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Determination of a novel thrombin receptor antagonist (SCH 530348) in human plasma: Evaluation of Ultra Performance Liquid Chromatography™–tandem mass spectrometry for routine bioanalytical analysis

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ABSTRACT

SCH 530348 is a safe and effective oral anti-platelet agent for patients with acute coronary syndrome. Clinical study results suggest that SCH 530348 dosage at 20 mg or 40 mg is feasible to achieve rapid maximum platelet inhibition following an acute coronary event or intervention procedure. To permit accurate determinations of circulating SCH 530348 in plasma following dosing, a method for measuring SCH 530348 concentrations in human plasma was validated using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The method utilized semi-automated 96-well protein precipitation with gradient chromatography using an ACQUITY™ UPLC BEH C_{18} (2.1 mm × 50 mm, 1.7 μm) column. The retention time of SCH 530348 was approximately 1.5 min. This method was validated for routine quantitation of SCH 530348 over the concentration range of 1.00–1000 ng/mL. Inter-run accuracy based on mean percent theoretical for replicate quality control samples was better than 95.2%. Inter-run precision based on percent relative deviation for replicate quality control samples was ≤3.3%. SCH 530348 quality control samples were stable in human plasma for up to three freeze/thaw cycles, for at least 467 days when frozen at −20 °C and for at least 7 h when stored at room temperature. The lower limit of quantitation was 1.00 ng/mL for a 100 μL plasma aliquot.

1. Introduction

Thrombosis can result in partial or complete blockage of the vasculature and is the underlying mechanism of acute coronary syndromes (ACS), including myocardial infarction and ischemic stroke. These acute vascular events are a leading cause of hospitalization and death in the developed world [1,2].

Thrombin is an integral protein in blood coagulation and promotes platelet activation by binding to the protease-activated receptor subtype 1, also known as PAR-1 or the thrombin receptor, thus inducing blood clotting [1,3]. Since platelets are activated at the site of atherosclerotic plaque rupture in arteries and release substances that initiate aggregation and clot formation, development of novel anti-thrombotic agents for patients with cardiovascular disorders has been challenging [1,4,5]. While much progress has been made in this area, patients often suffer from hemorrhagic side effects when taking standard anti-thrombotic therapies. Therefore, innovative agents that safely and effectively inhibit thrombin continue to be an unmet medical need [6–8].

Schering-Plough Research Institute (part of Merck Research Laboratories) has developed a novel orally active anti-thrombotic agent, SCH 530348 (herein referred to as vorapaxar), which is selective toward the thrombin receptor to reduce thrombin-induced platelet activation. Vorapaxar has a complex structure with seven stereogenic centers and is chemically referred to as ethyl[3aR,4aR,8aR,9aS)-9(S)-[(E)-2-[5-(3-fluorophenyl)-2-pyridinyl]ethenyl]dodecahydro-1(R)-methyl-3-oxonaphtho[2,3-c]furan-6(R)-YL]carbamate (Fig. 1).

In order to support the various clinical trials required for drug registration, a method for monitoring plasma levels of vorapaxar in patients was needed. In order to provide adequate assessment of vorapaxar pharmacokinetics in humans, the anticipated limit of quantitation required was 1.00 ng/mL. Therefore, the use of liquid chromatography coupled with tandem mass spectrometric detection (LC–MS/MS) would afford the best opportunity for successfully developing a robust quantitative method [9–11]. More recently, techniques such as Ultra Performance Liquid Chromatography™ (UPLC) that utilize sub-2 micron particles have offered substantial improvements in performance compared to conventional liquid chromatographic techniques. While the use of sub-2 micron particles requires the use of LC systems that can operate at

