:v) formic acid. The low organic solvent autosampler needle wash solvent contained Optima LC/MS grade 90:10 (v:v) water: acetonitrile containing 0.1% (v:v) formic acid. The pump seal wash solution was comprised of 90:10 (v:v) Optima LC/MS grade water: HPLC grade methanol. The autosampler was maintained at 10.0°C. An autosampler injection volume of 4.00 µl was employed. A Waters Xevo TQS tandem quadrupole mass spectrometer was employed for detection utilizing statistically optimized parameters. The MRM transitions used were 556.38 >324.26 and 560.50 >328.40 for PF-06882961 and PF-06974801, respectively. Full scan and product ion mass spectra for PF-06882961 and PF-06974801 are provided in Supplementary Figure S1. Proposed product ions of PF-06882961 and PF-06974801 are illustrated in Supplementary Figure S2.

2.6. Plasma matrix sample extraction

Control plasma, plasma calibration standards, plasma validation samples, or study samples were thawed at ambient temperature and vortexed. Aliquots (200 µl) of control matrix, calibration standards, validation samples or study samples were added to the wells of a 96-well polypropylene block. IS working solution (200 µl) in aqueous 100 mM ammonium acetate (pH 5.5) was added to each well (excluding double blank and carryover samples). Aqueous 100 mM ammonium acetate (pH 5.5) was added to the double blank and carryover samples. The 96-well plate was capped and vortexed, allowing the samples to equilibrate to a pH of 5.5. The samples were transferred to a supported liquid extraction (SLE) plate (Biotage, Isolute® SLE+ 400 µl Array Wells (2 ml Wells), catalog no. 820-0400-P01). The samples were allowed to absorb to the SLE bed for 5 min. Each well of the SLE plates was eluted with 0.9 ml of MTBE twice and the eluent was collected in a 2 ml SiliGuard non-binding coated polypropylene 96 well plate. 5 min was allowed for each MTBE elution. Vacuum was applied for approximately 30 s to complete elution. The eluent was dried under nitrogen at 40°C. To ensure complete removal of residual MTBE, 100 µl of acetonitrile was added to each well and allowed to dry at 40°C. The samples were reconstituted with 50.0 µl of 90:10 (v:v) water: acetonitrile containing 0.1% (v:v) formic acid and mixed. The injection plate was centrifuged at 1250 x *g* for 1 min and injected onto the microflowLC-MS/MS system.

2.7. LC-MS/MS data analysis

Peak areas of analyte and IS were determined by the MassLynx data processing software (version 4.1). These responses were imported into Watson laboratory information system (LIMS) (version 7.5) and a calibration curve was constructed using peak area ratios of the calibration samples and applying weighted ($1/x^2$) linear least squares regression analysis. All concentrations were calculated using this calibration curve.

2.8. Statistical mass spectrometer tuning

The mass spectrometer source tuning parameters included: capillary (kV), cone (V), source temperature (°C), cone gas flow (L/h), nebuliser (Bar), and nanoflow (Bar). Additional mass spectrometer parameters included: Low Mass (LM) resolution 1, High Mass (HM) resolution 1, ion energy 1, LM resolution 2, HM resolution 2, ion energy 2, collision gas flow (ml/min) and collision energy (eV). Initial parameter scouting experiments were conducted using a 10 µl/min infusion of PF-06882961 in 1:1 (v:v) water: acetonitrile containing 0.1% (v:v) formic acid. The following mass spectrometric parameters were identified as critical to the analyte S:N ratio: capillary (kV), cone (V), cone gas (L/Hr), nanoflow gas (Bar), collision gas flow (mL/Min), collision energy (eV), and source temperature (°C). A central composite design of 38 unique experimental conditions plus replicates was created taking into consideration the critical parameters [16]. For efficient execution, source temperature was held fixed in blocks of experiments creating a split-plot design structure. Table 1 summarizes the investigated electrospray source and collision cell parameters and ranges, juxtaposed with the Waters default mass spectrometer settings. Additionally, a visual representation of the study design is provided in Figure 2. The experiment was conducted by injecting PF-06882961 standard (10.0 pg/ml) prepared in monkey K_3EDTA plasma in triplicate for each mass spectrometer parameter set evaluated. Using those data, empirical tuning parameter models were constructed to simultaneously maximize S:N and peak height.

Waters MassLynx™ software contains an automatic tuning function, Intellistart™, which determines optimal cone voltage (2–100 V), collision energy (2–80 V) and MRM transitions while the compound is being infused into the mass spectrometer source [17]. This autotune feature of MassLynx was evaluated relative to the statistically optimized mass spectrometer parameters by performing replicate injections (n = 3) of extracted LLOQ standard (0.200 pg/ml PF-06882961 in K_3EDTA monkey plasma) using the IntelliStart and statistically optimized mass spectrometric parameters.

Table 1. Statistical tuning design summary illustrating the electrospray source and collision cell parameters investigated.

Parameter	Waters default setting	Investigated low	Investigated high
Capillary (kV)	3.5	3.0	5.0
Cone (V)	30	0	150
Source temperature (°C)	120	90	150
Cone gas flow (L/H)	150	20	150
NanoFlow (Bar)	0.10	0.1	0.2
Collision gas flow (ml/min)	0.15	0.10	0.15
MSMS Mode Collision Energy (eV)	4	25	30
Nebuliser (Bar)	5.0	Not investigated	
LM resolution 1	3.0		
HM resolution 1	15.0		
Ion energy 1	0.5		
LM resolution 2	3.0		
HM resolution 2	15.0		
Ion energy 2	0.5		

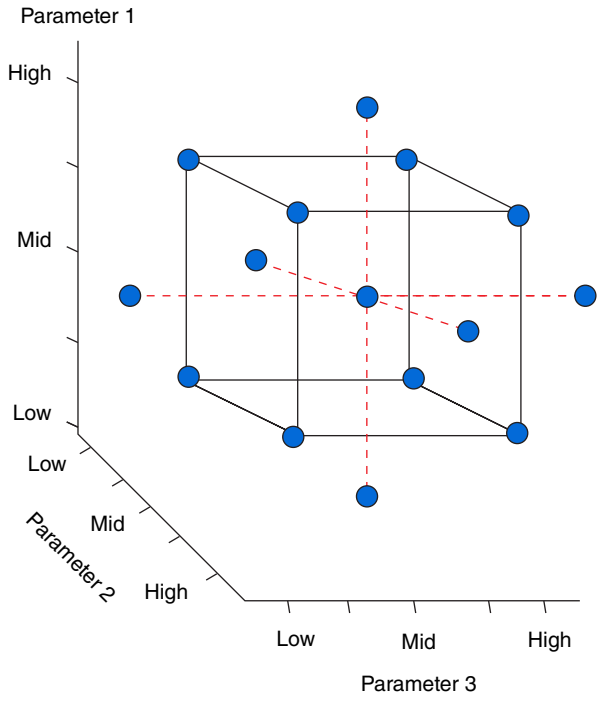

Figure 2. Central composite design visual representation over three parameters.

2.9. Validation

The microflowLC-MS/MS assay was validated via quantitation of PF-06882961 concentrations in cynomolgus monkey K_3EDTA plasma using the mass spectrometric parameters that were statistically optimized.

2.9.1. Analyte & IS stock solution stability

The stability of PF-06882961 was evaluated by comparison of a freshly prepared stock solution to the stored stock solutions. Stability was demonstrated if the percent difference between peak area ratio of fresh and stored stock solution was $\leq$10%. The stability of the stable labeled IS, PF-06974801, was assumed to be the same as the unlabeled analyte compound, PF-06882961.

2.9.2. System suitability test

A separate extracted monkey K_3EDTA plasma sample prepared at the LLOQ concentration of (0.200 pg/ml) was designated as the system suitability test (SST) sample. The SST sample was analyzed and evaluated prior to the start of each validation and sample analysis run. The retention time and sensitivity of each analyte in the SST sample were evaluated prior to the start of each run.

2.9.3. Precision & accuracy

Three validation batch runs were performed. Intra- and inter-assay precision (percent coefficient of variation [%CV]) and accuracy (percent relative error [%RE]) were evaluated for three validation batch runs. The acceptance criteria was: CV values were $\leq$15% (except $\leq$20% at LLOQ) and the RE were within $\pm$ 15% (except $\pm$ 20% at LLOQ) for PF-06882961.

2.9.4. Selectivity

Selectivity was evaluated for six independent sources of cynomolgus monkey K_3EDTA plasma (three male and three female monkeys) by extracting blank matrix (double blank) and blank matrix with IS (control blank). Area responses with a similar retention time to the analyte were compared with the analyte area response in the LLOQ calibration standard (0.200 pg/ml). The acceptance criterion for the analyte is any response with a similar retention time to the analytes are $\leq$20% of the analyte response in the LLOQ calibration standard (0.200 pg/ml). Area responses of the IS in the control blanks were compared with the IS response in the control blank prepared from pooled monkey plasma. The acceptance criterion for the IS is that any response with a similar retention time to the IS was $\leq$5% of the response for the IS peak in the control blank.

2.9.5. Matrix effects

The matrix factor (MF) is the quantitative measure of matrix effects for a given bioanalytical LC-MS/MS assay. The MF is defined as the ratio of peak area response in the presence of matrix ions to the analyte peak response in the absence of matrix ions. A MF value of one signifies no matrix effects are present. A value less than one or greater than one suggests that matrix effects may be present respectively attributed to ionization suppression or enhancement. The acceptance criterion is the IS normalized MF variability ≤15% CV. Six individual lots of cynomolgus monkey plasma (three male and three female) were evaluated for matrix effects.

2.9.6. Recovery

Analyte and IS peak areas from extracted (n = 6) validation samples (0.600 pg/ml [QC low], 3.00 pg/ml [QC middle], 16.0 pg/ml [QC high]) were compared with those obtained from samples spiked at the same final concentration into blank extracts (post-extract). The use of post-extract spikes as the recovery controls allows compensation for matrix effects and allows the extraction analyte and IS recovery to be evaluated.

2.9.7. Process efficiency

Analyte and IS peak areas from extracted (n = 6) validation samples (0.600 pg/ml [QC low], 3.00 pg/ml [QC middle], 16.0 pg/ml [QC high]) were compared with neat recovery solutions at the same final concentrations.

2.9.8. Carryover

Carryover was evaluated in each validation batch run. The response for analyte in the carryover sample was compared with the analyte response of the LLOQ calibration standard (0.200 pg/ml). Additionally, the response for the IS in the carryover sample was compared with the IS in the control blanks. Carryover was evaluated in each validation batch and sample analysis run. The acceptance criteria for the analyte in the carryover sample was ≤20% of the response of the analyte in the LLOQ calibration standard (0.200 pg/ml). The acceptance criteria for the IS in the carryover sample was ≤5% of the response for the IS in the control blank.

