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Developing a robust ultrafiltration-LC–MS/MS method for quantitative analysis of unbound vadimezan (ASA404) in human plasma

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ABSTRACT

Ultrafiltration of human plasma in combination with LC–MS/MS has been increasingly used in the quantitative analysis of the free fraction of drug candidates for PK/efficacy assessment. In addition to controlling the pre-incubation and centrifugation temperatures, some important factors that must be investigated and addressed include: (1) possible nonspecific binding, (2) possible impact of freeze/thaw cycles of plasma samples and extended storage of plasma samples at room temperature on the analyte recovery prior to ultrafiltration, and (3) identification of the appropriate assay dynamic range to avoid unnecessary dilutions. These factors were explored in the development and validation of a robust LC–MS/MS assay for the quantitative analysis of unbound vadimezan (ASA404) in human plasma. First, to mimic human physiological conditions, all plasma samples were incubated at ~37 °C for a minimum of 30 min after thawing and prior to centrifugation to obtain the ultrafiltrate. Second, by passing the calibration standards and QC samples in plasma ultrafiltrate through the ultrafiltration membrane, the observed non-specific binding of the analyte due to the membrane was corrected. Third, the effects of multiple freeze/thaw cycles and/or storage at room temperature for various periods (4, 8, 16 and 24 h) were evaluated to determine the impact on analyte concentrations in the ultrafiltrate from the plasma QC samples. Fourth, the appropriate dynamic range was established to accommodate the expected incurred sample free analyte concentrations. The validated assay has a dynamic range of 30.0–30,000 ng/ml for ASA404 in human plasma ultrafiltrate using a sample volume of 30 μl. Quality control pools containing the analyte were prepared at concentrations of 30.0–22,500 ng/ml to cover the assay calibration range. The intra-assay and inter-assay precision and accuracy were ≤15% (CV) and within ±15% (bias) of the nominal values, respectively, for all measured QC concentrations, including the LLOQ. Freeze/thaw for up to three cycles of the plasma samples and/or the extended human plasma sample exposure to room temperature for up to 24 h were confirmed to have no impact on the assay results for the free analyte. The validated method was successfully implemented to support clinical studies for the compound.

1. Introduction

It is well known that many drug molecules are loosely bound to plasma proteins such as albumin, glycoprotein, etc., forming an equilibrium ratio between bound and unbound drugs *in vivo*. Binding of drug molecules to plasma protein limits their pharmacological actions since only the unbound fraction of the drugs in plasma is available for many pharmacokinetic (PK) and pharmacology (efficacy) processes, such as membrane permeation and receptor binding, etc. Therefore, the free drug concentration measurement of clinical human plasma or serum samples is often considered one of the best approaches for understanding the PK/efficacy of a pharmacologically active molecule [1–3].

The most commonly used methods for quantitative analysis of unbound analyte in plasma are equilibrium dialysis, ultracentrifugation and ultrafiltration [4]. Each of the methods has some advantages over the others. Ultrafiltration utilizes a pressure gradient forcing the aqueous component of plasma containing the free drug molecules through a permeability selective membrane. In general, the method has an advantage over equilibrium dialysis or ultracentrifugation for being less time consuming, and therefore, likely to have a higher analysis throughput [4,5]. The disadvantage of the method is that it can be susceptible to variable non-specific binding (NSB) of the test compounds to the polymer-constructed membranes of the devices, depending on the hydrophobicity of the test compounds and the selection of the membrane materials. The higher hydrophobicity the test compound is, the higher NSB may occur, resulting in a deviation of the measured plasma ultrafiltrate

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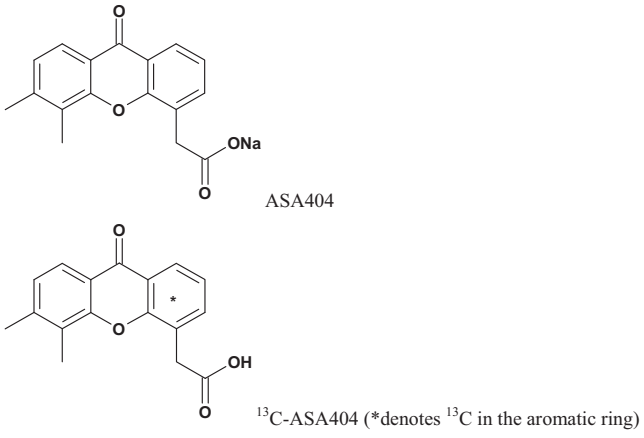

Fig. 1. Chemical structures of ASA404 and $^{13}C_6$-ASA404 (internal standard).

concentration from the true *in vivo* unbound analyte concentration [5,6]. In practice, the unbound analyte often is measured after the total (unbound + bound) analyte concentration measurement using the same set of plasma samples [7–10]. In this case, the plasma samples might have gone through multiple free/thaw cycles and/or extended exposure to room temperature. The possible analyte loss in the ultrafiltration process due to NSB and the possible impact of freeze/thaw cycles or extended sample exposure to room temperatures could significantly affect the outcome of the assay.

