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Investigation of high-throughput ultrafiltration for the determination of an unbound compound in human plasma using liquid chromatography and tandem mass spectrometry with electrospray ionization

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Abstract

A high-throughput ultrafiltration method with a direct injection assay has been developed to determine unbound concentrations of a high-protein binding compound, an $\alpha_v\beta_3$ bone integrin antagonist (**I**), in human plasma for a clinical pharmacokinetic study. The 96-well MultiScreen® filter plate with Ultracel-PPB membrane was evaluated for the separation of unbound from protein-bound compound **I** by ultrafiltration. The sample preparation was automated using a Packard MultiPROBE II EX liquid handling system to transfer the plasma samples to the 96-well PPB plate for centrifugation and to prepare ultrafiltrate samples for analysis. Using on-line extraction with a column-switching setup for sample clean-up and separation, the ultrafiltrate samples were directly injected onto a reversed-phase HPLC system and analyzed using a mass spectrometer interfaced with an electrospray ionization (ESI) source in the positive ionization mode (LC/ESI-MS/MS). The performance of the ultrafiltration using Ultracel-PPB 96-well plate for unbound **I** analysis was evaluated and optimized with respect to sample volume, centrifugation temperature, speed and time, and the relationship of the well positions of the PPB plate versus filtrate volumes and concentrations. The assay intraday accuracy and precision were between 93.9 and 104.8 and <7.3% (CV), respectively. The linear range of the calibration curve for the assay was 0.1–500 ng/mL on a Finnigan TSQ Quantum LC/ESI-MS/MS system. Evaluation and validation of the unbound plasma assay demonstrated it to be rapid, sensitive and reproducible.

Keywords: Unbound drug; Ultrafiltration; Ultracel-PPB 96-well plate; Packard liquid handling system; Human plasma; LC–MS/MS

1. Introduction

Compound **I** (Fig. 1) is a novel, orally active $\alpha_v\beta_3$ bone integrin antagonist developed for the treatment and prevention of osteoporosis by inhibiting bone resorption [1,2]. It is a highly protein-bound drug (>95%). Protein-binding drugs are loosely bound to plasma proteins such as albumin and α-acid glycoprotein, forming an equilibrium ratio between bound and unbound drugs [3]. Binding of a drug to protein limits its concentration in tissues and its pharmacological action, since only the unbound drug is in equilibrium across membranes. The free drug concentration is often considered the best quantitation of a pharmacologically active drug [4,5]. Analysis of an unbound drug in plasma is important for pharmacokinetic evaluation of the compound in pre-clinical and clinical studies.

Ultrafiltration is a reliable and efficient technique used for the determination of protein binding and free drug concentration in plasma. In ultrafiltration, a pressure gradient forces the aqueous component of plasma containing the free drug through a permeability selective membrane [6]. Ultrafiltration membrane filters at the nominal molecular weight limit (NMWL) of 10,000 Da have been used for over 20 years to separate free drugs from protein-bound drugs [7]. The major drug binding proteins are albumin (MW 67,000 Da) and α-acid glycoprotein (MW 42,000 Da), which are captured, along with other endogenous large macromolecules, from plasma by the ultrafilter. Since most drugs are small molecules (<500 Da), they pass freely through the 10,000 NMWL ultrafiltration membrane; drug recovery in the ultrafiltrate is high and non-specific binding to the membrane and device is low.

A number of available ultrafiltration devices able to process one sample at a time have been used for monitoring free-drug in clinical applications [8]. To some degree, automation of these unit devices has been successful using sample transfer

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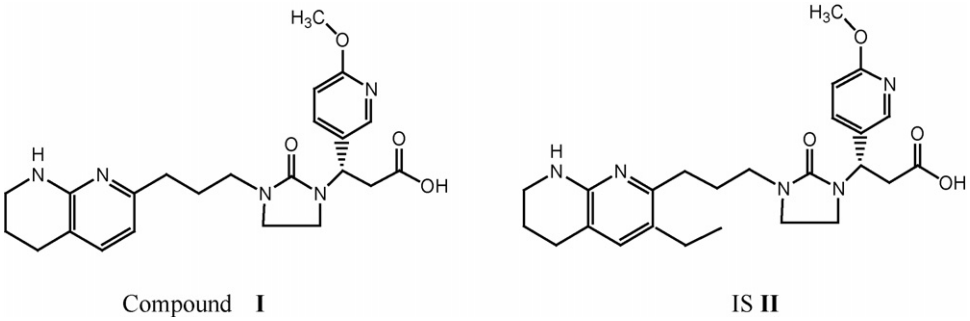

Fig. 1. Chemical structures of compound **I** and IS **II**.

instrumentation such as the Packard MultiPROBE II [9]. However, using these individual tube-based devices is inefficient and time consuming. For higher throughput, a 96-well plate with a 10,000 NMWL low-binding ultrafiltration membrane (MultiScreen filter plate with Ultracel-PPB membrane from Millipore Corp.) has been specifically developed for separating unbound from bound drug in plasma. A high-throughput assay for the quantitation of compound **I** in unbound plasma has been developed and validated using a 96-well PPB ultrafiltration plate and a direct injection LC–MS/MS assay. The 96-well PPB ultrafiltration plate used with the available centrifuge system in our laboratory was characterized for compound **I** ultrafiltration process. The performance of the PPB plate for the feasibility of the simultaneous determination of unbound **I** over 96 wells was investigated regarding the well positions across the whole plate. The ultrafiltration conditions used in the centrifugation process, e.g., centrifuge speed, spin time and temperature, evaporation, non-specific binding effects, etc., were optimized for the quantitation of unbound **I** by minimizing an identified "edge effect" on the receiving ultrafiltrate volume across the plate.

The Packard MultiPROBE II EX liquid handling system was utilized to transfer the plasma samples for ultrafiltration and prepare collected ultrafiltrate samples for free drug analysis. The ultrafiltrate sample was directly injected onto a reversed-phase high performance liquid chromatography system where a column-switching technique was employed for on-line extraction and separation, and analyzed on a Finnigan TSQ Quantum tandem mass spectrometer interfaced to an electrospray ionization (ESI) source in the positive ion mode (LC/ESI-MS/MS). The assay was validated over the concentration range of 0.1–500 ng/mL with a 5 μL injection volume and 4-min run time. The assay has been applied for unbound plasma sample analysis in a clinical study. Together with results from a total plasma assay for compound **I** [10], a pharmacokinetic study of percent unbound drug can be accomplished.