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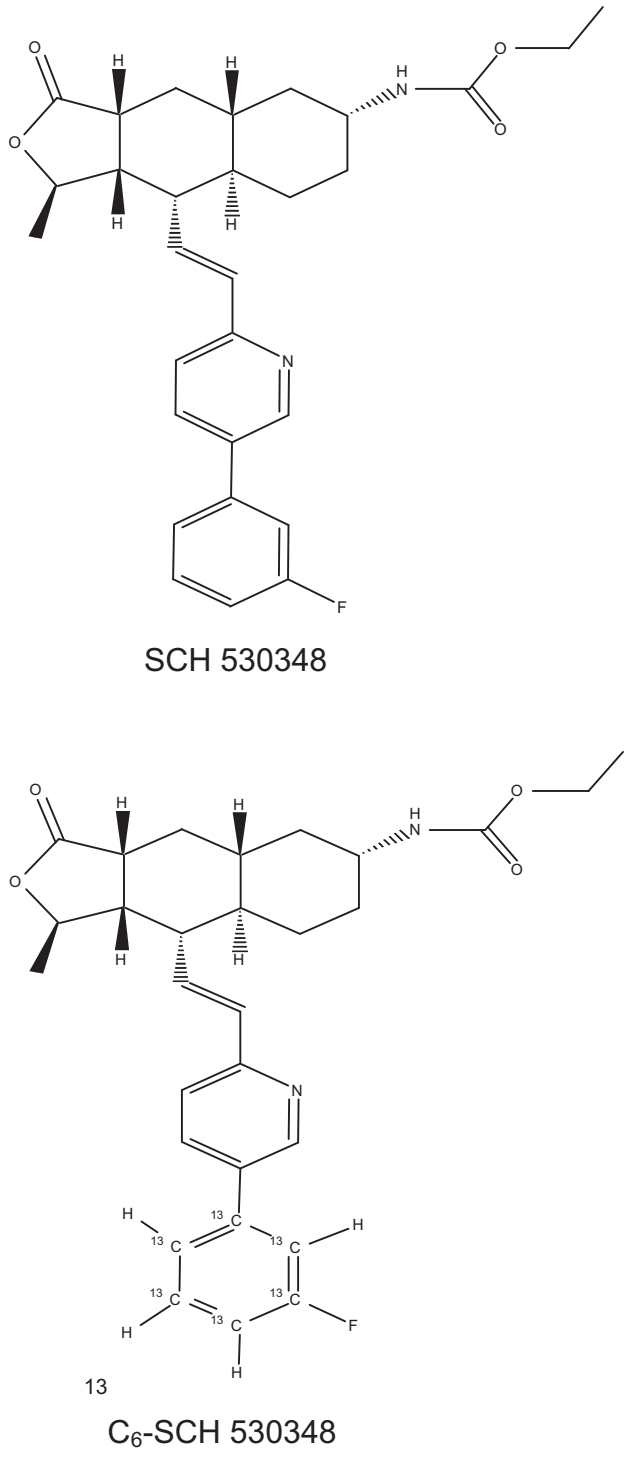

Fig. 1. Chemical structure of SCH 530348 and its IS, $^{13}C_6$-SCH 530348.

high back-pressures, the benefits of resolution, speed and sensitivity, particularly when coupled with mass spectrometers, are readily apparent for high-throughput bioanalytical applications [12].

In this article, we present a 96-well sample preparation and UPLC–MS/MS method that has been utilized to support a number of clinical studies requiring quantitation of SCH 530348. In addition to the significant gain in sensitivity and reduction in cycle time achieved by using UPLC, the method is sufficiently robust to support ongoing and future large scale clinical trials.

Table 1
Acquity™ UPLC gradient program.

Time (min)	% Mobile phase B	% Mobile phase A	Flow rate (mL/min)	Curve
Initial	40	60	0.500	Initial
2.00	98	2	0.500	6
3.00	98	2	0.500	6
3.01	40	60	0.500	6

2. Experimental methods

2.1. Reference materials

SCH 530348 (vorapaxar) and its internal standard (IS), $^{13}C_6$-SCH 530348 (Fig. 1) were synthesized at Schering-Plough Research Institute (Kenilworth, NJ, USA) with purities of 99.9% and 99.2%, respectively.

2.2. Biological matrix

Human plasma with dipotassium ethylenediaminetetraacetic acid (K_2 EDTA) as the anticoagulant was obtained from Bioreclamation Inc. (Westbury, NY, USA). The blank matrix was used as received for the preparation of calibration standards (STD) and quality control (QC) samples without further treatment.

2.3. Reagents

Optima-grade methanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA) ACS grade acetic acid was obtained from Sigma–Aldrich (St. Louis, MO, USA) Extra dry dimethyl sulfoxide with molecular sieves was obtained from Acros Organics (Morris Plains, NJ, USA) Ultra-pure type 1 water was generated by using a Millipore Milli-Q® water system (Bedford, MA, USA) All other chemical reagents used were reagent grade or higher and used without further purification.