Vadimezan (ASA404, Fig. 1) is a novel tumor-vascular disrupting agent that induces irreversible tumor vascular collapse, hemorrhagic necrosis of the tumor core, and cytokine production while enhancing cell-mediated cytotoxicity. In early clinic trials, a synergistic effect was reported in the treatment of non-small cell lung cancer patients with the compound in combination with a carboplatin and paclitaxel chemotherapy regimen [11–13]. The compound has a molecular weight of 282 Da and highly binds to plasma proteins in a concentration dependent manner [14,15].

An assay for unbound ASA404 was necessary to study its pharmacokinetics/efficacy. Zhou et al. [15] previously reported an HPLC-UV method for the unbound analyte via ultrafiltration of plasma samples with a 20 kDa molecular weight cut-off (MWCO). The method was not comprehensively validated although assay recovery, selectivity, linearity, precision, and accuracy were tested using *in vitro* samples. In the current work, a list of evaluations were conducted for the development and validation of a robust LC–MS/MS method for quantitative analysis of unbound analyte in human plasma samples using ultrafiltration with a 10 kDa MWCO. These evaluations include (1) ultrafiltration device selection, (2) NSB, (3) human plasma incubation at 37 °C, (4) centrifugation time and temperature (~37 °C), (5) freeze/thaw cycles and the extended sample exposure to room temperature, and (6) long term storage of human plasma samples. Incurred sample reanalysis (ISR) assessment was conducted as part of assay method validation.

2. Experimental

2.1. Materials and reagents

ASA404 (Fig. 1) and the ^{13}C-labeled internal standard ([M+6]ASA404) were, respectively, synthesized at Technical Research & Development of Novartis AG (Basel, Switzerland) and Drug Metabolism & Pharmacokinetics of Novartis Pharmaceuticals Corporation (East Hanover, NJ, USA). Trisma base was obtained from Mediatech (Herndon, NJ, USA). Acetic acid (AA), isopropyl alcohol (IPA), formic acid (FA), methanol (MeOH), acetonitrile (ACN), all in HPLC grade, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was deionized (DI) and purified on an in-house ELGA Lab Water system (Lowell, MA, USA). Tris solution (0.1%) was prepared by mixing 2.0 g of Trisma base with 2000 ml DI water and stored at room temperature. Human plasma (Li-heparin as anticoagulant) and the corresponding ultrafiltrate (with 10 kDa MWCO) were obtained from healthy donors (Bioreclamation Inc., Westbury, NY, USA). Microcon Ultracel YM-10 centrifugal filter tube with 10 kDa cut-off (can sustain up to 14,000 × *g* centrifugation force) was obtained from Millipore (Billerica, MA, USA). Centrifugation was conducted on a Beckman model Avanti J-E centrifuge (Beckman Coulter, Indianapolis, IN, USA).

2.2. Chromatography

An integrated Shimadzu liquid chromatography system consisting of a model SCL-10Avp controller, a multi-channel mobile phase degasser (DGU-14A), an LC-10ADvp and a LC-10AD pump (Shimadzu, Columbia, MD, USA) and an Onyx Monolithic C_{18} (50 mm × 2.0 mm, Phenomenex, Inc., Torrance, CA, USA) HPLC column held at room temperature was used for the chromatographic separation of ASA404 and the internal standard from the matrix components. The autosampler was an HTS-PAL from Leap Technologies (Carrboro, NC, USA). Mobile phase A was water (containing 0.1% formic acid, v/v) and mobile phase B was acetonitrile (containing 0.1% formic acid, v/v). Gradient elution at a flow rate of 0.500 ml/min was employed at the following program: 30% B from 0 to 0.20 min; 30% B to 70% B from 0.20 to 1.80 min; hold at 70% B until 2.00 min; 70% B to 90% B from 2.00 to 2.20 min; hold at 90% B until 2.80 min; 90% B to 30% B from 2.80 to 3.00 min; hold at 30% B until 4.60 min. The column effluent was directed to the MS source between 0.9 and 1.8 min.

2.3. MS/MS detection

A Sciex API3000 tandem mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a Heated-Source-Induced-Disassociation device (HSID, Ionics, Concord, ON, Canada) was employed for the MS/MS detection of both the analyte and internal standard. The optimized instrumental conditions were as follows: nebulization gas: 15 units; curtain gas: 11 units; collision associated dissociation (CAD) gas: 6 units; ion spray voltage: 5500 V; source temperature: 550 °C; HSID temperature: 250 °C; declustering potential: 35 V; focusing potential: 50 V; entrance potential: 9 V; collision energy: 40 eV; collision cell exit potential: 20 V; dwell time: 300 ms for each MS transition (m/z 283.1 → 243.1 for ASA404 and 289.1 → 243.1 for the ISTD). The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.7 Da) for both the first quadrupole and the third quadrupole.