2. Experimental

2.1. Material

Compounds **I** (purity 99.9%) and the internal standard (IS) **II** (purity 98.0%, an analog of **I**, Fig. 1) were obtained from Merck Research Laboratories (West Point, PA, USA). The MultiScreen filter assembly with Ultracel-PPB membrane in a 96-well format was purchased from Millipore Corp. (Bedford, MA, USA). The assembly consists of an extended centrifugal cover to minimize sample evaporation (a standard cover was used in place of the extended cover due to the size limitation of the centrifuge bucket), a 96-well plate with Ultracel-PPB 10,000 Da (NMWL) ultrafilter, and a 96-well collection plate with conical wells. A Centricon Plus-20 centrifugal filter device (PL-10, 10,000 Da NMWL) was also purchased from Millipore for generation of the control ultrafiltrate from human control plasma. Human control plasma (sodium heparin as anticoagulant) was purchased from Biological Specialty Co. (West Point, PA, USA). Phosphate buffered saline (PBS, sterilized) solution was purchased from Merck Lab Service (West Point, PA, USA). Water was purified by a Milli-Q ultra-pure water system from Millipore. Methanol, acetonitrile, formic acid (90%), ammonium formate, dimethyl sulfoxide and isopropyl alcohol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were used as received.

2.2. Instrumentation

A Packard MultiPROBE II EX automated liquid handling system (Meriden, CT, USA) was used to perform sample preparation in the unbound assay. A Sigma 4K15C centrifuge (Rotor #09100, bucket #09366 for 96-well plates, from Qiagen, CA, USA) was used to perform ultrafiltration. LC–MS/MS was performed on an Agilent HP1100 binary pump system with a Perkin-Elmer Series 200 micro LC pump (Norwalk, CT, USA) and a 96-Well Plate Autosampler (HTS PAL System from LEAP Technology, Carrboro, NC, USA), coupled to a TSQ Quantum triple quadrupole mass spectrometer with an electrospray ionization interface (Thermo Finnigan, San Jose, CA, USA). The data were collected and processed through Xcalibur v1.3 software.

2.3. Chromatographic conditions

Extraction and separation of the ultrafiltrate samples were performed on-line using a column-switching technique with direct 5 μL sample injection. A Cyclone HTLC column (50 mm × 0.5 mm, 60 μm) from Cohesive Technologies Inc. (Franklin, MA, USA) and a BDS Hypersil C_{18} column (30 mm × 2.1 mm, 3 μm) from ThermoHypersil-Keystone (Bellefonte, PA, USA) were used as the extraction and analytical columns, respectively. Three mobile phases were used in

Table 1
Gradient program for extraction and washing in on-line extraction LC/ESI-MS/MS assay

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
0.00	0.70	100	0.0
1.60	0.70	100	0.0
1.61	1.50	20	80
2.20	1.50	0	100
3.20	1.50	0	100
3.21	1.50	70	30
3.60	1.50	100	0
3.90	0.70	100	0
4.00	0.70	100	0

the analysis: (A) the extraction mobile phase composed of 5% acetonitrile and 95% 2 mM ammonium formate (pH 3.0) (v/v); (B) column washing solvent composed of 80% acetonitrile, 10% dimethyl sulfoxide and 10% 0.1% formic acid (v/v/v); and (C) analytical mobile phase composed of 80% acetonitrile and 20% 2 mM ammonium formate (pH 3.0) (v/v), which was degassed in a sonicator for 10 min.

A six-port switching valve equipped on the TSQ Quantum system was setup with a time profile to divert the mobile phases for on-line extraction and separation, including column washing and equilibration. Each sample was loaded onto the extraction column at the inject valve position (INJECT) for 0.60 min using mobile phase A. At 0.6 min, the valve was switched to the load position (LOAD) for approximately 1.0 min to deliver the mobile phase C at 0.2 mL/min through the extraction column onto the analytical column. The analytes extracted on the front end of the extraction column were back-flushed off the extraction column onto the analytical column; then, the valve was switched back to INJECT at 1.60 min, and various combinations of mobile phases A and B with a step gradient profile (as listed in Table 1) were used to wash the extraction column for 2.6 min. This wash was followed by a second switching step to repeat the switching from INJECT to LOAD and back to INJECT within 0.05 min; then, equilibrate the system for 0.8 min before the next injection. Mobile phases A and B were delivered using the Agilent HP1100 Binary pump, and the mobile phase C was delivered using the Perkin-Elmer 200 series micro pump. The retention times for compounds **I** and **II** were approximately 1.7 and 1.9 min, respectively. Two solutions of acetonitrile/0.1% formic acid (5:95, v/v) and acetonitrile/isopropyl alcohol/0.1% formic acid (1:1:1, v/v/v) were used as needle washing solvents. The autosampler had a 20 μL injection loop and was refrigerated at 4 °C during analysis.

2.4. Mass spectroscopy conditions

The LC system was interfaced via an electrospray (ESI) inlet to the Finnigan TSQ Quantum triple quadruple mass spectrometer with the operating software Xcalibur (Version 1.3). The mass spectrometry analyses for **I** and **II** were conducted in a positive ionization mode. Precursor ions as protonated molecular ions $[M+H]^+$ for **I** and **II** were determined from Q1 spectra obtained during the infusion of 1 μg/mL neat solution in methanol/water (50:50, v/v) for each analyte into the mass spectrometer using Quantum Tune Master (Version 1.0 SR1) with the collision gas off. The predominant precursor ions for **I** and **II** were observed at *m/z* 440.1 and 468.4, respectively. A product ion scan was performed for each of the precursor ions using the collision-induced dissociation (CID) to obtain fragmentation ions for each analyte. Representative product scan spectra for the protonated molecule $[M+H]^+$ of **I** and **II** are shown in Fig. 2. The major product ions selected for the analysis were *m/z* 261.0 for **I** and *m/z* 289.1 for **II**, respectively. The tune file parameters and instrument settings were optimized to maximize the response for **I** precursor → product ion transition at *m/z* 440.1–261.0. The spray voltage was set at 3500 eV, the temperature of the capillary transfer tube was maintained at 350 °C, the collision energy unit was 28 for **I** and 32 for **II**, and the collision cell pressure was 1.2 mTorr (argon). The sheath gas flow rate (N_2) was set at 50 units, and the auxiliary gas flow rate (N_2) was 12 units. The angle of the nebulizer probe to the orifice in the source was approximately 70°, which directed the spray away from the orifice in order to minimize contamination from the matrix. The analytes were detected by monitoring the precursor → product ion transitions using selected reaction monitoring (SRM) scan mode with 250 ms dwell time with scan width set at 0.5 *m/z* for each transition. The SRM was performed at *m/z* 440.1–261.0 for **I** and *m/z* 468.1–289.0 for **II**.