2.4. Liquid chromatography–tandem mass spectrometry

The Waters ACQUITY™ Ultra Performance LC system (Waters, Milford, MA, USA) consisted of a binary solvent manager, a sample manager and accompanying 96-well plate sample organizer. The detector was an Applied Biosystems (MDS Sciex, Concord, Ontario, Canada) API 4000™ triple quadrupole mass spectrometer. The column was a Waters ACQUITY™ UPLC (Waters, Milford, MA, USA) BEH C_{18} 2.1 mm × 50 mm, 1.7 μm particle size. A gradient elution program (Table 1) was used to separate vorapaxar and its internal standard from the bulk of the endogenous matrix components. Mobile phase A consisted of water:acetic acid (100:0.1, v:v) solution and mobile phase B consisted of acetonitrile:methanol:acetic acid (70:30:0.1, v:v:v). The retention time for both vorapaxar and its stable-labeled IS was approximately 1.5 min. The sample injection volume was 10-μL. The cycle time of the method was 3.0 min per injection.

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization was by TurboIonSpray™ in the positive ion mode. The ion spray voltage was set to 4500 V. The declustering potential was set to 96 V, and the collision energy was set to 35 eV. The probe temperature was set to 450 °C. The collision gas was set to maintain a pressure of approximately 3.5×10^{-5} Torr. To assay the analyte and IS, the mass spectrometer was operated at unit mass resolution and set to select the MRM transitions for the $[M+H]^+$ ions of m/z 493 → m/z 447 for SCH 530348 and m/z 499 → m/z 453 for the IS. The dwell time for the analyte and IS MRM transitions was 50 ms.