2.4. Data analysis

Data was processed using Watson LIMS version 7.2.0.01 (Thermo Fisher Scientific, Philadelphia, PA, USA). The calibration curves (analyte peak area/ISTD peak area versus analyte concentration) were constructed using the least squares linear regression model $y = ax + b$ with a weighting factor of $1/x^2$. According to the current industry practice and internal procedures, the acceptance criteria were established to be >0.98 for the calibration curve coefficient of determination (r^2) with the observed QC concentrations within ±15% bias of the nominal concentration (accuracy) and ≤15% CV (precision) for the intra-day and inter-day assay at all levels, except at the LLOQ, where the intra-day and inter-day assay accuracy and precision limits were within ±20% bias and ≤20% CV.

2.5. Plasma ultrafiltrate calibration standards (Cs) and quality controls (QCs)

Two primary ASA404 stock solutions were prepared in 0.1% Tris solution, each at a concentration of 20.0 mg/ml in 20-ml vials. The stock solutions were stored at 2–8 °C. For validation purposes, the stock solutions from the two weighings must have LC–MS/MS responses within 5% of each other. The stock solution was serially diluted with 0.1% Tris solution to prepare the standard working solutions at the desired concentrations. An internal standard working solution containing 500 ng/ml of [M+6]ASA404 was prepared from the internal standard stock solution using 0.1% Tris solution.

Eight non-zero calibration standards were prepared in human plasma ultrafiltrate at concentrations of 30.0, 60.0, 300, 1500, 3000, 15,000, 24,000 and 30,000 ng/ml via series dilution of an intermediate at a concentration of 600,000 ng/ml to ensure a minimum percentage of non-matrix in the standard samples. Each calibration standard concentration was assayed in duplicate in each assay run. Blank and zero (blank + ISTD) samples were also assayed, but not included in the calibration regression. QC samples were prepared in human plasma ultrafiltrate at 5 concentration levels in the range of 30.0 ng/ml (LLOQ) to 22,500 ng/ml (HQC).

2.6. Plasma quality controls (QCs)

Pooled quality control (QC) plasma samples were prepared by spiking appropriate amounts of the stock solution into pools of the human plasma blanks, followed by series dilution using human plasma. QC samples at concentrations of 2500, 10,000, 75,000 and 150,000 ng/ml were prepared. These concentrations were selected based on the total analyte concentration profiles from the patients administered intravenously with 1800 mg/m^2 of test article. The pooled human plasma QC samples were employed for the various assessments of sample preparation procedure for the unbound analyte.

2.7. Sample preparation

Sample was prepared using protein precipitation extraction with a Hamilton MicroLab®AT2 plus system and a Quadra 96 TomTec system. Calibration standards and QCs were pre-prepared using human plasma ultrafiltrate. Prior to sample preparation, all calibration standards, QCs, human plasma ultrafiltrate blanks and study plasma samples were thawed at room temperature. A 125 μl aliquot of each calibration standard and QC sample or a 250 μl aliquot of each study plasma sample was pipetted into the appropriate Microcon Ultracel YM-10 centrifugal filter tube. The tubes were placed in a centrifuge with the temperature pre-set to ~37 °C or a water bath (at ~37 °C) and allowed to equilibrate for 30 min prior to centrifugation at a speed of ~6100 × *g* for 1 h. Using the Hamilton system, a 30 μl aliquot of ultrafiltrate of each calibration standard, QC and study plasma sample was pipetted from the individual collection tubes into the appropriate wells of a 96-well assay plate. A 100 μl aliquot of 0.1% Tris solution (containing the internal standard at 500 ng/ml) was added to all wells except for the blanks, to which a 100 μl aliquot of 0.1% Tris solution was added. To all wells, a 50 μl aliquot of 1% FA in MeOH was added. A 250 μl volume of ACN was added to each well, the plate covered, vortex-mixed at a speed setting of 3 for about 2 min and centrifuged at approximately 3000 rpm (~1000 × *g*) for about 5 min at room temperature. Using a TomTec Quadra 96 model 320, 100 μl of water followed by 200 μl of the sample extract were transferred to a clean 1-ml 96 well plate and mixed using the TomTec via aspirating and dispensing the samples three times. Depending on LC–MS/MS system suitability test result, a fixed volume between 5 and 10 μl of sample extract was injected onto the HPLC system connected to a Sciex API3000 tandem mass spectrometer for analysis.

2.8. Assay method validation

Three validation batches were used to assess the precision and accuracy of the method. Each batch was processed on a separate day and had two replicates of each calibration standard and six replicates of each QC sample concentration (30.0, 90.0, 2250, 9000 and 22,500 ng/ml). The QC samples and other test samples were interspersed between the two replicates of calibration standards. A blank sample was always placed immediately after the upper limit of quantification (ULOQ) standard to evaluate the carry-over of the LC–MS/MS system.