2.9.9. Capability of dilution

Capability of dilution is established when the mean (n = 6) sample concentration of the dilution validation samples demonstrates both %CV ≤15% and %RE within ± 15% of the nominal concentration. A 100 pg/ml (5x upper limit of quantitation) dilution validation sample was prepared and diluted 10x (n = 6) with cynomolgus monkey control K_3EDTA plasma.

2.9.10. Run size evaluation

The trends over long injection periods were evaluated for a run size of 96 injections. The acceptance criteria is %CV values ≤15% (except ≤20% at LLOQ) and the %RE within ± 15% (except ± 20% at LLOQ) for PF-06882961.

2.9.11. Processed sample stability

The stability of processed samples that are stored for a period of time and not previously injected is determined by injecting previously extracted QC samples along with fresh standards and qualifying QC samples. Stability is demonstrated if stability processed samples meet intra-assay acceptance criteria.

2.9.12. Reinjection reproducibility

The reproducibility of reinjecting processed samples is determined by reinjecting a previously-assayed acceptable accuracy and precision run. Stability is demonstrated if reinjection reproducibility samples meet intra-assay acceptance criteria.

2.9.13. Benchtop stability

The stability of PF-06882961 is evaluated by storing QC samples at ambient temperature. Stability is demonstrated if the mean concentration of the stored QC sample is within ± 15% compared with nominal concentration and the CV is ≤15%. The stability of PF-06882961 was evaluated by storing validation samples (0.600 pg/ml (QC low) and 16.0 pg/ml (QC high)) at ambient temperature. Stability is demonstrated for PF-06882961 if the validation sample mean concentration was within ± 15% compared with nominal concentration and the CV was ≤15%.

2.9.14. Stability of analyte in matrix to freezing & thawing

The stability of PF-06882961 is evaluated by subjecting validation samples (0.600 pg/ml [QC low] and 16.0 pg/ml [QC high]) to freeze/thaw cycles. Stability is demonstrated if the mean concentration of the freeze/thaw QC sample is within ± 15% compared with nominal concentration and the CV is ≤15%.

2.9.15. Stability of analyte in frozen matrix

The stability of PF-06882961 is evaluated by storing validation samples (0.600 pg/ml [QC low] and 16.0 pg/ml [QC high]) at -70°C. Stability is demonstrated if the mean concentration of the stored QC sample is within ± 15% compared with nominal concentration and the CV was ≤15%.

2.10 Preclinical pharmacokinetics studies

All activities involving animals were carried out in accordance with federal, state, local and institutional guide-

lines governing the use of laboratory animals in research in AAALAC accredited facilities and were reviewed and approved by Pfizer's Institutional Animal Care and Use Committee. Monkey studies were conducted at Pfizer (CT, USA). Male Cynomolgus monkeys were purchased from Covance (NJ, USA), Charles River Laboratories, Inc. (MA, USA), or Envigo Global Services (IN, USA); subjects 3–8 years of age were used in pharmacokinetics studies.

A microdose study was conducted by dosing 0.005 mg total of PF-06882961 intravenously to male cynomolgus monkeys (n = 3) by the saphenous vein. PF-06882961 was administered as a solution in 5% (v/v) polyethylene glycol 400:95% (v/v) [12% (w/v) sulfobutylether-β-cyclodextrin in deionized water] with a dose volume of 0.5 ml per animal. Serial blood samples were collected via the femoral vein before dosing and at specified time points (0.083, 0.25, 0.5, 1, 2, 4, 7 and 24 h) post-dose into K_3EDTA vacutainers. The blood was centrifuged, and plasma was removed and frozen at -20°C or lower.

3. Results & discussion

3.1. Method development

The microflowLC-MS/MS system was operated in positive ion mode, taking into consideration the physicochemical properties of PF-06882961. A tabulation of the physicochemical properties of PF-06882961 is provided in Supplementary Table S2. The physicochemical properties were estimated utilizing ACD Labs Percepta software [18]. A plot of Log D vs pH calculated using this software is illustrated in Supplementary Figure S3.

The optimal SLE extraction pH of 5.5 was chosen based on ACD Lab Log D analysis. The pH was controlled by addition of 100 mM ammonium acetate, pH 5.5 buffer to the plasma sample at a ratio of 1:1 (v:v). At this pH, PF-06882961 is expected to remain in its neutral form, providing the best partitioning into the organic phase and resulting in optimal recovery. The use of MTBE as the SLE extraction eluent provided adequate recovery and minimal matrix effects. However, it was found that residual MTBE in the final injection sample caused analyte peak fronting. Therefore, an additional rinse of acetonitrile was performed after the MTBE dry-down to remove residual solvent.

Analyte carry-over was observed in the double blank (blank plasma without IS). The source of the carry-over was contributed to un-eluted analyte trapped on the analytical column after the gradient wash step. Therefore, an additional wash gradient was added to the analytical gradient in order to minimize the column carryover.

3.2. Statistical mass spectrometer tuning results

The study design was conducted with n = 41 experimental runs, of which 38 were unique parameter combinations that included the Intellistart conditions. Replicates were placed at the beginning, middle, and end of study to confirm no experimental drift. The PF-06882961 peak height and S:N ratio were obtained for each injection of the 10.0 pg/ml PF-06882961 standard prepared in monkey K_3EDTA plasma using MassLynx software. Using those data, empirical tuning parameter models were constructed to simultaneously maximize S:N and peak height. Optimizing over both responses ensures optimal solutions do not include artificially high S:N values from non-representative peaks. Initially, the S:N measurements were obtained from a baseline region immediately before the PF-06882961 peak. However, interfering peaks were observed immediately prior to the PF-06882961 peak, not allowing a consistent S:N to be calculated. Therefore, the central composite design was repeated using a region of the baseline immediately after the PF-06882961 peak, which produced a more consistent S:N ratio between replicates and mass spectrometer conditions. Supplementary Table S3 provides a tabulation of the monkey plasma central composite design and data using S:N immediately after the PF-06882961 peak.

Standard response surface methodology was used to build predictive relationships that mapped variation in both S:N and peak height against deliberate changes in the tuning parameters. Empirical model building was accomplished by fitting a full quadratic model to the median of the triplicate data for each experimental condition and employing a backward selection algorithm to include only those terms that meet a threshold significance level of $p < 0.05$ while preserving model hierarchy. For both responses, residual diagnostics such as normal probability plots, scaled residuals, and Box-Cox transformations were used to verify standard error distribution assumptions are satisfied, such as normality or constant variance, and identify if any outliers with respect to the model are present. Data transformations were applied if deemed appropriate by the Box-Cox method for power transformations, and the resulting model fit significantly improved. Regarding outliers, experimental conditions that contained data greater than 3 standard deviations away from the model prediction were identified as discordant, removed and the subset of data were refit. Stat-Ease Design Expert software, version 12 was employed for all statistical modeling, diagnostic checking and visualization. Using those data and the methodology outlined above, the final empirical tuning parameter models were constructed that simultaneously maximized peak height and S:N (immediately after PF-06882961 peak).

Not surprisingly, all parameters demonstrated some influential, statistical impact in predicting peak height and S:N through main parameter effects, multi-parameter interactions, or quadratic terms. Final ANOVA tables and predictive models are shown in Supplementary Table S4. Figure 3 illustrates example predictive surface plots for each modeled response to reveal the functional relationship over two of the seven key variables. The various R^2-type statistics indicate particularly good predictive capability to locate candidate optima (~81% for S:N and ~96% for peak height). From the modeling exercise, we infer that the predicted optimal settings are non-obvious; unlikely to be found via univariate parameter scanning, and quite different from the Waters Intellistart auto-tune algorithm recommended setting.

A small confirmatory study was performed to verify potential optima, where some parameters were fixed at their optimal settings while others were varied to ensure method robustness. The potential optima are described in Supplementary Table S5. The potential optimal tuning parameters evaluation data is presented in Supplementary Table S6. The final confirmed statistically tuned parameters and the mass spectrometer values produced by the IntelliStart feature of MassLynx are tabulated in Table 2. The statistically optimized tune parameters produced a S:N approximately threefold greater than the Waters auto-tune algorithm. Supplementary Table S7 tabulates a comparison of statistically optimized and IntelliStart mass spectrometer parameters using the monkey plasma LLOQ calibration standard (0.200 pg/ml). Supplementary Figure S4 illustrates this comparison between S:N of PF-06882961 of the statistically optimized and Waters IntelliStart mass spectrometric parameters. Saxena et al. achieved a LLOQ of 0.100 ng/ml to support a multiple ascending dose human phase one clinical trial [7]. The LLOQ of 0.200 pg/ml presented in this work represents a 500x improvement in sensitivity.

The optimal tuning parameter conditions were used to perform a validation over the range of 0.200 to 20.0 pg/ml PF-06882961 in cynomolgus monkey K_3EDTA plasma and analyze a monkey microdose pharmacokinetics study.

3.3. Validation results

The microflowLC-MS/MS assay was validated to quantitate PF-06882961 in cynomolgus monkey K_3EDTA plasma. All validation criteria were met, including selectivity, calibration standards and parameters, precision, accuracy, matrix effects, carryover, capability of dilution and run size evaluation. Stock and matrix stability assessments were also successfully performed. Supplementary Figures S5 & S6 provide representative chromatograms of a monkey K_3EDTA plasma double blank (no IS added) and LLOQ standard (0.200 pg/ml PF-06882961 in monkey K_3EDTA plasma), respectively.

3.3.1. Analyte & internal standard stock solution stability

PF-06882961 and its internal standard, PF-06974801, were found to be stable for 112 and 29 h at 4°C and at ambient temperature, respectively.

3.3.2. System suitability test

The retention time and sensitivity of PF-06882961 in the system suitability test (SST) sample (10.0 pg/ml PF-06882961 in K_3EDTA monkey plasma) were evaluated prior to the start of each run and found to be acceptable.

3.3.3. Calibration standards & calibration parameters

The calibration standards of the three validation batch runs provided acceptable precision and accuracy corresponding to concentration %CV values of $\leq$4.5 and %RE values ranging from -0.9 to 3.9, respectively. The precision of the slope values was 11.9%. R-squared values were $\geq$0.9983. Supplementary Table S8 provides a tabulation of the calibration standard and calibration parameters,

3.3.4. Precision & accuracy

Three validation batch runs were performed. Intra- and inter-assay precision (%CV) and accuracy (%RE) and were evaluated for three validation batch runs. The %CV values were $\leq$15% (except $\leq$20% at LLOQ) and the %RE were within $\pm$ 15% (except $\pm$ 20% at LLOQ) for PF-06882961. Table 3 tabulates the mean intra/inter precision and accuracy data of the validation samples. Supplementary Table S9 provides individual replicate data for intra/inter precision and accuracy.