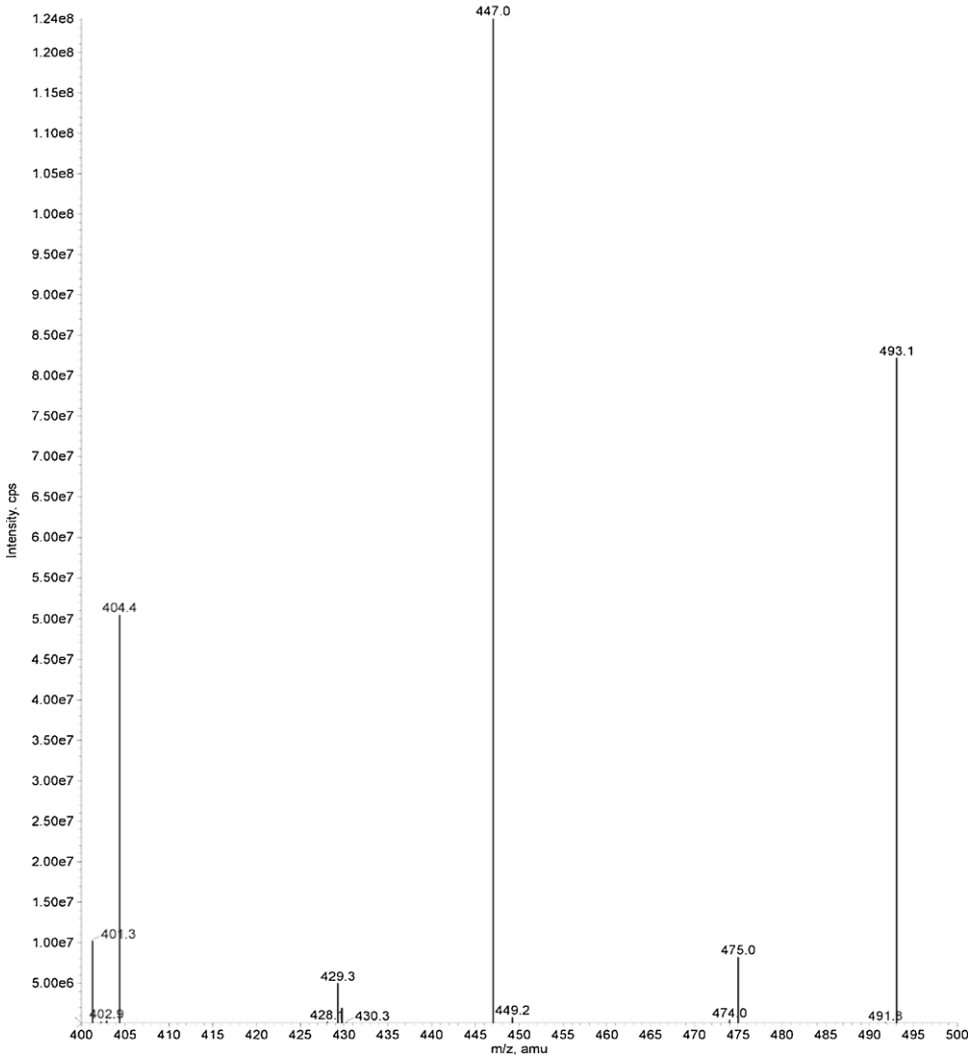

Fig. 2. Product ion mass scan of vorapaxar.

MRM data were acquired and integrated by using the Sciex Analyst™ software package (version 1.3.1). Data regression was performed by using the Watson™ (Thermo LabSystems, Philadelphia, PA, USA) drug metabolism laboratory information management system (version 6.4.0.03).

2.5. Sample preparation

Fresh stock solutions of vorapaxar and $^{13}C_6$-SCH 530348, along with the vorapaxar intermediate solution, were prepared in dimethyl sulfoxide at concentrations of 1.0 mg/mL for each stock, respectively, and 10.0 μg/mL for the intermediate. IS working solution was prepared in acetonitrile:acetone (95:5, v:v) at a concentration of 40.0 ng/mL. Ten vorapaxar calibration standards ranging in concentration from 1.00 ng/mL to 1000 ng/mL were prepared in blank human K_2 EDTA plasma by serial dilution at nominal concentrations of 1.00, 2.00, 4.00, 10.0, 20.0, 50.0, 100, 400, 850 and 1000 ng/mL. Four levels of vorapaxar quality control samples were prepared in blank human K_2 EDTA plasma by serial dilution at nominal concentrations of 1.00 ng/mL (lower limit of quantitation; LLOQ), 3.00 ng/mL [QC of low concentration (QCL)], 80.0 ng/mL [QC of medium concentration (QCM)] and 800 ng/mL [QC of high concentration (QCH)]. All samples were stored frozen at −20 °C until use.

On the day of analysis, a 100-μL aliquot of the appropriate sample was pipetted into a 96-well format dilution tube. A 500-μL aliquot of IS working solution was added to all samples except matrix blanks, to which a 500-μL aliquot of acetonitrile:acetone (95:5, v:v) was added. Samples were capped and mixed on a multi-tube vortexer, then refrigerated for approximately 10 min to accelerate precipitation of the plasma proteins. The samples were then centrifuged at 4000 rpm in an Eppendorf 5810 R centrifuge (Brinkman Instruments, Westbury, NY, USA) for 10 min at a temperature setting of 5 °C. By using a Tomtec (Tomtec Corp., Hamden, CT, USA) Quadra96® liquid handling system, 200 μL of the supernatant from each sample was transferred into a 2-mL 96-well collection plate, from which a 10-μL aliquot was injected for analysis.

2.6. Study design

Method validation was conducted in accordance with the FDA Guidance for Industry for the validation of bioanalytical methods [13]. Three analytical core runs were performed to establish