The matrix effect of the method was determined by comparing the LC–MS/MS response of extracted blank plasma ultrafiltrates fortified post-extraction with neat solutions of the analyte at low (90.0 ng/ml), medium (2250 ng/ml) and high (22,500 ng/ml) concentrations with the response obtained from the neat solutions of the analyte at the same concentrations. Recovery was determined by comparing the LC–MS/MS response of extracted QC samples at concentrations of 90.0, 2250 and 22,500 ng/ml with the response obtained from extracted blank plasma ultrafiltrates fortified post-extraction with the same analyte concentrations.

For the short-term stability assessment, plasma ultrafiltrate QC samples at 90.0 and 22,500 ng/ml were subjected to three cycles of freeze–thaw (free–thaw stability) or storage on the laboratory bench at room temperature for approximately 24 h (bench-top stability) and processed together with calibration standards and regular QC samples. As part of the stability assessment, one batch of extracted samples was stored in the auto-sampler for approximately 6 days before re-injection onto the LC–MS/MS system to determine the storage and re-injection reproducibility of the processed samples.

3. Results and discussion

3.1. Method development

3.1.1. Selection of ultrafiltration devices

Early feasibility test was carried out by using a MultiScreen 96-well ultrafiltration assembly (10 kDa Ultracel, Millipore) to separate the unbound analyte from the plasma with an intent for a higher analysis throughput. However, due to the configuration of the 96-well ultrafiltration plate, different centrifugal forces were noticeably applied to the outer wells vs. the inner wells of the assay plate, resulting in a significant difference in the ultrafiltrate volume collected across the plate, e.g. ~40 μl from the middle wells vs. ~120 μl from the outer wells of the plate when a 250 μl volume of plasma was employed. Although the changes in free drug concentrations were reportedly insignificant with the changes in the ultrafiltrate volumes [16–18], this phenomenon, known as "edge effect" [18], rendered it impossible for the repeat analysis because of the uncontrolled variability of the obtained ultrafiltrate volumes. Attempts to minimize the 'edge effect' were unsuccessful. In the current experiment, individual ultrafiltration tubes [Microcon Ultracel YM-10] with both sample reservoir and ultrafiltrate compartment were all placed in the outer columns of a 48-well centrifugation rack in order to obtain consistent ultrafiltrate volumes in the entire analysis process. Approximately 120 μl volume of ultrafiltrate could be obtained from a ~250 μl plasma sample after 1 h centrifugation (×6100 *g*) at 37 °C.

3.1.2. Centrifugation force and duration

It has been suggested that the ultrafiltration yield should be lower than 35% of the plasma volume for a minimum distur-

bance to the protein-binding equilibrium [18]. In the course of ultrafiltration, all nonfiltrable components present in the plasma compartment become increasingly concentrated. This may result in a so-called 'sieve effect' [6,19,20] under the assumption that the membrane may not act as a perfect molecular sieve but instead discriminates between water molecules and drug molecules if the drug molecule is relatively large, e.g. >500 Da [5,6]. As a result of the 'sieve effect', the obtained ultrafiltrate from the later stage of ultrafiltration may have been diluted compared to those obtained from the early stage. Accordingly, a relatively large plasma volume (e.g. 2 ml) is often used for ultrafiltration via a higher MWCO (30 kDa, instead of 10 kDa) and a relatively short centrifugation duration, i.e. 30 min. Unfortunately, limited plasma sample volumes were available in the present study. Considering the nature of the anticoagulant (Li-heparin) and the relatively small molecule of the analyte (282 Da), a centrifugation force at 6100 × *g*, instead of ~3000 × *g* [5,6,18] or 12,000 rpm [8], was employed to ensure that there is enough ultrafiltrate volume (e.g. ~100 μl) to be obtained for the initial analysis and at least two more repeats (30 μl each) for any plasma sample. The duration of centrifugation was carefully evaluated by measuring the free analyte concentration in the ultrafiltrate receiving chamber at 0.5, 1 and 2 h post ultrafiltration for the plasma QC samples at concentrations of 2500, 10,000, 75,000 and 150,000 ng/ml. No apparent difference was seen for the measured free analyte concentrations in the receiving chamber. The measured analyte concentrations from the ultrafiltrate obtained after a 2-h centrifugation were slightly (3–7%) higher than that obtained after a 1-h centrifugation.

3.1.3. Nonspecific binding due to ultrafiltration membrane and associated correction

Although it is generally compound dependent, NSB tends to be high for compounds with high molecular weight (e.g. MW > 500 Da) because of the potential molecular sieving effect. The NSB also increases with the extent of protein binding [5,6]. Ultrafiltration of plasma ultrafiltrate containing analyte at concentrations of 30.0, 90.0, 2250, 9000 and 22,500 ng/ml was employed for evaluating the NSB. The LC–MS/MS responses of the analyte with and without passing through the membrane were compared. Devices with Ultracel YM regenerated cellulose membranes showed the least NSB with analyte loss from ~15% at the LLOQ (30 ng/ml) to ~10% at the HQC (22,500 ng/ml) in a slight concentration dependent manner. Other membranes tested showed greater NSB with observed analyte loss up to 60% (details not shown). Pre-treatment of ultrafiltration membranes with Tween-80 or benzalkonium chloride might be feasible in modulating NSB [5]. However, it is not practical when working with a large number of clinical samples. In the current work, in order to correct the observed loss of analyte in the plasma ultrafiltrate due to NSB, the plasma ultrafiltrate calibration standards and QC samples (125 μl at each concentration level) were used as controls and passed through the membrane along with the study plasma samples during the sample preparation process. The percentage of non-matrix components in the ultrafiltrate calibration standard and QC samples was minimized as even small quantities of non-matrix solvent may interfere with the correction of the observed NSB in the ultrafiltration process.