3.3.5. Selectivity

The response of any peaks with a similar retention time to PF-06882961 in the K_3EDTA monkey plasma double blanks (no IS added) and K_3EDTA monkey plasma control blanks (containing IS) did not exceed 2.3% of the response for the respective LLOQ calibration standard. Any response with a similar retention time to the IS, PF-06974801, did not exceed 0.2% of the response for the IS peak in the K_3EDTA monkey plasma control blanks (containing IS).

3.3.6. Matrix effects

The matrix factor (MF) is defined as the ratio of peak area response for the analyte, PF-06882961, in the presence of matrix ions to the analyte peak response in the absence of matrix ions. The acceptance criteria is that the IS normalized MF variability must be $\leq$15% CV. The %CV in the IS normalized MFs, did not exceed 2.5% CV.

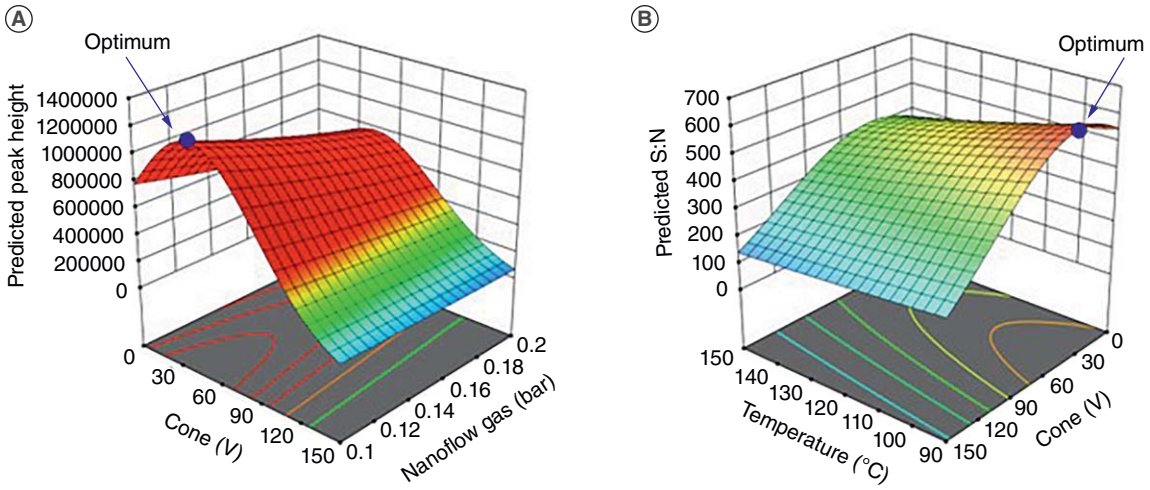

Figure 3. Response surface plots for each analyzed response across two of the seven investigated tuning parameters. **(A)** Illustrates the relationship between predicted peak height, cone (V), and nanoflow gas (bar) and **(B)** illustrates the relationship between predicted S:N, temperature (C), and cone (V).

Table 2. Optimized mass spectrometer parameters.

Parameter	Statistically optimized setting	IntelliStart optimized setting
Capillary (kV)	3.10	2.93
Cone (V)	45.00	30.00
Source temperature (°C)	90	120
Cone gas flow (L/H)	Off	150
NanoFlow (Bar)	0.10	0.20
Collision gas flow (ml/min)	0.10	0.15
Nebuliser (Bar)	7.00	7.00
LM resolution 1	2.8	2.8
HM resolution 1	15.0	15.0
MSMS Mode Collision Energy (eV)	28.00	20.00
Ion energy 1	0.5	0.5
LM resolution 2	2.7	2.7
HM resolution 2	15.1	15.1
Ion energy 2	1.1	1.1

Table 3. Intra/inter precision and accuracy of validation samples.

	Validation samples			
	QC LLOQ (0.200 pg/ml)	QC low (0.600 pg/ml)	QC middle (3.00 pg/ml)	QC high (16.0 pg/ml)
Validation batch run no. 1				
Intra-run mean (n = 6)	0.213	0.572	2.77	15.3
Intra-run %CV	11.5	4.2	1.3	1.4
Intra-run %RE	6.5	-4.7	-7.7	-4.4
Validation batch run no. 2				
Intra-run mean (n = 6)	0.173	0.580	3.13	17.6
Intra-run %CV	4.1	3.9	3.3	1.0
Intra-run %RE	-13.5	-3.3	4.3	10.0
Validation batch run no. 3				
Intra-run mean (n = 6)	0.203	0.582	2.87	16.7
Intra-run %CV	5.3	5.7	2.5	7.7
Intra-run %RE	1.5	-3.0	-4.3	4.4
Inter-run mean (n = 18)	0.197	0.578	2.92	16.5
Inter-run %CV	11.7	4.4	5.9	7.4
Inter-run %RE	-1.5	-3.7	-2.7	3.1

3.3.7. Recovery

The recovery values of PF-06882961 ranged from 77.5 to 90.7% and the recovery values of the IS PF-06974801 ranged from 55.3 to 63.0%. The recovery values of the analytes and IS were consistent across the 0.600 pg/ml (QC low), 3.00 pg/ml (QC middle) and 16.0 pg/ml (QC high) concentration range, considering recovery %CV values of 7.9 and 7.1 were obtained for the analyte and IS,

respectively. This precision is less than intra-run %CV criterion of 15% required for the replicate analysis of validation samples.

3.3.8. Process efficiency

The process efficiency values of PF-06882961 ranged from 67.9 to 79.7%. The process efficiency of the IS PF-06974801 ranged from 50.2 to 56.0%. The process efficiency values of the analytes and their respective internal standards were consistent across the 0.600 pg/ml (QC low), 3.00 pg/ml (QC middle) and 16.0 pg/ml (QC high) concentration range. This is evident considering process efficiency %CV values of 8.8 and 5.9 were obtained for the analyte and IS, respectively. This precision is less than intra-run %CV criterion of 15% required for the replicate analysis of validation samples.

3.3.9. Carryover

The response for the analyte, PF-06882961, in the carryover sample was $\leq$20% of the response of the analyte in the LLOQ calibration standard (0.200 pg/ml). The response for the IS, PF-06974801, in the carryover sample was $\leq$5% of the response for the IS in the control blank. The acceptance criteria for both the analyte and IS were met.

3.3.10. Capability of dilution

The mean (n = 6) sample concentration of the dilution validation samples demonstrated both %CV $\leq$15% and %RE within $\pm$ 15% of the nominal concentration for PF-06882961, successfully validating a 10x dilution factor.

3.3.11. Run size evaluation

The trends over long injection periods were evaluated for a run size of 96 injections. Intra-assay batch run acceptance criteria of %CV values $\leq$15% (except $\leq$20% at LLOQ) and the %RE within $\pm$ 15% (except $\pm$ 20% at LLOQ) were met.

3.3.12. Processed sample stability

Stability was demonstrated for processed samples for 45.4 h at 10°C. Intra-assay batch run acceptance criteria of %CV values $\leq$15% (except $\leq$20% at LLOQ) and the %RE within $\pm$ 15% (except $\pm$ 20% at LLOQ) were met.

3.3.13. Reinjection reproducibility

The reproducibility of reinjecting processed samples stored 57.8 h at 10°C met the intra-assay batch run acceptance criteria of %CV values $\leq$15% (except $\leq$20% at LLOQ) and the %RE within $\pm$ 15% (except $\pm$ 20% at LLOQ).

3.3.14. Benchtop stability

PF-06882961 was found to be stable in K_3EDTA monkey plasma at ambient temperature for 92 h.

3.3.15. Stability of analyte in matrix to freezing & thawing

PF-06882961 was found to be stable in K_3EDTA monkey plasma four 4 freeze/thaw cycles at -70°C.

3.3.16. Stability of analyte in frozen matrix

PF-06882961 was found to be stable in K_3EDTA monkey plasma four for 14 days at -70°C.

3.4. Microdose study

Three cynomolgus monkeys were dosed with 0.005 mg total PF-06882961 by intravenous bolus, corresponding to an average dose of 0.0007 mg/kg. The 24-h time point was below the limit of quantitation (BLOQ) of $<$0.200 pg/ml. Table 4 presents pharmacokinetic parameters for the monkey microdose study. Supplementary Figure S7 illustrates a representative microdose study K_3EDTA monkey plasma 0.083-hour (5 minute) chromatogram. Figure 4 provides individual and mean (n = 3) pharmacokinetic plots for the microdose IV study.

Utilizing microflowLC-MS/MS in conjunction with statistical mass spectrometer tuning allowed a pharmacokinetic profile to be constructed, demonstrating that sufficient sensitivity was obtained for a possible human microdose study to be conducted using microflow-LC-MS/MS as opposed to AMS. A limitation that should be considered is that the microdose study performed in this paper was administrated by intravenous bolus, whereas a human microdose study would typically be dosed orally. The oral dose may lead to additional constraints around oral absorption and therefore a further decrease in plasma exposure if oral absorption is incomplete. However, this work demonstrates that a considerably low limit of quantitation can be obtained by microflowLC-MS/MS, alleviating the requirement for AMS.

4. Conclusion

A microflowLC-MS/MS assay was successfully developed and validated to quantitate PF-06882961 with an LLOQ of 0.200 pg/ml in monkey K_3EDTA plasma. This method was applied to a monkey microdose intravenous bolus pharmacokinetics study (0.005 mg total dose) to successfully demonstrate the possibility of a human plasma microdose study with unlabeled PF-06882961 using microflowLC-MS/MS as opposed to AMS, thereby reducing cost and eliminating synthesis and volunteer exposure to ^{14}C labeled material. Additionally, response surface methodology statistically optimized the tuning

Table 4. Pharmacokinetic parameters for monkey 0.005 mg total microdose iv. bolus (mean ± standard deviation, n = 3).