2.5. Preparation of standard solutions and quality control (QC) samples

Primary stock solutions of **I** and **II** were prepared at 100 and 25 μg/mL in methanol/water (50:50, v/v), respectively. Working standard solutions of **I** at the concentrations of 1–5000 ng/mL in methanol/water (50:50, v/v) were prepared by serial dilutions from the stock solution. The working standard solution of **II** at 1 ng/mL was obtained by dilution of **II** stock in acetonitrile/0.5% formic acid (2:3, v/v). All stock and working standard solutions were stored in 10-mL glass tubes at −20 °C. A primary QC stock solution of **I** was prepared at 500 μg/mL in methanol/water (50:50, v/v) from a separate weighing. Working QC standards at 100 μg/mL, 10 μg/mL and 50 ng/mL in methanol/water (50:50, v/v) were prepared by a serial dilution from the QC stock solution.

Plasma ultrafiltrate (UF) control matrix was prepared from human control plasma using a Centricon Plus-20 centrifugal filter device (PL-10, 10 kDa) centrifuged at about 3000 × *g* RCF, 10 °C for 60 min. UF calibration standards were prepared daily by adding 30 μL of working standards into 270 μL of control UF to provide final concentrations of **I** in UF ranging from 0.1 to 500 ng/mL; then 90 μL of 1 ng/mL working IS was added to a 30 μL aliquot of each UF standard for analysis.

Ultrafiltrate QC (UF-QC) samples were prepared by adding appropriate volumes of the stock and working QC solutions into assigned volumetric flasks and diluting to the mark with pooled control UF to obtain QC concentrations of 0.3, 40 and 400 ng/mL for low, medium and high (L, M and H) QCs, respectively. To monitor the ultrafiltration process for unbound plasma sample analysis, an additional set of plasma

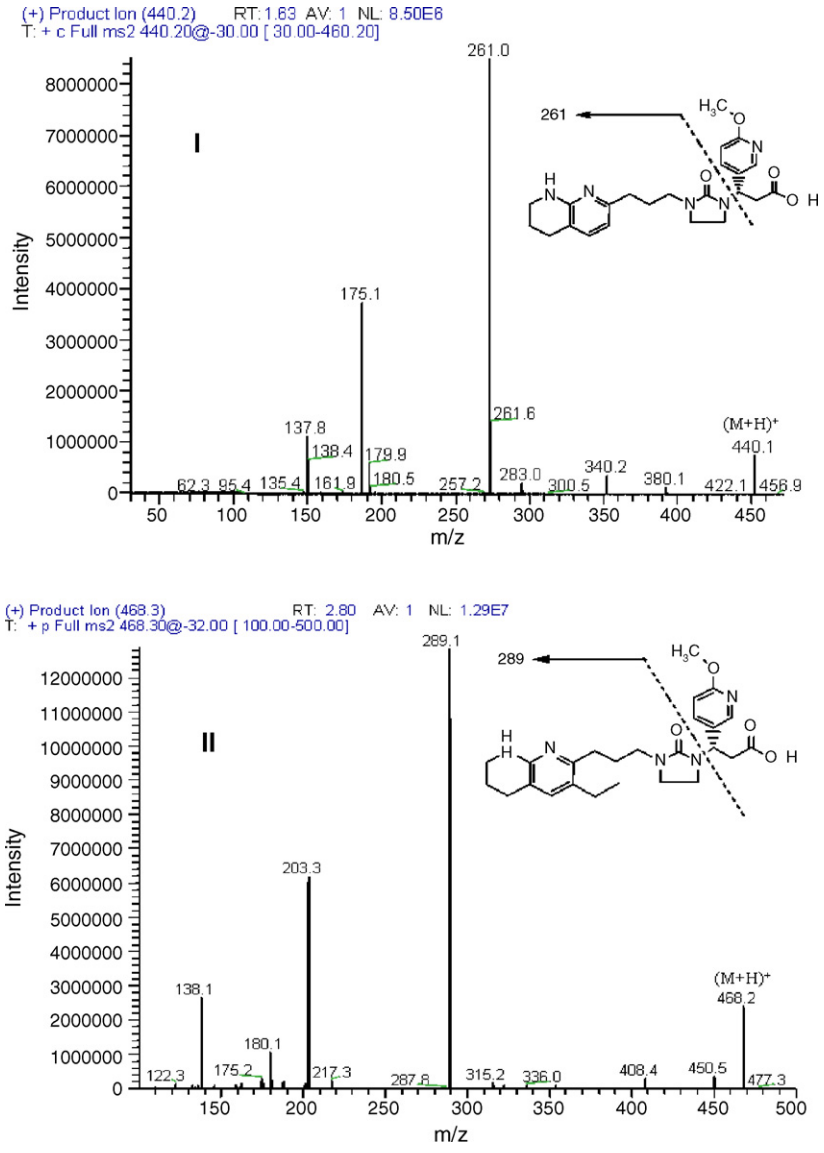

Fig. 2. Representative product ion mass spectra for compound **I** and IS **II**.

QC (PL-QC) samples was prepared in pooled fresh control plasma at 50, 1000 and 6000 ng/mL for L, M and H plasma QCs, respectively; these were subsequently incubated at 37 °C for 30 min. Both types of QC samples were aliquoted into 2-mL polypropylene (pp) micro tubes and then stored at −20 °C.

Neat standard solutions were prepared in a phosphate buffer saline solution at the three UF-QC levels, respectively, for evaluation of non-specific binding (NSB) in the ultrafiltration process using the Ultracel-PPB plate.

2.6. Preparation of ultrafiltrate samples for unbound analysis

Control UF, UF-QC, PL-QC and clinical plasma samples were thawed at room temperature, mixed by vortex, and centrifuged at about 3000 × *g* RCF, 10 °C for 15 min. An aliquot of 300 μL of PL-QC and clinical plasma samples were transferred from the tubes into the assigned well positions (balanced well position) on the Ultracel-PPB assembly plate using the Packard MultiPROBE II EX. The assembly, which consists of the filter plate, the collection plate and a standard cover, was centrifuged using the Sigma 4K15C at an optimized speed profile with maximum speed at 3000 × *g* RCF, 25 °C for 30 min. After centrifugation, the UF collection plate was detached from the PPB assembly and placed on the deck of the Packard workstation for UF volume measurement, as needed, and prepared for analysis.