3.1.4. Possible impact of incubation and centrifugation temperature (37 °C) on the analyte stability in human plasma

Plasma QC samples at concentrations of 300, 2500, 10,000, 75,000 and 150,000 ng/ml were incubated in a water bath at 37 °C for different period of time followed by analysis with a set of plasma calibration standard (100–200,000 ng/ml) and QC samples (300, 2500, 10,000, 75,000 and 150,000 ng/ml) for the total analyte concentrations. The results showed that the analyte is stable in human plasma at 37 °C (in water bath) for at least 2 h.

3.1.5. Possible impact of the extended storage at room temperature (RT) and multiple freeze/thaw (F/T) cycles of plasma samples on the accuracy of free analyte determination

The possible impact of the extended room temperature (RT) stay of plasma samples on the accuracy of the free analyte determination was evaluated by placing the thawed plasma QC samples (at concentrations of 2500, 10,000, 75,000 and 150,000 ng/ml) on laboratory bench (~22 °C) over 4, 8, 16 and 24 h period followed by incubation at 37 °C for at least 30 min, ultrafiltration and LC–MS/MS analysis using ultrafiltrate calibration standards and QC samples. The measured analyte concentrations in the ultrafiltrates from the plasma QC samples as above were compared with the values measured from the ultrafiltrates obtained from the same set of plasma QC samples at time zero (immediately after thawing followed by a 30 min incubation at 37 °C). The observed bias (%) ranged from −8.9 to 8.5%, indicating that the extended stay of human plasma samples at room temperature has no impact on the yield of the unbound analyte.

The possible impact of multiple freeze/thaw (F/T) cycles of plasma samples on the accuracy of the free analyte determination was evaluated by analyzing the plasma QC samples (at concentrations of 2500, 10,000, 75,000 and 150,000 ng/ml) that experienced three cycles of freeze–thaw for unbound analyte concentrations together with one set of ultrafiltrate calibration standards and QC samples. The measured analyte concentrations in the ultrafiltrates from the above plasma QC samples were compared with the values measured from the ultrafiltrates obtained from the same plasma QC samples without going through the freeze/thaw cycles. The observed bias (%) ranged from −1.0 to 2.2%, indicating that multiple freeze/thaw cycles has no impact on the yield of the unbound analyte.

3.1.6. Dynamic range adjustment

In general, dilution of plasma samples before ultrafiltration should be avoided because of the laborious nature of the ultrafiltration process using individual tubes and the possible error associated, especially when the analyte of interest has a relatively high free fraction in plasma or if analyte protein binding is concentration dependent. Although dilution of the resulting ultrafiltrate using blank matrix is feasible, it increases the already time-consuming and labor intensive sample preparation activity. Our approach was to adjust the assay dynamic range to cover the entire range of projected therapeutic concentrations. In cases where measured free analyte concentration is above the upper limit of quantification, dilution of the obtained ultrafiltrate, instead of the plasma sample, should be made for repeat analysis. To validate the dilution integrity of the method, a 30 μl volume of human plasma ultrafiltrate QC sample at a concentration of 500,000 ng/ml was passed through the filter membrane, which was followed by a 10-fold dilution with blank human plasma ultrafiltrate before LC–MS/MS analysis along with a set of ultrafiltrate calibration standards and QC samples that passed through the membrane in the same fashion as the dilution QCs. The observed CV (%) and bias (%) from the results of six replicate measurements were 1.2% and 13.4%, respectively. In contrast, the observed bias was only 1.6% for the same dilution QCs in a separate experiment where all ultrafiltrate calibration standards and QCs did not pass through the filter membrane. The relatively high bias (13.4%) for the former further suggested a slight concentration dependency of the NSB.

Table 1
Intra-day and inter-day accuracy and precision of the assay method.