AUC (0-tlast) (pg*h/ml)	307 ± 114
AUC (0,inf) (pg*h/ml)	312 ± 120
AUC extrapolated (%)	1.60 ± 0.93
C_0 (pg/ml)	1110 ± 424
$t_{1/2}$ (h)	2.40 ± 0.49
Vd_{ss} (l/kg)	1.76 ± 0.69
CL_{plasma} (ml/min/kg)	39.1 ± 6.3

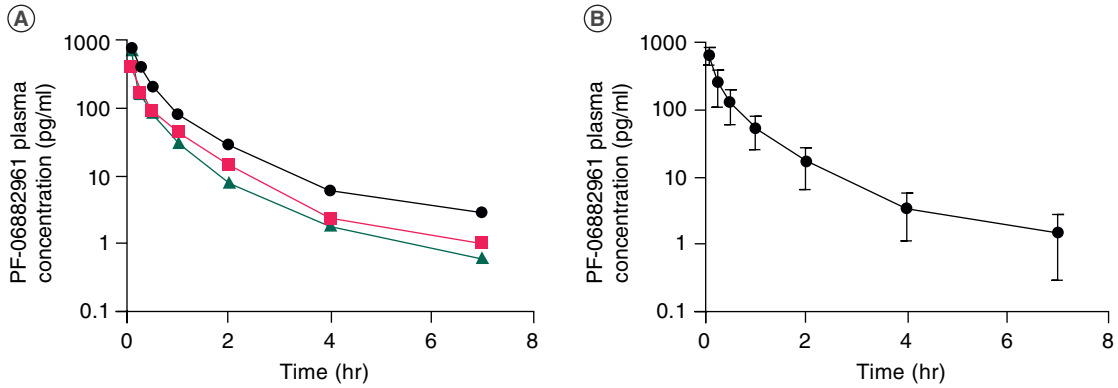

Figure 4. Pharmacokinetic plots for monkey microdose iv. bolus study. **(A)** Depicts individual plasma concentrations and **(B)** depicts mean concentrations with error bars representing standard deviations.

parameters to produce a S:N approximately threefold greater than the Waters auto-tune algorithm.

Article summary

- The purpose of this work was to determine the possibility of supporting a human plasma microdose study for a small molecule agonist of the human GLP-1R using microflowLC-MS/MS in lieu of accelerator mass spectrometry.
- Mass spectrometer optimization via response surface methodology was employed in conjunction with microflowLC-MS/MS to achieve the required LLOQ of 0.200 pg/ml PF-06882961.
- A monkey microdose study was conducted using a 0.005 mg total dose of PF-06882961 by intravenous bolus.
- The statistically optimized tune parameters produced a S:N approximately 3x greater than the Waters auto-tune algorithm.
- A microflow LC-MS/MS assay was successfully developed and validated to quantitate PF-06882961 with an LLOQ of 0.200 pg/ml in cynomolgus monkey K_3EDTA plasma.
- This work determined that microdose plasma samples for PF-06882961 could potentially be analyzed by microflow LC-MS/MS, instead of accelerator mass spectrometry.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity 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.

Writing disclosure

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

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Waters™

Productivity, Throughput, and Data Quality in Discovery and Regulated Bioanalysis

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Waters Corporation, Milford, MA USA[a]
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SUMMARY

Liquid chromatography combined with electrospray tandem quadrupole mass spectrometry (MS) is the backbone of quantitative bioanalysis to support drug discovery, preclinical development, and human safety/efficacy trials. Over the last 30 years, MS developments have focused on improving sensitivity and throughput. More recently, however, the attention has moved to improving instrument robustness, up-time, and operational efficiency.

This paper evaluates the robustness and long-term performance of Waters™ Xevo™ TQ Absolute XR Mass Spectrometer equipped with StepWave™ XR Ion Guide technology combined with liquid chromatography for bioanalysis of plasma-derived samples. More than 30,000 injections of rat plasma extract were analyzed with exceptional data quality (> 99.65% of all QCs were within the acceptance criteria, the precision > 90% and bias less than 10%), minimal maintenance, and no instrument downtime, demonstrating suitability for high-throughput discovery and regulated bioanalysis and potential for large batch applications.

INTRODUCTION

Since its commercialization in the early 1990s, electrospray ionization (ESI) tandem quadrupole MS has become the technology of choice for quantitative bioanalysis, especially when combined with high performance liquid chromatography.[1] This is due to the specificity, selectivity, and sensitivity of the technique when operated in multiple reaction monitoring (MRM) mode, facilitating sub-pg/mL sensitivities in biofluids, even for challenging compounds *e.g.*., inhaled steroids.[2]

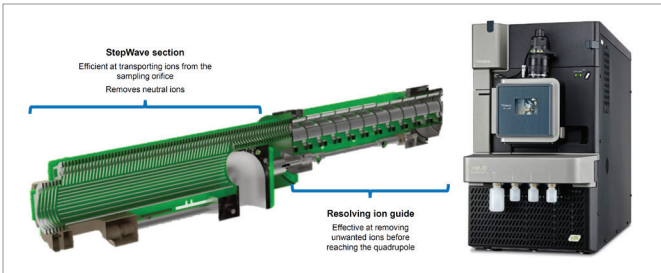
In addition to improving assay detection limits, the exquisite sensitivity and selectivity of ESI-MRM acquisition has enabled a significant reduction in chromatographic run times, typically sub-5 min, compared to high-performance liquid chromatography (HPLC) with UV or fluorescence peak detection, where analysis times of 15–20 min were common. The commercial introduction of sub-2 µm particle ultra high-performance liquid chromatography (UHPLC) in 2004, when combined with ESI-MRM, not only increased assay sensitivity but also facilitated 2- to 3-fold reduction in analysis times.[3]

The extra specificity and selectivity of the MRM acquisition process also reduced the need for complex, comprehensive sample preparation (clean up). Such that solid-phase extraction (SPE) and liquid - liquid extraction (LLE), which were once commonplace for biofluid analysis[4], were replaced by simple protein removal, either by filtration or by precipitation with an organic solvent. Additionally, these advances in MS performance, LC speed, and sample preparation enabled a significant daily increase in the number of samples which could be processed, with the processing of 200–400 samples per instrument per day now feasible. With this increase in throughput, many bioanalytical laboratories can implement full automation - achieving 24/7 operation.[5]

ADDRESSING CHALLENGES TO MS PERFORMANCE

However, the combination of limited sample clean-up and short chromatographic run times has resulted in an increase in the mass of co-extractive matrix material entering the MS source and vacuum region of the mass spectrometer. This matrix material progressively deposits on the ion guide, focusing regions and quadrupoles in the mass spectrometer, resulting in a gradual reduction in signal response, eventually requiring cleaning of the ion optics to restore the MS performance to original levels. This is a time-consuming process, as it necessitates venting and subsequent pumping down of the mass spectrometer, which can take more than one day to complete before study samples can be analyzed. Ultimately, this leads to delays in project support, reduced data quality, inefficient instrument utilization, and loss in revenue (up to $20,000 based on 4 microtiter plates per day and a cost of $50–100 per sample).

To address this challenge, MS manufacturers have developed and introduced new technologies to mitigate instrument fouling issues. One example of this is the StepWave XR slotted band pass ion guide which effectively mitigates Q1 quadrupole contamination. This new ion guide combines resolving direct current (DC) and an axial field to create a bandpass filter, protecting downstream ion optics from contamination by preventing unwanted high mass ion transmission into the MS1 quadrupole. Filtering out high *m/z* ions from biological matrices prevents quadrupole charging and associated losses in sensitivity observed from the accumulation of these ions on the MS1 quadrupole rods.

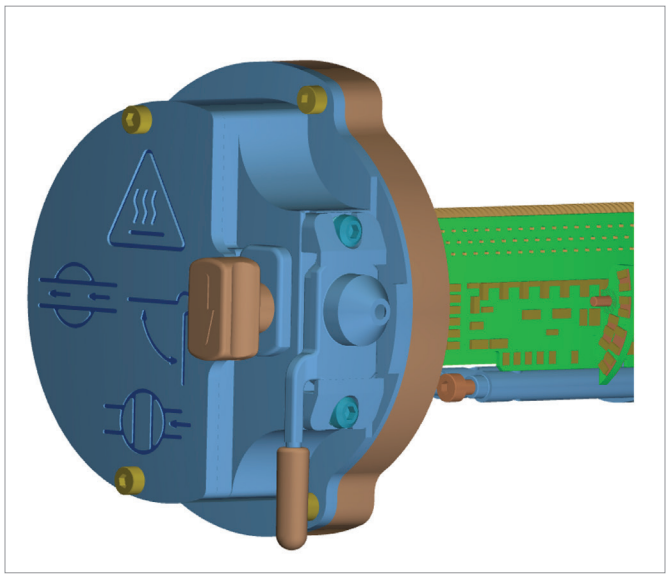


New StepWave XR Ion Guide in the Xevo TQ Absolute XR Mass Spectrometer.

IMPACT OF TECHNOLOGY

The advantage of this new ion guide technology was illustrated in the operation of a bioanalytical assay for naltrexone, a synthetic opioid antagonist, and its metabolite 6-beta-naltrexol, in rat plasma over 30,000 injections (performed by Alderley Analytical, UK). Using a previously validated assay, a multi-point calibration line was generated over the range of 0.2–100 ng/mL along with quality controls (QCs) at 5 levels, including lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) in control rat plasma. Samples were analyzed in 96 well plate batches which consisted of reagent blanks, 1st calibration curve, 3 QC sets, extracted matrix blanks, 3 more QC sets, and a 2nd calibration curve followed by system suitability test samples. The samples were prepared by simple protein precipitation with organic solvent containing the stable isotope-labeled versions of both analytes.

The resulting samples were analyzed using a 2-minute reversed–phase gradient separation with detection by ESI+ MRM using an ACQUITY™ Premier UPLC System coupled to Xevo TQ Absolute XR Mass Spectrometer. Over the course of the study, more than 21 mL of plasma was injected onto the LC-MS/MS system, and a total of 64 liters of mobile phase were used. The system was continuously operated for 55 days with the source block and sampling cone cleaned once a week (every ≈3300 injections). The unique design of the ZSpray™ ESI Source allowed the sampling cone to be removed and cleaned in just a few minutes without the need to break the instrument vacuum.



Waters ZSpray ESI Source.

Each one of the 96-well plates passed the FDA M10 Bioanalytical Method Validation and Study Sample Analysis6 acceptance criteria, using a 1/x2 weighting of the calibration line. The reproducibility of the data is illustrated by the variation in the LLOQ QCs (0.2 ng/mL) results for both naltrexone and 6-beta-naltrexol shown in Figure 1. The data shows that 99.65 % of the naltrexone LLOQ QCs and 99.70% of the 6-beta-naltrexol LLOQ QCs were within the acceptance criteria, from a total of 1,980 LLOQ QCs, across the entire 30,000 injection study. A box whisker plot analysis of the data showed that the calculated concentrations of all the QCs at each of the levels were closely distributed with only a few outliers at each concentration - none of which exceeded +/- 15% of the nominal concentration as illustrated by the dashed red line in Figure 2.