The ultrafiltrate volumes can be determined automatically on a Packard using a customized volume measurement program and a gravimetric balance (Mettler SAG285/L Balance–Gravimetric Performance Evaluation Option, Packard). The automated procedure to measure ultrafiltrate volumes was evaluated across the full plate using water over the range 25–100 μL (from the center to the edge columns of the plate). The accuracy and precision ($n = 8$ for each volume level) ranged from 96.9 to 101.8% of nominal and 0.4 to 3.6% (CV), respectively.

UF calibration standards were prepared on the Packard by adding 30 μL of the appropriate working standards into assigned 2-mL micro tubes containing 270 μL aliquot of control UF matrix, mixed well by vortex to provide the final concentrations of **I** in UF ranging from 0.1 to 500 ng/mL; then, 90 μL of 1 ng/mL working IS was added to 30 μL of each UF standard for analysis. For the analysis of unbound **I**, 30 μL of UF standard, UF-QC, collected UF of PL-QC (UF-PL-QC), and UF of plasma samples were transferred into a destination 650-μL polypropylene 96-well plate (Orochem Technologies, IL, USA). Then, 90 μL of 1 ng/mL IS in acetonitrile/0.5% formic acid (2:3, v/v) was added to each well (including the single UF blank). After mixing by vortex, the plate was ready for LC/ESI-MS/MS analysis.

3. Results and discussion

3.1. Evaluation on uniformity of unbound drug concentration across the PPB plate

3.1.1. "Edge effect" with the PPB plate orientation during centrifugation

The orientation of the PPB plate during centrifugation in the Sigma 4K15C centrifuge was "landscape" configuration (see Fig. 3). According to the relative centrifugal field equation, $RCF = 1.12\, r\,(RPM/1000)^2$, wells in the outer columns 1–3 and 10–12 at the edge of the plate experienced enhanced centrifugal force, which was somewhat tangential to the membrane compared to the center lines of centrifugal force, with a relatively longer radius of rotation [11,12]. With the same initial plasma sample volume, the different centrifugal forces applied across a "landscaped" 96-well plate will result in major well-to-well differences in collectable ultrafiltrate volumes from center to edge of the plate. The variation in filtrate volumes observed across the plate resembling a smiling face, is called the "edge effect" in this paper.

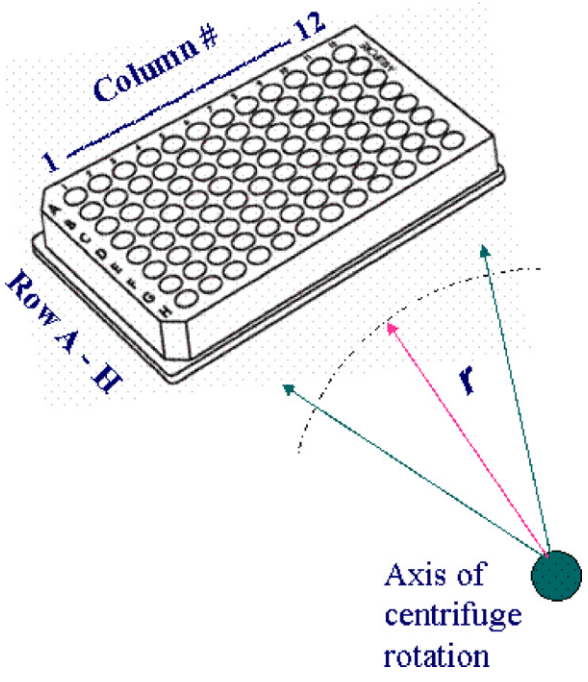

Fig. 3. Centrifugal force experienced across a PPB plate in centrifugation using Sigma 4K15C centrifuge with a landscape configuration.

Given this "edge effect", a major concern regarding the determination of free drug using the PPB 96-well ultrafiltration plate was a potential change of the free drug concentration corresponding to varied ultrafiltrate volumes across the plate. Based on the general findings and predictions by Bowers et al. [6,13] on ultrafiltrate volumes, the changes in free drug concentrations are not significant with changes in the ultrafiltrate volumes. However, the recommended percent of UF volume to plasma sample volume in each well is <20%; 20–35% is acceptable, with minimum disturbance to the protein-binding equilibrium [14].

In experiments to determine the effect of well position on the percent protein binding (%PPB) of several radiolabeled drugs [11,12], rather consistent %PPB was observed across the same type of PPB plate used in our present study. Unlike the present study, those experiments used a "portrait configuration" for centrifugation using a Jouan CR422 centrifuge where the variation in force is across rows A1–H1 (8 well positions) instead of across columns A1–A12 (12 well positions). Compared to the "landscape configuration" in our current centrifugation setup, the "portrait configuration" translates to less variation in centrifugal force across the plate, thus, less "edge effect". Our challenge was to evaluate the feasibility of quantitative analysis for unbound **I** using the PPB plate with respect to uniformity, accuracy, and precision. The centrifugation settings have been optimized with respect to centrifugal speed, spin time, temperature and plasma sample volume, respectively.

3.1.2. Optimization of centrifugation settings versus well position

3.1.2.1. Speed profile. The centrifugal speed profile, the centrifugal speed over spin time, has a significant impact on UF volume variation across the PPB plate, and affects the extent of the "edge effect". Using a standard linear speed profile for centrifugation of the PPB membrane plate at a maximum speed of 3000 × *g*, 25 °C for 28–30 min, the UF volume observed from an initial 300 μL of fresh plasma sample per well, which was incubated at 37 °C for 30 min, ranged from 23 to 100 μL across a plate. In order to minimize the "edge effect", the centrifugal profile was customized and optimized with a ramped speed from 0 to 4310 rpm (3000 × *g* RCF) over 30 min. The optima condition of the customized speed profile is shown in Fig. 4. Under the

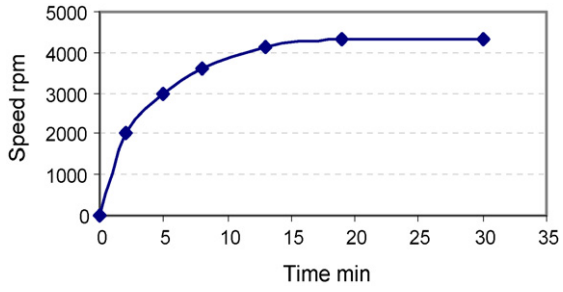

Fig. 4. Optimized centrifuge speed profile for the ultrafiltration.

same centrifugation conditions using the optimized speed profile instead of the standard linear speed profile, the UF volume found across a plate ranged from 38 to 90 μL. By optimizing the centrifugal profile, the "edge effect" on ultrafiltration volume was reduced, resulting in a more uniform volume across the plate. Similar effects across the plate were observed from 400 μL plasma samples at similar centrifuge conditions, with slightly higher UF volumes received in comparison with UF volumes collected from the 300 μL plasma samples. Since an aliquot of 400 μL plasma in the well of the plate was quite full, the 300 μL aliquot of plasma sample was selected for the assay to reduce the risk of well-to-well cross contamination during the sample transfer and centrifugation process.