Items	Run	LLOQ (30.0 ng/ml)	Low (90.0 ng/ml)	Mid (2250 ng/ml)	Mid (9000 ng/ml)	High (22,500 ng/ml)
Intrarun mean		32.2	91.1	2410	9810	24,700
Intrarun SD		1.25	5.67	150	639	1220
Intrarun %CV	Run 1	3.9	6.2	6.2	6.5	4.9
Intrarun %bias		7.3	1.2	7.1	9.0	9.8
n		6	6	6	6	6
Intrarun mean		31.8	93.0	2450	9650	23,700
Intrarun SD		1.29	2.79	116	216	1090
Intrarun %CV	Run 2	4.1	3.0	4.7	2.2	4.6
Intrarun %bias		6.0	3.3	8.9	7.2	5.3
n		6	6	6	6	6
Intrarun mean		32.6	91.0	2330	9010	22,800
Intrarun SD		2.07	2.85	128	403	564
Intrarun %CV	Run 3	6.3	3.1	5.5	4.5	2.5
Intrarun %bias		8.7	1.1	3.6	0.1	1.3
n		6	6	6	6	6
Mean conc. found (ng/ml)		32.2	91.7	2390	9420	23,600
Inter-run SD		1.52	3.88	135	557	1120
Inter-run %CV		4.7	4.2	5.7	5.9	4.7
Inter-run %bias		7.3	1.9	6.2	4.7	4.9
n		18	18	18	18	18

3.2. Validation of the final method procedure

The free concentration of an analyte of interest in *in vivo* samples is not only dependent on its total plasma concentration and protein binding, but also on the plasma concentrations of various binding proteins as they can be different from one subject to the other or at different stage of a disease for a given subject. Unfortunately, no plasma sample with a known free analyte concentration is available for assay validation. The accuracy and precision determination reported here therefore does not involve the ultrafiltration of plasma matrix, but is simply the analytical result of spiked blank plasma ultrafiltrate samples that pass through the filter membrane to mimic the plasma ultrafiltration process. In addition to conducting assay validations according to the FDA guidance of 2001, incurred sample reanalysis (ISR) and incurred sample stability (ISS) of the plasma samples for the free analyte and other evaluations were conducted according to the AAPS/FDA Crytal III meeting of 2006 outcomes and the follow-up discussions to demonstrate the robustness of the current LC–MS/MS method.

3.2.1. Specificity, selectivity, sensitivity and carryover

Under the LC–MS/MS conditions, ASA404 was well separated from interferences in blank plasma ultrafiltrates. Analysis of six lots of the ultrafiltrate blanks showed no co-eluting endogenous peaks with the analyte and ISTD in the LC–MS/MS chromatograms (figure not shown). Analysis of six lots of ultrafiltrate blanks individually spiked with the ISTD showed no detectable peaks in the retention time region of the analyte (figure not shown).

The current assay has an analyte LLOQ of 30.0 ng/ml using a 30.0 μl volume of plasma ultrafiltrate. Reliable precision (CV 4.7%) and accuracy (bias 7.3%) was obtained from the analysis of six replicates of LLOQ samples (Table 1) along with two sets of calibration standards and six replicates of QCs at low (90.0 ng/ml), medium (2250 and 9000 ng/ml) and high (22,500 ng/ml) concentration levels in each of three validation runs. A representative LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 2. Injection of an extracted blank ultrafiltrate sample immediately after the ULOQ sample shows no carryover (figure not shown) of the LC–MS/MS method.

3.2.2. Matrix effect and recovery

The matrix effect was estimated by spiking neat solutions of the analyte (90.0, 2250 and 22,500 ng/ml, $n = 3$) into the extracted blank plasma ultrafiltrate samples and comparing their mean analyte peak areas with those from the corresponding neat solutions. The overall matrix effect ranged from 0.963 to 1.01 across the three concentration levels.

The recovery was assessed by comparing the mean analyte peak areas from the extracted plasma ultrafiltrate QC samples at concentrations of 90.0, 2250 and 22,500 ng/ml with those from the extracted blank plasma ultrafiltrate samples, to which the analyte was post-spiked at the same concentrations as above. The overall recovery was estimated at 80.8–95.0% across the three concentration levels.

3.2.3. Precision and accuracy

The accuracy and precision of the method were determined by analyzing six replicates of each plasma ultrafiltrate QC sample concentration level (90.0, 2250, 9000 and 22,500 ng/ml) along with two sets of each plasma ultrafiltrate calibration standard (30.0 to 30,000 ng/ml) for the analyte in each of the three validation runs. The accuracy of the method was obtained by calculating the bias (%) and the precision by calculating CV (%). Table 1 summarizes the accuracy and precision of the three validation runs for the analyte in human plasma ultrafiltrate with the bias(%) ranging from 1.9 to 6.2% and the CV(%) ranging from 4.2 and 5.9% over the concentration range evaluated.

3.2.4. Stability

The stability of the analyte in the reconstituted samples stored in the autosampler at 2–8 °C was assessed for 6 days after the initial analysis. The analyte was stable as demonstrated by the acceptable bias (%) that ranged from 2.7 to 6.0% for both the low (90.0 ng/ml) and high (22,500 ng/ml) plasma ultrafiltrate QC concentrations tested. The bench-top stability of analyte in the ultrafiltrate was evaluated at ambient temperature (~22 °C) over a 24-h period using plasma ultrafiltrate QC samples at 90.0 and 22,500 ng/ml. The measured concentration of analyte in these QC samples sitting at room temperature for 24 h was compared to the nominal values with bias (%) ranging from −8.3 to −5.3%, indicating that the analyte was stable for at least 24 h in ultrafiltrate when stored at ambient temperature. The freeze–thaw stability of plasma ultrafiltrate QC samples at 90.0 and 22,500 ng/ml concentration levels experiencing three cycles of freeze–thaw were analyzed together with one set of plasma ultrafiltrate calibration standards and regular QC samples. The %bias for these results ranged from −6.1 to −2.7%. The long term stability (LTS) of the analyte in the plasma ultrafiltrate QC samples stored at ≤−60 °C immediately following preparation and