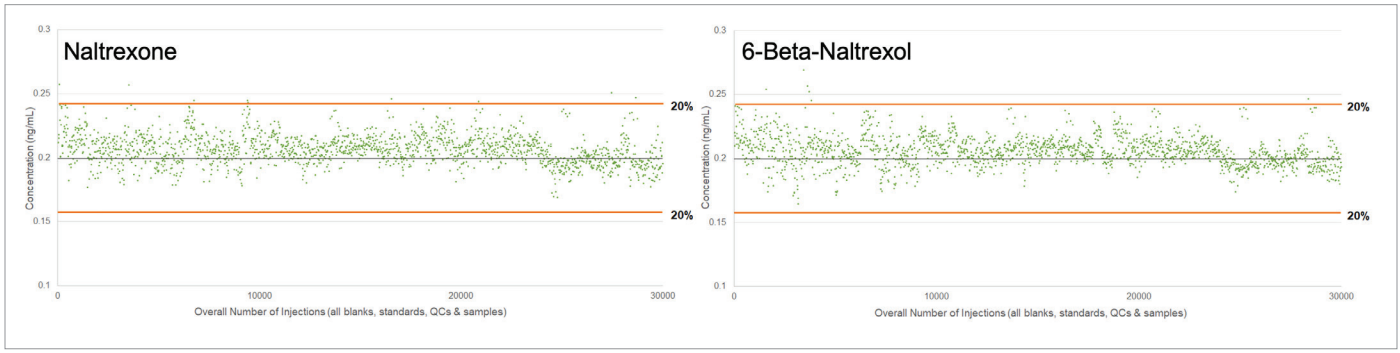


Figure 1. LLOQ QC concentration plot for 30,000 injections of naltrexone and 6-beta-naltrexol.

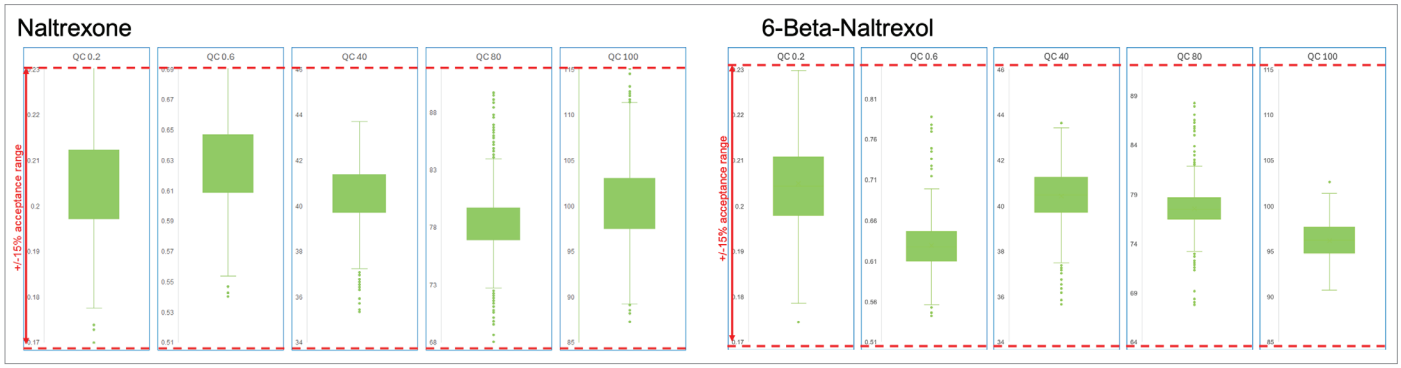


Figure 2. Box whisker plot for QCs of naltrexone and 6-beta-naltrexol.

Bias, precision, and accuracy are key attributes of any bioanalytical method and are used to show the applicability of a methodology or LC-MS/MS system for the measurement of a drug and or metabolite in biological fluid over the course of the study. They are particularly important at the low concentration levels, as they give an indication of the accuracy of the method at the LLOQ, which can be particularly important when defining the pharmacokinetic elimination phase of a drug, when the concentration levels are typically at their lowest.

The better the bias and precision, the more reliable the results. The analytical bias of the Xevo TQ Absolute XR Mass Spectrometer, seen in Figure 3, was evaluated using the LLOQ QCs for both naltrexone and 6-beta-naltrexol for the entire 30,000 injection study. The bias across the study was extremely low, with the majority of the LLOQ QC samples showing a bias of less than ± 10% and only a few individual samples having values outside of the ± 20% range.

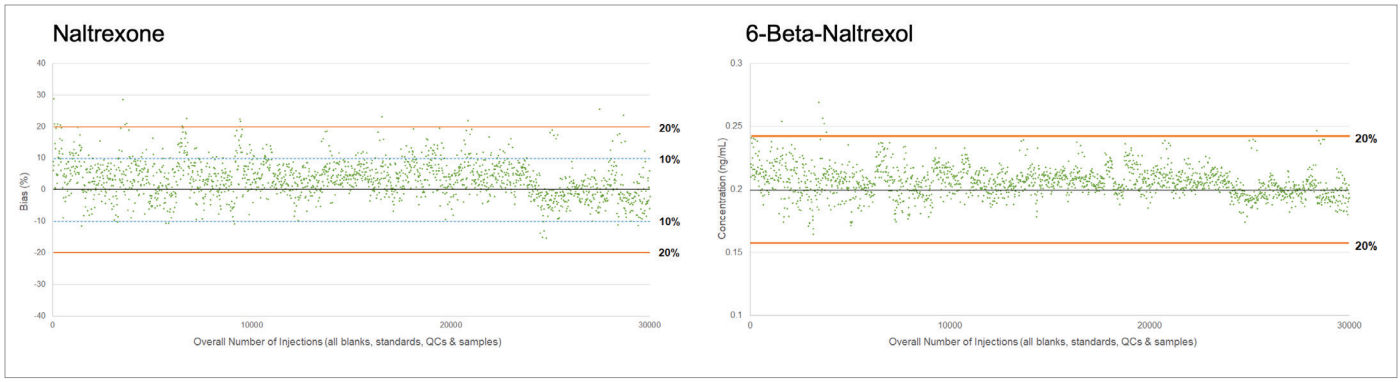


Figure 3. LLOQ QC bias plots for naltrexone and 6-beta-naltrexol across the entire 30,000 injection study.

Precision is another key indicator of the quality of a bioanalytical method and can be significantly affected by any change in performance, such as reduction in sensitivity or variation in peak response, over an analytical batch or study. The precision of the QCs across the analytical range was evaluated over the course of 30,000 injections. The data displayed in Figure 4 shows the variation in precision for five QC concentrations for the duration of the study. All individual QC samples for each of the concentrations showed a precision within 10% of the nominal value, except for one 0.6 ng/mL QC at the 13,900 injections point.

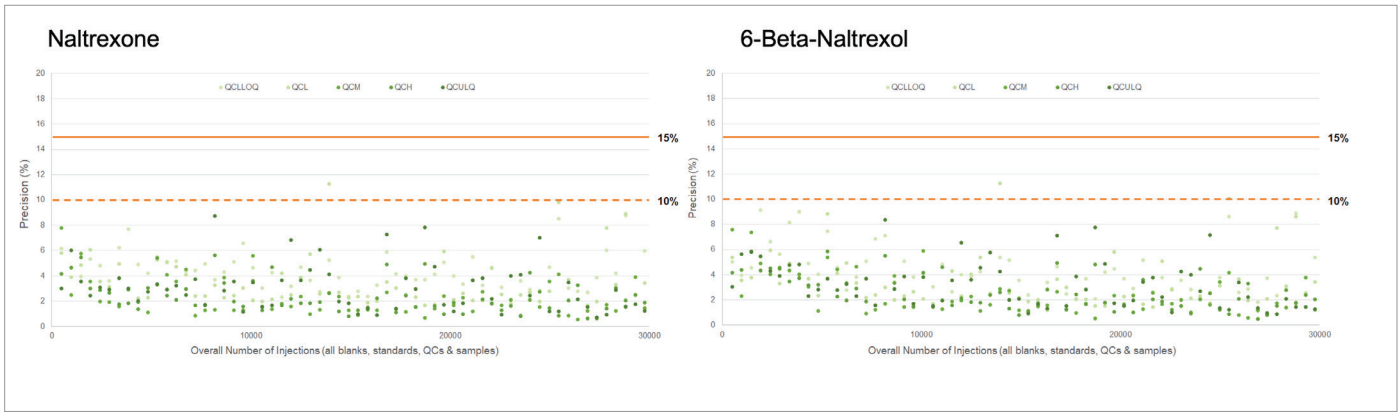


Figure 4. Variation in QC bias for naltrexone and 6-beta-natrexol across the duration of the study batch.

These results illustrate that the LC-MS/MS system showed exceptional robustness and reproducibility over the course of 30,000 injections. The precision and bias values generated by the system were well within the acceptance criteria of the M10 guidelines.[5] The extreme robustness produced by modern tandem quadrupole mass spectrometers equipped with these advanced ion guides has allowed for the continuous operation of the instrument for over 10 weeks with no requirement to break the vacuum, clean the ion optics, and pump down the instrument. Thus, eliminating several days of downtime, increasing laboratory efficiency and data quality.

IMPACT ON LARGE BATCH ANALYSIS

Within the bioanalytical community there is a growing interest in the application of larger size batches beyond the conventional 96-well plate. This is achieved via the combination of multiple sample extraction plates into one larger batch, or by using a 384-well plate.[7,8] However, the use of such large batch sizes is not without its challenges, especially if the sample has not undergone an analyte extraction and clean-up process like SPE or LLE. Addressing this issue often requires the use of multiple QC sets distributed throughout the run to monitor for any change in instrument performance, thus reducing the number of revenue-generating study samples that can be analysed during the batch.

The extra robustness and stability demonstrated by the new Xevo TQ Absolute XR Mass Spectrometer offers the possibility of larger batch size analysis without the need for extra QC sets. This concept was investigated by processing four 96-well plates from the middle of the 30,000-injection study using the calibration line from the beginning of one 96-well plate and the last calibration line from the 96-well plate, four plates later. A total of 363 extracted samples were analyzed from injection number ~22,500 to ~24,000, with a total of 1510 bracketed injections simulating the analysis of 4 x 384 extracted well plates in one continuous batch.