No difference was observed in the UF volumes between two different lots of fresh plasma. There were only small variations observed for UF volumes between fresh and 1 freeze–thaw (F/T) cycle plasma samples over the corresponding well positions. Representative mean UF volumes for each column ($n = 8$) across the plate from the center to the edge ranged from 48 to 88 μL and 50 to 96 μL for fresh and single F/T plasma samples, respectively.

3.1.2.2. Temperature. The filtrate volume, also found to be temperature related, was evaluated at 4, 25 and 37 °C for the "edge effect" across a plate. A smaller "edge effect", in general, was observed with lower temperatures. Though a more uniform volume was found across the full plate at 4 °C, the absolute volume level collected in the center of the plate at this temperature was not sufficient for analysis, and the difference between 4 °C and physiological temperature was too great. UF volumes obtained at 25 °C were about 10 μL less than at 37 °C with less "edge effect"; the minimum volume was above 38 μL, and the percent of UF volume to initial plasma sample volume ranged from 17 to 32% across the plate.

3.1.2.3. Evaporation. The possible loss of the total UF and plasma sample volume by evaporation during the centrifugation at the higher temperatures could affect a true sample concentration; for this reason, evaporation tests for a PPB assembly with the cover were conducted over the centrifugation process by weighing the assembly before and after the centrifugation. The calculated percent weight loss was about 8 and 4% at 37 and 25 °C, respectively. Considering the results of the evaporation test, the availability of the UF volumes for analytical analysis and roughness of the centrifugation process for routine analysis, 25 °C was selected for the assay instead of 37 °C.

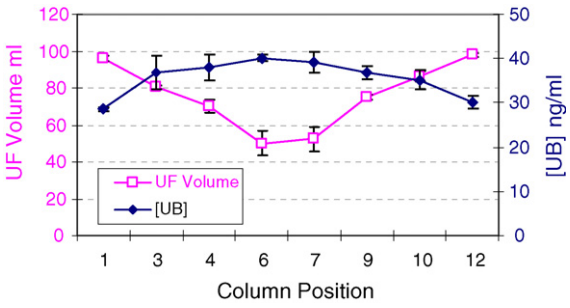

Fig. 5. Unbound concentration [UB] and receiving UF volume vs. the well positions across a PPB plate.

3.1.3. Evaluation of unbound drug concentration versus well position

To evaluate the collected ultrafiltrate (UF) volumes and unbound concentrations ([UB]) versus well positions across the plate, a 300 μL aliquot of a 1000 ng/mL plasma QC sample prepared from fresh control plasma was incubated at 37 °C for 30 min in the PPB plate. The plate was centrifuged at 3000 × *g* RCF, 37 °C for 30 min. The UF samples from the same concentration PL-QC samples in 32 wells located in columns 1, 3, 4, 6, 7, 9, 10 and 12 over four rows (A, C, F and H) on the plate were assayed for [UB] of **I**. The effect of well position on [UB] and UF volume across the plate is shown in Fig. 5. The variability of the UF volume and [UB] was low for matched wells in each row against the center within the plate, as well as for matched wells between two plates centrifuged with balanced weight and the sample well positions in one centrifuge run. The mean variability of UF volume and [UB] correlate to the percent of unbound (%UB) variability across the plate is listed in Table 2. The %UB in the table was calculated using [UB] over the nominal concentration of 1000 ng/mL. Across the column position (1–12) on the plate as shown in the table, there is significant variation in UF volumes ranging from 50 to 98 μL with the mean standard deviation (S.D.) of 18.1 μL and the coefficients of variation (CV) of 23.9%. However, there is less variation in according [UB] of 28.6–40.1 ng/mL with the mean S.D. of 4.5 ng/mL and CV of 12.5%. As shown, the "edge effect" resulted in a large variation in UF volume but in relatively less variation in corresponding [UB]. In fact, there is much less variation in [UB] between columns 3 and 10 with the according percent of unbound (%UB) ranging from 3.5 to 4.0% while the according percent of UF volume to the initial plasma sample volume ranging from 16.7 to 28.7% (<30%); with the percent of UF volume to the initial plasma sample volume

Table 2
Representative unbound concentration [UB] in 300 μL fresh 1000 ng/mL plasma sample vs. receiving UF volume across the PPB plate

	Column position numbers							
	1	3	4	6	7	9	10	12
PL concentration (ng/mL)	1000	1000	1000	1000	1000	1000	1000	1000
UF volume received (μL) (±S.D.)	96 (1.6)	80 (1.2)	70 (3.4)	50 (6.6)	52 (6.6)	75 (1.4)	86 (2.7)	98 (0.9)
% UF volume of initial PL volume	32.1	26.8	23.4	16.7	17.5	25.0	28.7	32.7
Mean [UB] ($n = 8$) over rows A–H (±S.D.)	28.6 (0.4)	36.8 (3.8)	38.1 (2.9)	40.1 (0.7)	39.1 (2.3)	36.9 (1.4)	35.2 (2.1)	30.2 (1.5)
Mean UB% ($n = 8$) over rows A–H	2.9	3.7	3.8	4.0	3.9	3.7	3.5	3.0

≤30%, the variation for the quantitation of unbound **I** is considered acceptable for the current ultrafiltration conditions. Given that the variation is relatively high at the outermost columns, only columns 2–11 of the plate were used for the study with 300 μL of plasma samples per well with the current ultrafiltration setup.

3.1.4. Evaluation of percent unbound (%UB) versus total plasma concentration for plasma QC level selection

In order to monitor the ultrafiltration process using the PPB plate, plasma QC samples spiked with compound **I** in control plasma (PL-QC) were prepared at concentrations that had been experimentally determined to provide unbound concentrations within the UF calibration range of 0.1–500 ng/mL.