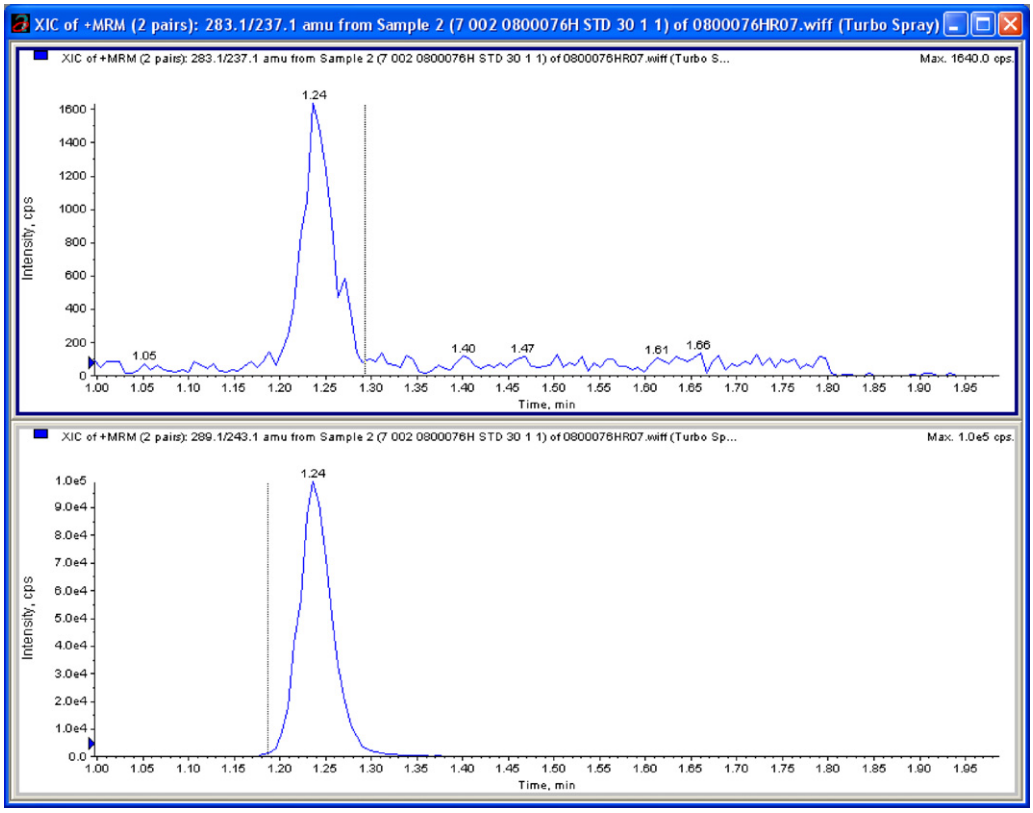

Fig. 2. Representative LC–MS/MS chromatogram of extracted human plasma ultrafiltrate LLOQ samples at a concentration of 30.0 ng/ml.

analyzed along with ultrafiltrate calibration standards and regular QCs showed that the analyte is stable in ultrafiltrate for at least 499 days when stored at ≤−60 °C.

Incurred sample stability (ISS) assessment was conducted for 21 randomly selected study plasma samples 183 days after the initial analysis for the unbound analyte. As per current industrial practice, the BLLOQ samples or samples with initially measured unbound analyte concentrations less than three times of LLOQ (30.0 ng/ml) were not selected for the assessment and 2/3 of the differences (%) between the initial and repeat results must be within ±30%. As shown in Table 2, all 21 ISS sample results met the acceptance criteria, demonstrating unaltered analyte plasma protein binding and/or unaltered physiochemical properties of proteins in the plasma samples after 183 days storage at ≤−60 °C.

3.2.5. *Incurred sample reanalysis*

As part of robustness evaluation of the assay method, incurred sample reanalysis (ISR) was conducted for 21 randomly selected clinical plasma samples within 4 weeks of the initial analysis. Again, the BLLOQ samples or samples with initially measured free analyte concentrations less than three times the LLOQ were not selected for evaluation. The ISR evaluation was considered acceptable if 2/3 of the differences (%) between the initial and repeat results were within ±30%. The results were summarized in Table 3 with the difference between the repeated and the initial data within ±30% for all 21 samples except for two samples with the difference at 31.7 and 31.5%.