Analysis of the QC data showed that all the QCs at each of the concentration levels for both analytes were within ±15 % nominal concentration. A box whisker plot of the data (Figure 5) shows that there was only one outliers for each of the analytes, which were distributed above and below the 2nd and 3rd quartile of the data. The results derived from this analysis suggest that the increased robustness the StepWave XR ion guide technology imparts on the Xevo TQ Absolute XR Mass Spectrometer can facilitate the successful operation of large batch analysis (*e.g.*,384 samples). This mode of operation would allow for the elimination of 6 calibration lines, approximately 60 samples, which could be replaced by revenue generating samples. Based on an average cost of $50–100 per sample this could be between $3,000 and $6,000 per batch.

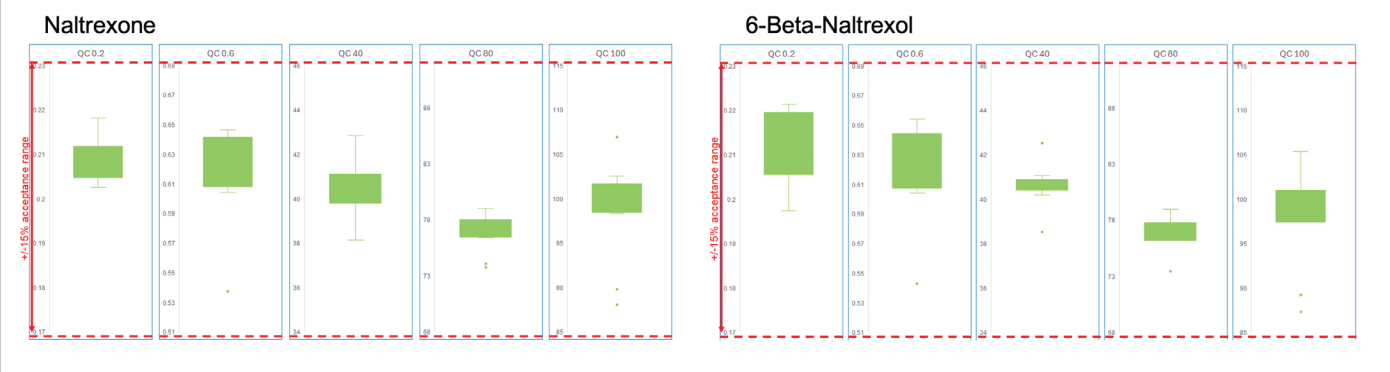


Figure 5. Box whisker plot of the variation in calculated QC concentrations for the 4-microtiter plate batch.

NEXT-LEVEL PERFORMANCE IN REGULATED BIOANALYSIS

Mass spectrometry coupled to liquid chromatography is the mainstay of bioanalytical laboratories supporting drug discovery and development DMPK. The use of fast chromatography combined with basic sample preparation *e.g.*, protein removal results in a significant mass of co-extractive matrix material entering the mass spectrometer. Without the appropriate transfer optics safeguards, this material can contaminate the ion optics in the Q1 region of the mass spectrometer-reducing instrument robustness and requiring time-consuming cleaning to restore MS instrument performance.

The implementation of an efficient ion guide filter, such as the StepWave XR Ion Guide employed in the Xevo TQ Absolute XR Mass Spectrometer, mitigates the contamination of the Q1 quadrupoles by preventing unwanted high mass ion transmission into the analyzer region of the mass spectrometer. The efficiency of this technology was demonstrated in a large-scale bioanalytical assay, performed by a commercial bioanalytical laboratory. Using simple protein precipitation of plasma and a conventional reversed–phase LC-MS/MS method, ver 30,000 injections were performed without the requirement to break the vacuum and clean the ion optics.

Over the course of the study, 21 mL of plasma were injected on to the LC-MS/MS system and 64 litres of mobile phase used. Statistical analysis of the data showed no significant temporal or batch trend across the entire course of the study, illustrating the robustness and stability of the system. Further analysis of a subset of the data suggested that the batch size could be extended to 384 samples with no loss in data quality or compliance, increasing the number of revenue-generating samples which could be acquired per sample batch. The Xevo TQ Absolute XR Mass Spectrometer with StepWave XR Ion Guide technology combined with liquid chromatography demonstrated excellent robustness and long-term performance throughout this study, showcasing these solutions as ideal for bioanalysis of plasma-derived samples.

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Bioanalysis

Development of LC–MS/MS method for cyanoenone triterpenoid determination to support CNS tissue distribution study

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Aims: Cyanoenone triterpenoids penetrate the CNS, exhibiting biological activity via the nuclear factor E2-related factor (Nrf2) pathway. This is the first report on methods for the quantification of cyanonenone triterpenoids' distribution in various CNS tissues by LC–MS/MS. **Materials & methods:** The analyte was extracted from brain tissue homogenate using protein precipitation and supported liquid extraction. **Results & conclusion:** The assay validated a quantification range of 3.00–3000 ng/g in brain tissue samples as low as 5 mg. All parameters, including interference (≤20% at LLOQ) and accuracy/precision (15%, with 20% at LLOQ), met acceptance criteria. This assay supported a CNS distribution study, analyzing more than 10 mouse brain regions successfully.

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Keywords: central nervous distribution • cyanoenone triterpenoid • LC–MS/MS • protein precipitation • supported liquid extraction

Semisynthetic oleanane triterpenoids are potent inducers of cytoprotective enzymes and inhibitors of inflammation via activation of the nuclear factor e2-related factor 2 (Nrf2) signaling pathways [1]. Omaveloxolone (SKYCLARYS®) was recently approved as the first semisynthetic oleanane triterpenoid Nrf2 activator for treatment of Friedreich ataxia [2–4]. Among numerous diseases characterized by inflammatory and oxidative stress, preclinical studies showed that semisynthetic oleanane triterpenoids were effective in most major organs, including brain, eye, heart, lung, liver and kidney [5]. Nrf2 has also been reported to inhibit the occurrence of neural tube defects in a mouse model and plays a protective role in developing neurons, indicating that Nrf2 activators may be used for treatment of neurological disorders, including Alzheimer, Parkinson and Huntington diseases [6].

Oleanane triterpenoids can penetrate the blood–brain barrier and exhibit pharmacodynamic activity within the brain, providing a protective effect against neurodegenerative diseases [6]. Identifying and quantifying oleanane triterpenoids in the CNS would be valuable for gaining insights into the mechanisms of action of these compounds. To explore the tissue distribution of a semisynthetic cyanoenone triterpenoid, TX102, in different brain regions, it is essential to develop a sensitive and reliable bioanalytical method capable of measuring low concentrations of TX102 in limited sample sizes. Despite many years of development of cyanoenone triterpenoids, specific and sensitive methods suitable for their determination in tissue samples have not yet been reported. Currently, LC–MS/MS is the predominant platform for quantifying small molecule drug candidates in plasma and tissue samples. In this study, a highly sensitive LC–MS/MS method was developed and validated for quantifying the semisynthetic cyanoenone triterpenoid, TX102, in various regions of mouse brain tissues.

Experimental section

Reference standard, internal standard, chemicals, reagents & instrumentation

TX102 and its stable isotopically labeled internal standard (IS), d_6-TX102 (Figure 1), were produced at Reata Pharmaceuticals (TX, USA). LC–MS-grade acetonitrile and methanol were obtained from EMD Millipore (MA, USA). Optima LC/MS-grade formic acid was obtained from Thermo Fisher Scientific (NJ, USA). LC–MS reagent-grade ethyl acetate was obtained from J.T. Baker (PA, USA). CD-1 (ICR) mouse brain homogenate (pooled) was

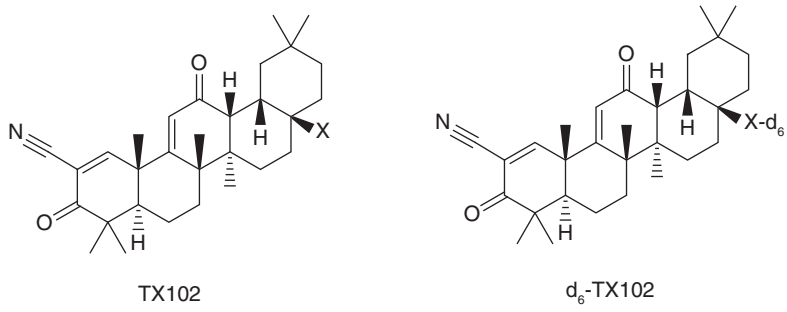

Figure 1. Structure of TX102 and the internal standard d_6-TX102.

purchased from BioIVT (NY, USA). Deionized water was produced in-house using a Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (Thermo Fisher Scientific). Novum supported liquid extraction (SLE) 96-well plate and 96-well collection plate (2-ml conical polypropylene) were purchased from Phenomenex (CA, USA). A 96 DeepWell Plate (1 ml), phosphate-buffered saline (PBS) 1x was obtained from Thermo Fisher Scientific (VA, USA).

The UPLC–MS/MS system consisted of a Waters Acquity™ UPLC system and Xevo TQ-S triple quadrupole mass spectrometer (MA, USA).

Preparation of standards, quality control samples & IS spiking solution

Brain homogenate standards at concentrations of 1.00, 2.00, 5.00, 15.0, 60.0, 250, 850 and 1000 ng/ml in methanol/water (1:1, v/v) were freshly prepared by spiking 0.015 ml of blank brain homogenate at a 1:1 ratio with eight levels of corresponding standard spiking solutions (2.00–2000 ng/ml). Pooled quality control (QC) samples at concentrations of 3.00 ng/ml (LQC), 30.0 ng/ml (MQC) and 825 ng/ml (HQC) were prepared by serially diluting a QC spiking solution (40,000 ng/ml) with blank brain homogenate. Standard spiking solution and quality spiking solution were prepared from separate stock solutions (0.5 mg/ml in methanol) generated from separate weighing using a microbalance (XPR26PC, Mettler-Toledo, OH, USA). An IS spiking solution (d_6-TX102) was prepared at a concentration of 25.0 ng/ml in methanol/water (1:1, v/v).

Study sample processing

The brain tissue sample was weighed (5.00–10.00 mg) then placed into a 1.5-ml polypropylene centrifuge tube. Next, the required volume of 1x PBS buffer was quantitatively add to the tube at a tissue-to-buffer ratio of 1:3 (w/v). The tissue sample was homogenized for 20 s using a Branson Ultrasonics™ Sonifier™ Homogenizer (Shanghai, China) and kept on ice before extraction.

Study sample extraction

An aliquot of 0.015 ml of blank brain homogenate, QC samples, or study samples was pipetted into the corresponding wells of a 96 DeepWell Plate (1 ml) following a predefined plate map. An aliquot of 0.015 ml of standard spiking solutions in methanol/water (1:1, v/v) was spiked into the wells corresponding to each standard. The remaining wells (blanks, QCs and study samples) were spiked with 0.015 ml of methanol/water (1:1, v/v), and the plate was thoroughly mixed by vortexing.