The highest total plasma concentration measured previously for **I** in plasma samples was about 10,000 ng/mL using the plasma assay [10]. To evaluate the profile of %UB in plasma up to 10,000 ng/mL, plasma samples with four replicates at five concentrations of 4, 50, 1000, 6000 and 8000 ng/mL in control plasma were prepared by spiking **I** in fresh human control plasma and then incubating them at 37 °C for 30 min. These plasma samples were centrifuged and analyzed at the optimized assay conditions, and [UB] were determined at 0.13, 1.8, 36.9 and 493 ng/mL, respectively. The %UB, calculated using [UB] over the nominal concentration value as shown in a semi-log plot, was 3.2–6.2% over the total range of 4–8000 ng/mL (Fig. 6). There was a slight increase of %UB from 3.2 to 3.7 for 4–1000 ng/mL, and a dramatic non-linear increase of %UB from 3.7 to 6.2 for 1000–8000 ng/mL. Since the [UB]s of 0.13 and 493 ng/mL were close to the lower and the upper limits of the UF calibration curve 0.1–500 ng/mL, the 50, 1000 and 6000 ng/mL plasma QC concentrations were selected as low, middle and high plasma QC levels, respectively.

3.2. Non-specific binding and recovery

The loss of compound **I** due to non-specific binding to the PPB device, particularly to the high surface area membrane, was assessed for the ultrafiltration process. Since compound **I** is highly protein bound, its levels in aqueous media are expected to be low. Therefore, the NSB loss of analyte can significantly affect the outcome of the assay.

The extent of the NSB was evaluated using neat standards prepared in PBS solution at the three UF-QC (L, M and H) levels. The PBS standard aliquot of 300 μL per well with five replicates for each level were run through the ultrafiltration plate under assay conditions. The analyte concentrations in both PBS solutions, before and after the centrifugation, were analyzed and compared. The analyte recovery was determined by normalizing the peak area of **I** in the PBS filtrate to the peak area of **I** in the original solution (without filtration step). The overall recovery against the original level was about 96 ± 5% (CV of 5.2%), from which the overall corresponding loss was calculated at about 4%. The NSB loss at the assay conditions should not affect the determination of [UB] significantly.

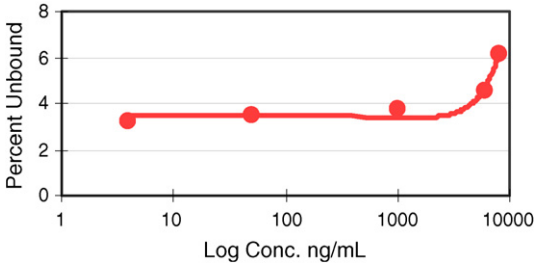

Fig. 6. Percent unbound of **I** in fresh plasma sample vs. total plasma nominal concentration.

3.3. LC/ESI-MS/MS method development and validation

There were several challenges in the development of the LC/ESI-MS/MS assay methodology. (1) As a highly protein bound compound, the free concentration of **I** is a small percent of its total plasma concentration and varies greatly between different subjects. (2) A characteristic of the PPB plate is that UF sample volumes vary across well positions due to the "edge effect". The maximum UF sample volume used for sample preparation is limited by the minimum receiving volume that can be accurately transferred using the MultiPROBE II, 30 μL. (3) The variability in the concentration of unbound **I** in the receiving ultrafiltrate sample required a highly sensitive assay with a wide linear dynamic range. (4) In addition, there was still a small amount of protein residuals remained in the ultrafiltrate matrix obtained through the PPB plate; these residuals could potentially accumulate and block the narrow diameter metal needle of the ESI source on a Finnigan Quantum. To meet the above challenges, a direct injection approach with on-line extraction using a column-switching valve was employed for ultrafiltrate sample analysis. This approach eliminated any possible loss involved in off-line sample clean up procedures. An aliquot of 30 μL of the UF samples was directly transferred from the UF collecting plate to the destination plate, acidified and followed by direct injection using on-line sample cleanup for LC–MS/MS analysis. The optimization of the LC–MS/MS method focused on enhancing assay sensitivity with a wide dynamic range and minimizing concurrent carryover effect by careful evaluation of each of the following steps: sample preparation, on-line extraction, separation, washing and re-equilibration.

3.3.1. Sample preparation

Preliminary results showed that the organic content in the final UF matrix had a positive effect on reducing carryover in the column-switching setup for on-line extraction. The acidifying solvent added to the UF sample was optimized for pH and the organic content. Different percentages of acetonitrile or methanol with 0.1, 0.5 and 1% of formic acid were evaluated; the final solvent used was acetonitrile/0.5% formic acid (2:3, v/v). To reduce the number of transferring steps during sample preparation, IS **II** was added in the acidifying solvent. The UF standards, QCs, and samples (except for double blank samples) were acidified with the solvent containing the working IS at a ratio of 1–3. In the final injection matrix, the percentage of formic acid and the organic content was about 0.2 and 31%, respectively.

3.3.2. On-line extraction chromatographic conditions

With column-switching for on-line extraction, the acidified UF sample was directly injected with an 5 μL injection volume on a narrow bore Cyclone HTLC column (50 mm × 0.5 mm, 60 μm), which provided better recovery than a Cyclone-P column with the same dimensions. At the loading step, the matrix components were rapidly washed off the column while analytes of **I** and **II** were retained at the front end of the column with the aqueous mobile phase A at 0.7 mL/min. The analytes were then back-flushed off the extraction column with mobile phase C at a flow rate of 0.2 mL/min onto an analytical column at the eluting step. Chromatographic separation was performed on a BDS Hypersil C18 column (30 mm × 2.1 mm, 3 μm). Acetonitrile (80%) was used as the organic modifier in the mobile phases, which gave a higher signal in the Quantum ESI source compared to methanol and reduced memory effects in the source. The extraction mobile phase A was composed of 5% acetonitrile and 95% 2 mM ammonium formate buffer at pH 3, which was also used in mobile phase C for the analytical column. The aqueous portion of the mobile phase in C and A had to be buffered in order to obtain a better and more stable peak shape in the eluting step.

While the chromatographic separation and analysis were performed on the analytical column, the extraction column was washed with acetonitrile/dimethyl sulfoxide/formic acid (80/10/10, v/v/v), which was optimized to eliminate the carryover introduced from the extraction column and switching valve. During the washing, an additional "quick-valve switching" between the two positions was performed within 0.05 min to provide an extra washing step for the divert valve to reduce possible carryover from it. No carryover peak was observed in a blank sample injected following the highest standard using the current assay conditions.