4. Application to clinical studies

The current method has been successfully applied to the determination of plasma ultrafiltrate ASA404 concentrations in pharmacokinetic studies, where ASA404 at a dose of 1800 mg/m^2 was administered as a 20-min infusion. A representative mean plasma unbound analyte concentration versus time profile for 11 patients randomly selected from several hundreds of treated patients is shown in Fig. 3. The overall plasma unbound analyte concentration is about 2–8% of the total analyte plasma concen-

Table 2
Summary of incurred plasma sample stability (ISS) for ASA404 in the ultrafiltrate from the plasma samples after 183 days of storage at ≤−60 °C.

First determination (ng/ml)	Repeat determination (ng/ml)	Normalized difference (%)	First determination (ng/ml)	Repeat determination (ng/ml)	Normalized difference (%)
26,000	26,200	0.8	12,100	14,200	17.4
15,400	15,700	1.9	14,000	15,900	13.6
12,100	12,200	0.8	14,900	17,100	14.8
7440	8180	9.9	18,300	17,500	−4.4
3640	3570	−1.9	7520	7960	5.9
20,700	18,800	−9.2	18,500	15,300	−17.3
10,600	9830	−7.3	8900	6910	−22.4
7510	6600	−12.0	19,700	18,200	−7.6
3850	3430	−10.9	6620	6660	0.6
1650	1860	12.7	21,200	19,600	−7.5
14,600	13,100	−10.3			

Table 3
Summary of incurred plasma sample reanalysis (ISR) for ASA404 in the plasma ultrafiltrate.

First determination (ng/ml)	Repeat determination (ng/ml)	Normalized difference (%)	First determination (ng/ml)	Repeat determination (ng/ml)	Normalized difference (%)
17,000	16,200	−4.7	7670	8120	5.9
14,200	12,000	−15.5	4110	5060	23.1
9740	10,000	2.7	1610	2090	29.8
5250	5320	1.3	934	1230	31.7[a]
1620	1690	4.3	18,400	18,300	−0.5
906	1050	15.9	20,700	20,900	1.0
13,800	15,100	9.4	13,000	17,100	31.5[a]
10,300	11,700	13.6	6410	8330	30.0
7930	8810	11.1	2050	2530	23.4
8630	9620	11.5	934	1100	17.8
9850	11,700	18.8			

[a] An ISR result that did not meet the acceptance criteria.

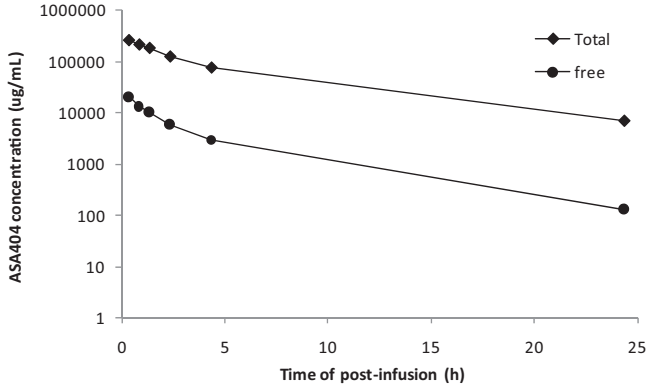

Fig. 3. Mean concentration–time profiles of the total (♦) and free (•) ASA404 measured from the plasma samples collected from randomly selected 11 patients administered with ASA404 at a dose of 1800 mg/m^2 via a 20-min IV infusion.

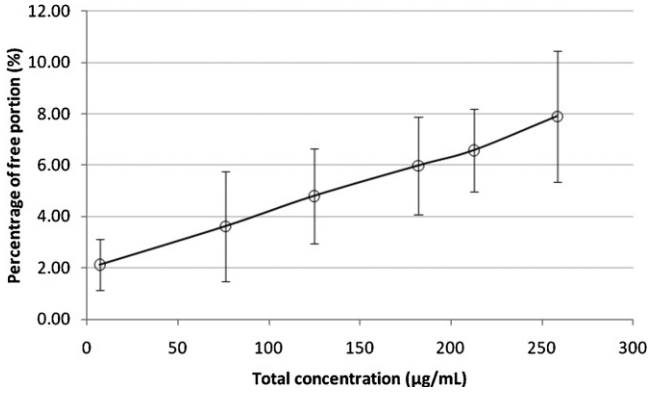

Fig. 4. Percentage ± SD (%) of the free ASA404 in the total ASA404 concentrations measured from the plasma samples collected from randomly selected 11 patients administered with ASA404 at a dose of 1800 mg/m^2 via a 20-min IV infusion.

tration with a clear trend toward a higher unbound fraction with rising total analyte plasma concentration (Fig. 4). This observation is in general agreement with the *in vitro* protein binding results previously reported [15].

5. Conclusion

A robust LC–MS/MS method was developed and validated for quantitative analysis of ASA404 in human plasma ultrafiltrate in support of clinical studies. The validated assay method is rugged as demonstrated by excellent intra- and inter-day precision and accuracy for the results of plasma ultrafiltrate QC samples during validation and very good incurred sample reanalysis (ISR) and incurred sample stability (ISS) results during study sample analysis. The evaluations conducted here can be extended to quantitative analysis of other small molecule drug candidates in plasma ultrafiltrate.

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