Next, an aliquot of 0.020 ml of 25 ng/ml d_6-TX102 in methanol/water (1:1, v/v) was added to each well using a repeater pipette, except for the blank sample, where 0.020 ml of methanol/water (1:1, v/v) was added. After brief mixing, 0.40 ml of methanol was added to each well using a repeater pipette. The plate was capped with a plate mat and mixed for 2 min by vortexing at medium speed. Subsequently, the plate was centrifuged at 4000 × *g* for 10 min at 4°C, and 0.25 ml of the supernatant was aliquoted to a new 96-well plate using a multichannel pipette. The plate was then dried at 40°C under a stream of nitrogen gas.

The dried sample was reconstituted with 0.40 ml of 0.2% formic acid in water and mixed well by vortexing for approximately 2 min. Using a multichannel pipette, transfer all content of each sample to the corresponding sample well of a Novum 96-well SLE plate. The SLE plate was placed on top of a 2-ml conical polypropylene 96-well plate in a vacuum manifold. The sample solution was allowed to be completely adsorbed into the sorbent bed for 5 min after the initiation of the process by briefly applying vacuum, followed by addition of 0.60 ml of

Table 1. Back-calculated concentrations (ng/ml) of TX102 calibration standards.

Run no.	Concentration (ng/ml)							
	1.00	2.00	5.00	15.00	60.0	250	850	1000
1	1.10	2.20	4.70	14.8	60.0	243	877	970
	0.90	1.90	5.10	14.6	62.1	250	900	979
2	1.15	2.20	5.10	15.3	58.1	244	879	1010
	1.00	2.20	4.80	14.4	59.9	245	974	987
3	1.00	2.10	4.90	15.1	54.2	251	842	988
	1.00	2.10	4.90	14.5	61.4	264	872	1010
Mean	1.03	2.12	4.92	14.8	59.3	249	874	991
SD	0.0880	0.117	0.160	0.354	2.85	7.69	18.7	16.4
%CV	8.6	5.5	3.3	2.4	4.8	3.1	2.1	1.7
%RE	2.5	5.8	-1.7	-1.4	-1.2	-0.3	2.8	-0.9
n	6	6	6	6	6	6	6	6

%CV: Percent coefficient of variation; %RE: Percent relative error; SD: Standard deviation.

ethyl acetate twice (0.60 ml × 2) to each well. The eluent was collected in the sample collection plate using gravity without vacuum which takes ~20 min. Then the sample collection plate was placed under a stream of nitrogen gas (40°C) until the sample was completely dried.

The dried sample was reconstituted with 0.40 ml acetonitrile/water/formic acid solution (40:60:0.1, v/v/v), capped with a silicon mat, mixed well by vortexing approximately 2 min. The plate was placed in the ultra-performance LC (UPLC) autosampler and an aliquot of the reconstituted sample was injected onto an LC–MS/MS system for quantitative analysis of TX102 using d_6-TX102 as the IS.

UPLC–MS/MS conditions

Chromatographic separation was conducted on an Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm) coupled with an Acquity Vanguard HSS C18 guard column (2.1 × 5 mm, 1.7 µm). The column temperature was set at 40°C and autosampler temperature was set at 4°C. Mobile phase A was water/formic acid (100:0.1, v/v) and mobile phase B was acetonitrile/formic acid (100:0.1, v/v), the total flow rate was set at 0.40 ml/min. The gradient started from 40% B and increased to 80% B in 1 min, to 95% B in 3 min. After maintaining 95% B for 1 min, the gradient returned to the initial condition of 40% B at 4 min and equilibrated for 1 min before next sample was injected.

The mass spectrometer operated in electrospray positive ion mode using the following optimized operating parameters: ion spray voltage: 3.5 KV; desolvation temperature: 65°C; desolvation gas flow: 1000 l/h; Corn voltage and collision voltage: 54 and 40 V. Multiple reaction monitoring transitions monitored were m/z 545.4 → 97.9 (TX102) and m/z 551.4 → 104.2 (d_6-TX102).

Tissue distribution study of TX102 in CD-1 mice

To assess the bioavailability and distribution of TX102 in the CNS of CD-1 mice, a total of 84 mice were used (three animals/group for vehicle control groups, six animals/group for dosed groups). The mice were orally administered TX102 consecutively for 5 days at doses of 0, 30, 50 and 100 mg/kg. On the fifth dosing day, the animals were sacrificed, and various regions of the brain, including the left and right cortex, hippocampus, striatum, hypothalamus and cerebellum, as well as the dorsal and lumbar spinal cord, were collected from three animals in each group. All tissue samples were stored at -80°C and subsequently shipped to the bioanalytical lab for analysis.

Results & discussion

Linearity & sensitivity

The linearity of the developed method ranges from 3 to 3000 ng/g. Linear regression analysis was performed with $1/x^2$ weighting, achieving a coefficient of determination (R^2) ≥0.9975 for accuracy and precision in all analytical runs. The lower limit of quantification (LLOQ) of the method in tissue is 3.00 ng/g (1.00 ng/ml in brain homogenate). For a validation run to be deemed acceptable, a minimum of 75% of the number of calibration standards in the calibration range could not deviate by more than ±15% from their nominal value. Table 1

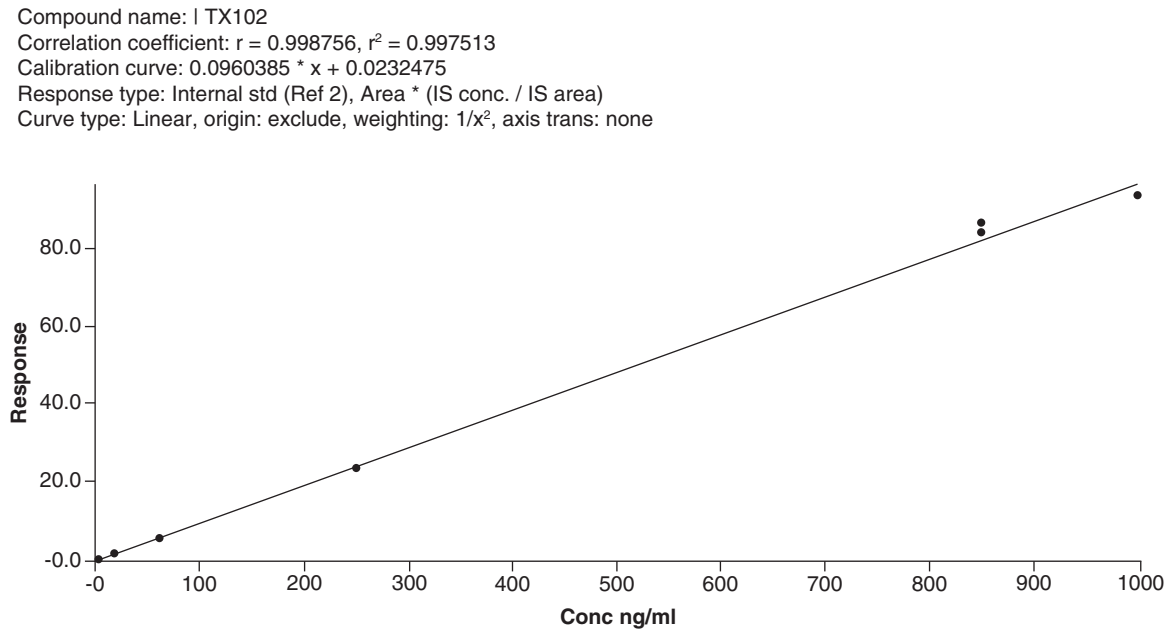

Figure 2. Representative standard curve regression for TX102 from accuracy and precision analytical run 1.
Conc: Concentration;; IS: Internal standard.

Table 2. Intrarun and interrun accuracy and precision of TX102 in brain homogenate.

Run no.	Concentration (ng/ml)			
	1.00	3.00	30.00	825
1	1.01	2.80	28.8	830
	1.02	2.80	29.1	841
	0.99	2.90	30.0	826
	1.00	2.90	30.4	816
	0.95	3.10	28.1	850
	0.97	3.00	31.6	855
2	1.21	2.70	28.7	813
	1.15	2.85	29.5	848
	1.12	2.90	28.4	844
	1.03	3.00	29.1	837
	1.20	3.00	30.9	851
	0.96	3.30	28.7	813
3	0.90	3.00	31.9	811
	0.90	3.00	29.9	861
	1.00	3.00	29.3	801
	1.20	3.10	29.8	797
	0.90	2.90	29.4	826
	1.00	3.00	31.2	832
Mean	1.03	2.96	29.7	831
SD	0.104	0.135	1.10	19.1
%CV	10.1	4.6	3.7	2.3
%RE	2.8	-1.4	-1.0	3.8
n	18	18	18	18

%CV: Percent coefficient of variation; %RE: Percent relative error; SD: Standard deviation.

summarizes the back-calculated calibration standard concentrations for the three accuracy and precision runs of TX102. Figure 2 shows an example of the calibration curve.

Accuracy & precision

Three concentration levels for QC were employed to assess the accuracy and precision of the method (Table 2). Accuracy was determined through replicate analyses (n = 6) of QC samples and expressed as percent relative

error (%RE). Acceptable accuracy (%RE) fell within ±15% of the theoretical value. Precision was assessed using percent coefficient of variation (%CV), and acceptable precision was set at ≤15% of the theoretical value. Intra-run and inter-run accuracy and precision all met the acceptance criteria, demonstrating the assay's excellent performance for routine sample analysis.

Selectivity & interference

Due to the unavailability of certain types of blank tissue, the method's selectivity was only assessed using commercially available whole brain homogenate, while different regions of tissue sample were not evaluated. The pooled brain homogenate exhibited no significant interference peaks at the retention time of TX102 and the IS when samples were extracted using the optimized protein precipitation and supported liquid extraction (SLE) method.

On the contrary, the interference peak at the retention time of TX102 could not be eliminated when samples were extracted by protein precipitation alone (Figure 3A). The combined use of protein precipitation and SLE allowed for the complete removal of the endogenous interference peak from the matrix (Figure 3B & C). Representative LC–MS/MS chromatogram of TX102 and the IS at the LLOQ is shown in Figure 3D.

Due to unidentified endogenous interference peak(s) in brain samples, sharing the same multiple reaction monitoring transitions as TX102 (m/z 545.4 → 97.9), an observed issue is the gradual increase in background over time. Another transition of m/z 545.4 → 189.1 is not sensitive enough for quantifying low concentrations of the analyte in limited tissue sample sizes. Despite the combined use of protein precipitation and SLE, the issue is not entirely eliminated. It is noteworthy that the guard column should be changed approximately every 500 injections, even though the HPLC column typically lasts more than 4000 injections.