3.3.3. Specificity, sensitivity and intra-day variability

The specificity of the UB plasma assay was assessed in five different lots of control ultrafiltrate matrices generated from five different lots of human control plasma. No interfering endogenous peak was observed in the retention time window of the analyte and IS under the assay conditions.

An assessment of intra-day variability of the assay was conducted with five calibration standard curves that were prepared by spiking compound **I** in five different lots of control UF over the calibration range of 0.1–500 ng/mL. The results of intra-day assay validation are presented in Table 3. Linearity was established over the range of 0.1–500 ng/mL with least-squares linear regression (weighing $1/x^2$); the intra-day assay accuracy ranged from 93.9 to 104.8% of nominal; the assay's precision, measured by coefficient of variation (%CV), was less than 7.3%. The mean coefficient of determination (R^2) was 0.997. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the analyte that could be analyzed with an accuracy of ±15% of the nominal value and a %CV ≤ 15%. The LLOQ for **I** was found to be 0.1 ng/mL, with an accuracy of 100.9% and a precision (%CV) of 7.3%. Representative chromatograms of a control UF double and single blanks, and an UF standard at 0.1 ng/mL (LLOQ) of **I** are shown in Fig. 7A–C, respectively.

3.3.4. QC validation

Two sets of QC samples prepared in control UF (UF-QC) and control plasma (PL-QC) were evaluated for the assay. The intra-day precision and accuracy for both sets of QC samples at three concentrations (L, M and H) are listed in Table 4.

The UF-QC samples were validated to assess the intra-day variability of the LC–MS/MS assay. The intra-day accuracy ($n = 5$) of the plasma UF QC samples at 0.3, 40 and 400 ng/mL averaged 105.8, 103.8 and 102.4%, respectively; precision (%CV) was 3.1, 1.1 and 2.1%, respectively.

The PL-QC samples were used to evaluate the ultrafiltration process using the PPB plate. The mean unbound concentrations

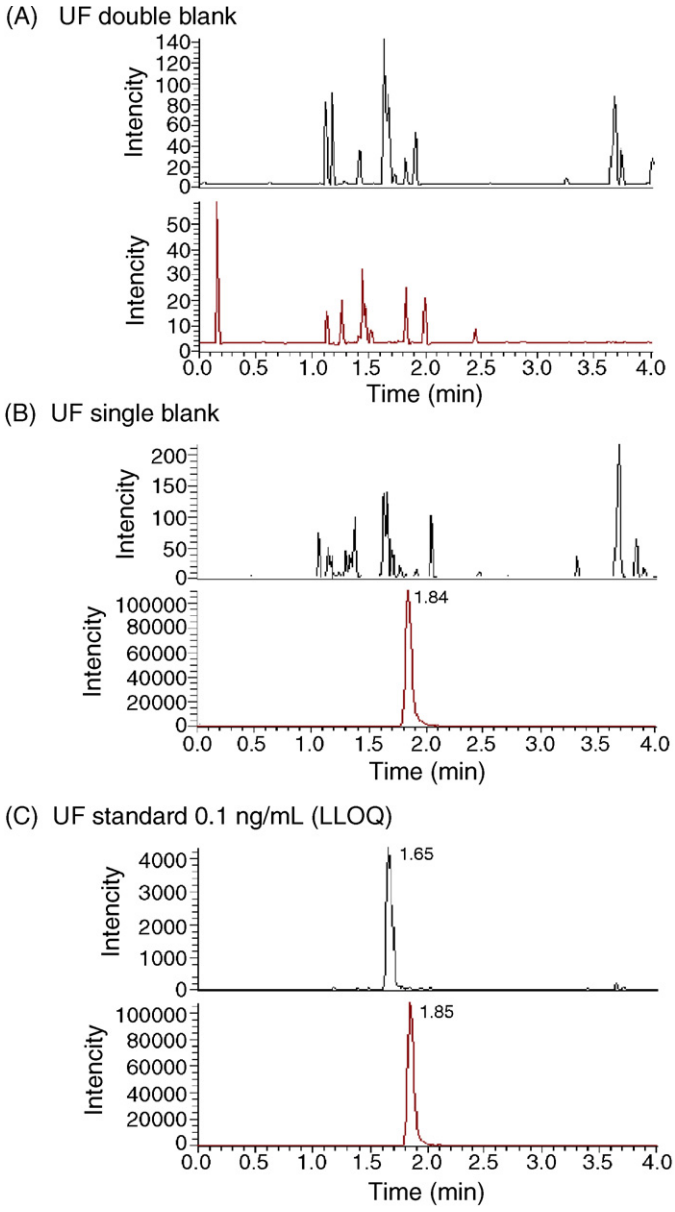

Fig. 7. Representative chromatograms of **I** in human control ultrafiltrate (UF) in unbound plasma assay on Finnigan TSQ Quantum. (A) UF double blank; (B) UF single blank; (C) UF standard 0.1 ng/mL (LLOQ). Each upper chromatogram represents compound **I** channel (*m/z* 440.1–261.0), and each lower chromatogram represents IS channel (*m/z* 468.1–289.0).

Table 3
Intra-day precision and accuracy of unbound assay for compound **I** on Finnigan TSQ Quantum

Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Mean accuracy[a] (%)	Mean precision[b] (%CV)
0.10	0.101	100.9	7.3
0.50	0.493	98.6	2.7
1.0	0.94	93.9	4.2
5.0	5.01	100.2	1.9
10.0	9.94	99.4	2.0
100.0	99.8	99.8	4.0
250.0	256.1	102.4	2.6
500.0	523.9	104.8	2.7

Linear regression $R^2 = 0.997$; slope = 0.325; intercept = 0.004.
[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100 ($n = 5$).
[b] Expressed as coefficient of variation (%CV) based on peak area ratios ($n = 5$).

Table 4
Intra-day precision and accuracy of unbound quality control (QC) samples of compound **I** in ultrafiltrate (UF-QC) and in plasma (UF-PL-QC)

Nominal conc. (ng/mL)	Mean conc. (ng/mL)	Accuracy[a] (%)	Precision[b] (%CV)
UF-QC ($n = 5$)			
0.3	0.32	105.8	3.1
40.0	41.5	103.8	1.1
400	409.8	102.4	2.1
UF-PL-QC ($n = 4$)			
50	1.23	Initial[c]	10.6
1000	25.1	Initial	4.7
6000	222.1	Initial	5.6

[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100 ($n = 5$).
[b] Expressed as coefficient of variation (%CV) based on peak area ratios ($n = 5$).
[c] Used as an initial value of unbound concentration in plasma QC samples for analysis.

($n = 4$) measured for the ultrafiltrate of the PL-QC samples at 50, 1000 and 6000 ng/mL were 1.23, 25.1 and 222.1 ng/mL, respectively. These values were used as the initial unbound concentrations for the plasma QC samples during sample analysis. The intra-day precision for the unbound concentrations of **I** in the PL-QC samples ranged from 4.7 to 10.6%, which reflected a relatively high variability during the ultrafiltration process. However, it was acceptable for monitoring the process.

A freeze–thaw (F/T) stability study for this unbound plasma assay was performed using the PL-QC samples to evaluate the stability of plasma samples under going the F/T process. The PL-QC samples at 50, 1000 and 6000 ng/mL were assayed after 1, 2 and 3 F/T cycles stored at −20 °C between cycles. The results, shown in Table 5, indicate insignificant change for the unbound concentrations after 3 F/T cycles.

Inter-day variability of the assay was determined using the plasma UF-QC samples, which were analyzed daily with samples and were used to accept a batch run. The PL-QC samples were used to qualitatively evaluate the ultrafiltration procedure. The inter-day accuracy and precision (%CV) of the assay over three runs as demonstrated by the UF-OC samples analyzed at L, M and H concentrations averaged from 99.6 to 104.6% and 3.3 to 4.3%, respectively. The inter-day analysis of [UB] in PL-QC samples at 50, 1000 and 6000 ng/mL showed the plate-to-plate variability of the ultrafiltration process averaged from 6.6 to 14.6% over three runs.

3.3.5. Assay recoveries, matrix effect and stability

On-line extraction efficiency for **I** and IS **II** was evaluated over the calibration range using five replicates of the neat

Table 5
Freeze–thaw (F/T) cycle stability, unbound concentration [UB] and theoretical percent unbound (%UB)[a] in plasma QC samples

PL-QC nominal conc. (ng/mL)	Mean values ($n = 4$)					
	F/T-cycle 1		F/T-cycle 2		F/T-cycle 3	
	[UB] (S.D.) (ng/mL)	%UB	[UB] (S.D.) (ng/mL)	%UB	[UB] (S.D.) (ng/mL)	%UB
50	1.23 (0.1)	2.5	1.25 (0.04)	2.5	1.28 (0.1)	2.6
1000	25.1 (1.2)	2.5	28.7 (1.7)	2.9	29.1 (2.7)	2.9
6000	222 (12.3)	3.7	227 (9.5)	3.8	228 (15.3)	3.8
Overall mean %UB (S.D.)		2.9 (0.7)		3.1 (0.7)		3.1 (0.6)

[a] Expressed as [mean unbound concentration/nominal plasma concentration] × 100.

Table 6
Recovery and matrix effect of unbound plasma using LC/ESI-MS/MS assay

Nominal concentration (ng/mL)	Mean recovery[a] (S.D.) ($n=5$)	Mean matrix[b] (S.D.) ($n=5$)
0.5	67.2 (3.4)	107.1 (2.9)
10	67.6 (2.3)	105.7 (5.2)
250	68.1 (1.9)	104.4 (3.0)
IS[c] ($n=15$)	64.7 (2.3)	104.0 (3.2)

[a] Expressed as ([d]ext-neat mean peak area/[e]direct-neat mean peak area) × 100 (%), where superscript "d" means neat sample went through on-line extraction using column-switching after injection and superscript "e" means neat sample was directly injected on analytical column without going through on-line extraction.
[b] Expressed as (ultrafiltrate mean peak area/neat mean peak area) × 100 (%).
[c] In the presence of compound **I** at three concentration levels.

standards at 0.5, 10 and 250 ng/mL. The extraction recovery was determined by comparing the absolute peak area of the same neat samples with and without the extraction column between the on-line extraction condition and the direct-injection condition. Since the on-line extraction involved a back flushing step, a segment of mobile phase A in the extraction column was transferred to the analytical column during the back flushing. The real component of mobile phase C for eluting at the analyte retention times could be different from the undisturbed mobile phase C regarding the methanol%. Under the direct-injection condition by directly injecting the neat samples on the analytical column using mobile phase C only, the analyte ionization in the source could be different from that at the real on-line extraction condition. Therefore, the recovery obtained using this approach was considered a relative value instead of an absolute. As shown in Table 6, the mean recoveries were about 67–68% for **I** and 65% for IS **II**, respectively. Matrix effect in plasma UF were determined by comparing the absolute peak area of ultrafiltrate standards with the neat standards through on-line extraction. The matrix effect averaged from 104 to 107% of nominal for **I** and IS **II** (Table 6), respectively. Compared to the intra-day calibration curve, the linear regression (slope, interception and R^2) of the neat standard curves obtained during the recovery and matrix effect analyses were basically the same.

The stability of processed UF samples in the autosampler with temperature control set at 4 °C was assessed by comparing the results of UF-QC (L, M and H) samples analyzed at the beginning and the end of the run. The mean percent remaining for **I** ($n=5$) over 23 h averaged from 99.7 to 102.2%.

3.4. Clinical application

The unbound assay was used to quantify unbound **I** in clinical unbound plasma samples collected from patients given 2 × 100 mg oral administration of **I**. The percent unbound of the clinical samples was calculated from the measured [UB] using the unbound assay over the total concentration of the same plasma sample using total plasma assay × 100 [10]. The calculated percent unbound ranged from 2.3 to 4.1% of total **I** in plasma.

4. Conclusions

A rapid, sensitive and reproducible high-throughput ultrafiltration assay using a PPB 96-well plate with a 10KDa ultrafilter membrane has been developed to determine unbound **I** in human plasma. The assay has been validated over a range of 0.1–500 ng/mL. The performance of the unbound assay has been tested on clinical subjects, and the UB% of **I** in both clinical samples and plasma QC samples were similar. The 96-well ultrafiltration plate is a robust device with high protein retention and low NSB for compound **I**. Several factors affecting volume and binding equilibrium should be considered when using the plate for unbound drug studies. Centrifugal temperature, speed, time and plate position are important variables that should be optimized. By keeping the ultrafiltrate volume to <20% (preferred <35%) of the original sample volume used, the effect of centrifugation on protein binding is minimized.

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