Lower limit of quantification

The sensitivity (LLOQ) of the assay in mouse brain homogenate was assessed using LLOQ QC (1.00 ng/ml) in three A&P runs. This LLOQ corresponds to 3.00 ng/g in tissue. The accuracy and precision of the LLOQ met the acceptance criteria, falling within ±20% (Table 2). Given that the tissue was homogenized in PBS buffer with a tissue to PBS ratio of 1:3 (w/v), an aliquot size of 0.015 ml corresponds to 5 mg of tissue.

Recovery & matrix effect & carryover

The recovery of the analyte and IS was assessed by comparing the mean response of the peak area ratio of analyte/IS and IS/analyte in QC samples (LQC, MQC and HQC) to the blank brain homogenate extract spiked post-extraction. The recovery of TX102 ranged from 104 to 107%, with %CV ≤13.3, and the IS recovery ranged from 93.9 to 97.0%, with %CV ≤12.4.

The IS-normalized matrix factor was evaluated by comparing the peak area ratio of analyte to IS in the extracted QC samples (n = 6) to that in the neat solution. No significant matrix effect (1.00∼1.01) was observed for the method, indicating minimal matrix-related impact on the quantification of TX102 in the brain extract.

There was no carryover observed for the method. The minimal interference peak at the retention time of TX102 and d_6-TX102 after the upper limit of quantitation sample (ULOQ) at 3000 ng/g in tissue was <20% of the LLOQ and <5% of the average of the internal standard peak.

Stability

Because all tissue samples are extracted and analyzed immediately after homogenization, only benchtop (on ice) and processed sample stability were evaluated. Benchtop stability was assessed by thawing LQC, MQC and HQC samples on the bench (on ice) and extracting them along with freshly prepared standards. Processed stability was evaluated by storing the extracted LQC, MQC and HQC samples (n = 6) in the autosampler for 78 h at 4°C and injecting them along with freshly extracted standards. Table 3 summarizes the results of 3-h benchtop stability (on ice) and processed sample stability (78 h), and all stability samples met the acceptance criteria.

Application to tissue distribution study

The developed assay was applied to support a central nervous distribution study of TX102 in CD-1 mice. A total of four analytical runs were conducted for the analysis of TX102 in different regions of brain tissues, and the analytical runs met acceptance criteria for standards and QC samples (%CV <15.0%; %bias within ±15.0%, 20% for LLOQ).

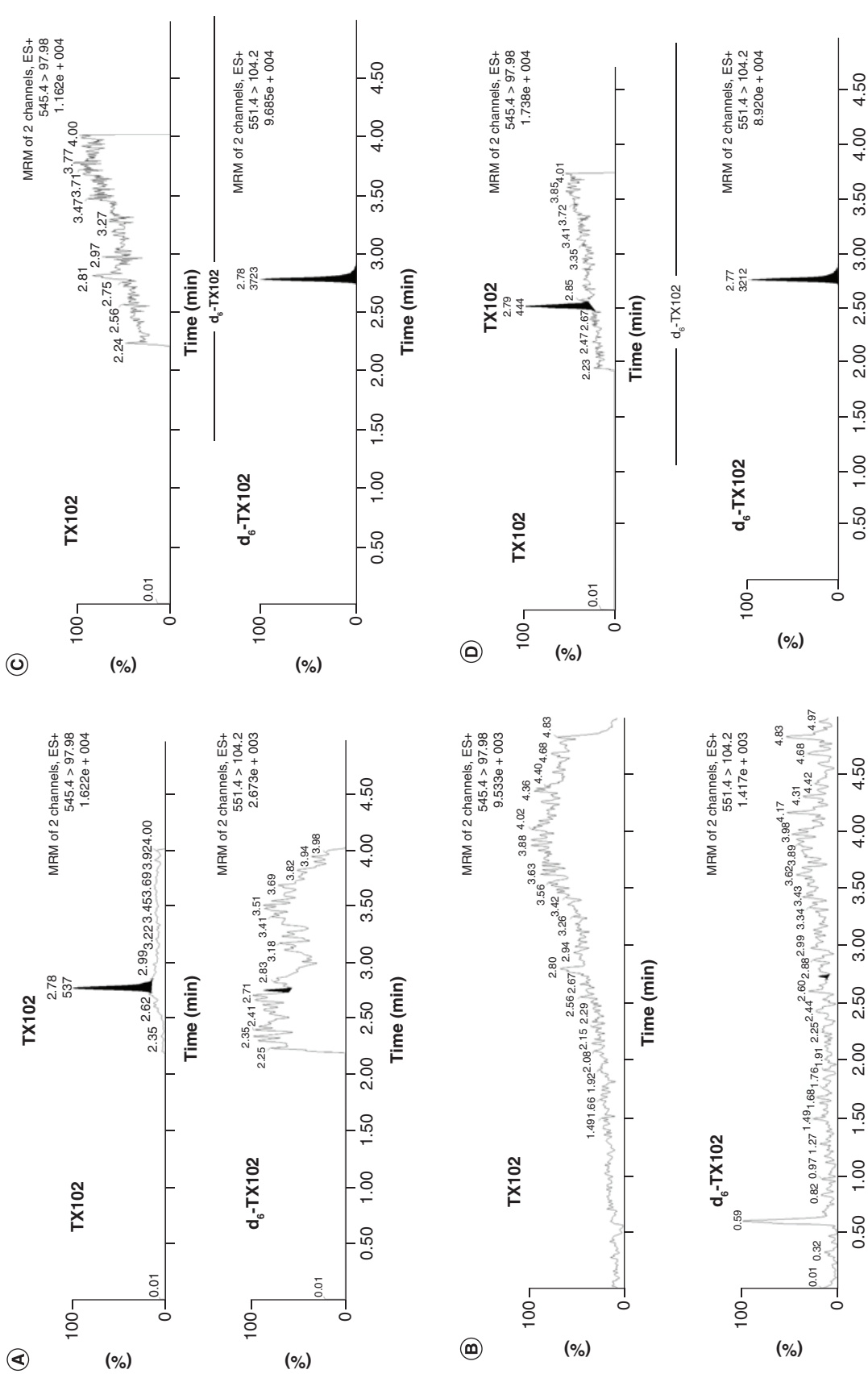

Figure 3. Representative LC–MS/MS chromatograms of TX102 and internal standard d_6-TX102. (A) Blank mouse brain homogenate extracted by protein precipitation and samples extracted by protein precipitation and supported liquid extraction. **(B)** Blank mouse brain homogenate. **(C)** Blank + internal standard. **(D)** Lower limit of quantification sample (3.00 ng/g).
ES: Electrospray; MRM: Multiple reaction monitoring.

Table 3. Stability of TX102 under different conditions.

Concentration (ng/ml)		3.00	30.0	825
Benchtop (on ice) stability 3 h	Mean ± SD	2.95 ± 0.235	32.3 ± 2.01	885 ± 67.3
	%CV	7.9	6.2	7.6
	%Bias	-1.7	7.7	7.3
	n	6	6	6
Autosampler stability (4°C 78 h)	Mean ± SD	2.65 ± 0.105	29.9 ± 1.16	858 ± 9.71
	%CV	4.0	3.9	1.1
	%Bias	-11.7	-0.2	4.0
	n	6	6	6

%CV: Percent coefficient of variation; SD: Standard deviation.

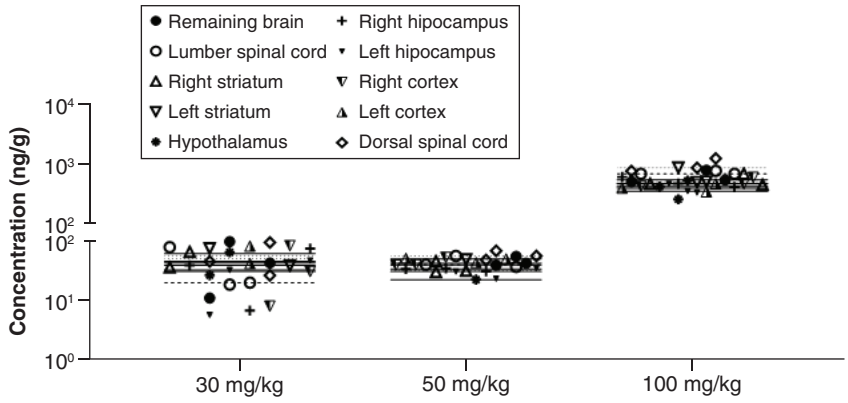

Figure 4. Concentration profiles of TX102 in different regions of the mouse brain following a 5-day administration of TX102 at doses of 30, 50 and 100 mg/kg.

Three levels of QCs (low, mid and high, n = 2 for each level QC per analytical run) were analyzed in each sample analytical run. The mean %bias ranged from -0.4 to 2.1%, and the mean %CV ranged from 1.6 to 9.4%, demonstrating the ruggedness of the assay for analyzing real study samples. Figure 4 shows the concentration levels of TX102 in different regions of the brain tissue for the 5-consecutive-day dosing group animals. The lack of an apparent difference in tissue concentrations between 30 and 50 mg/kg could be attributed to intra-animal variability in the 30 mg/kg group, stemming from dosing variability.

Conclusion

The developed method for determining TX102 in limited brain tissue samples demonstrated high selectivity, sensitivity, precision, accuracy, and ruggedness. The assay range was established as 3.00–3000 ng/g. This novel LC–MS/MS assay can reliably determine TX102 in different regions of the mouse brain to support the tissue distribution study.

Summary points

- A novel LC–MS/MS assay was developed and validated to quantify a semisynthetic cyanoenone triterpenoid, TX102, in brain homogenate, with a quantitation range of 3.00–3000 ng/g.
- This assay is suitable for quantifying low-concentration analytes in limited sample sizes with an aliquot volume of 0.015 ml, equivalent to 5 mg of the sample homogenized in phosphate-buffered saline at a ratio of 1:3 (w/v).
- Samples were extracted through protein precipitation followed by supported liquid extraction before LC–MS/MS analysis.
- The method underwent assessment for linearity, sensitivity, accuracy, precision, selectivity, interference, recovery, matrix effect and stability.
- The validated LC–MS/MS assay was successfully employed to support a mouse central nervous distribution study of TX102.

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Competing interests disclosure

W Lafon, Q Tian and E Tamer are employed by Reata Pharmaceuticals, Inc., and hold stock options and restricted stock with the company. L Tian is a chemistry under-graduate student at Stony Brook University conducting a summer internship at Reata Pharmaceuticals. The authors have no other competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript apart from those disclosed.

Writing disclosure

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

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