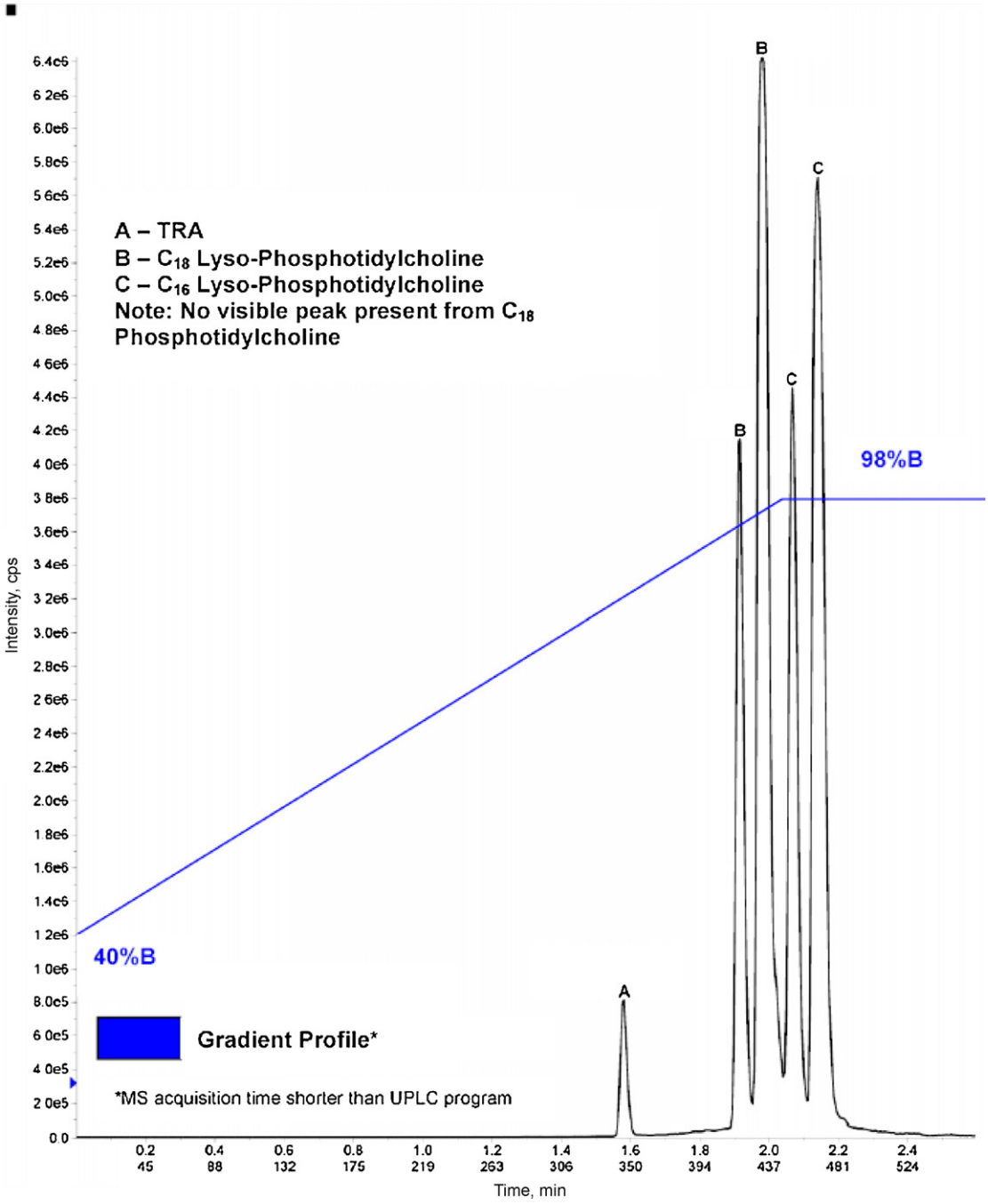

Fig. 3. Separation of vorapaxar and its IS, $^{13}C_6$-SCH 530348, from 3 common phosphotidylcholines.

ruggedness, reproducibility and sensitivity. Each of the three analytical core runs included calibration standards prepared at ten concentrations ($n = 2$ at each concentration) and quality control (QC) samples prepared at four concentrations ($n = 6$ at each concentration). Each run also contained two zero standards (blank human plasma samples with IS) and three control blanks (blank human plasma samples without IS). In order for each core run to be considered acceptable, at least three-quarters of the calibration standards must have individual accuracy within ±15% of the nominal value (±20% at the LLOQ). For each of the three core analytical runs, at least one of the two calibration standards at both the LLOQ and upper level of quantitation (ULOQ) must meet this criterion. If a calibration standard does not have accuracy within ±15% of the nominal value (±20% at the LLOQ), it is omitted from the calibration curve regression. For each validation run, the coefficient of determination (r^2) of the calibration curve must be ≥0.98. For each core validation run, at least two thirds of the QC samples must have individual accuracy within ±15% (±20% at the LLOQ) and at least 50% of each level should meet this criterion. The intra-run (within-run) and inter-run (between-run) precision of these runs should be ≤15% (≤20% at the LLOQ).

Selectivity of the method was assessed by screening six sources of blank plasma for interference at the retention time of vorapaxar and $^{13}C_6$-SCH 530348. The selectivity of the method was also assessed to ensure that there was no interference between

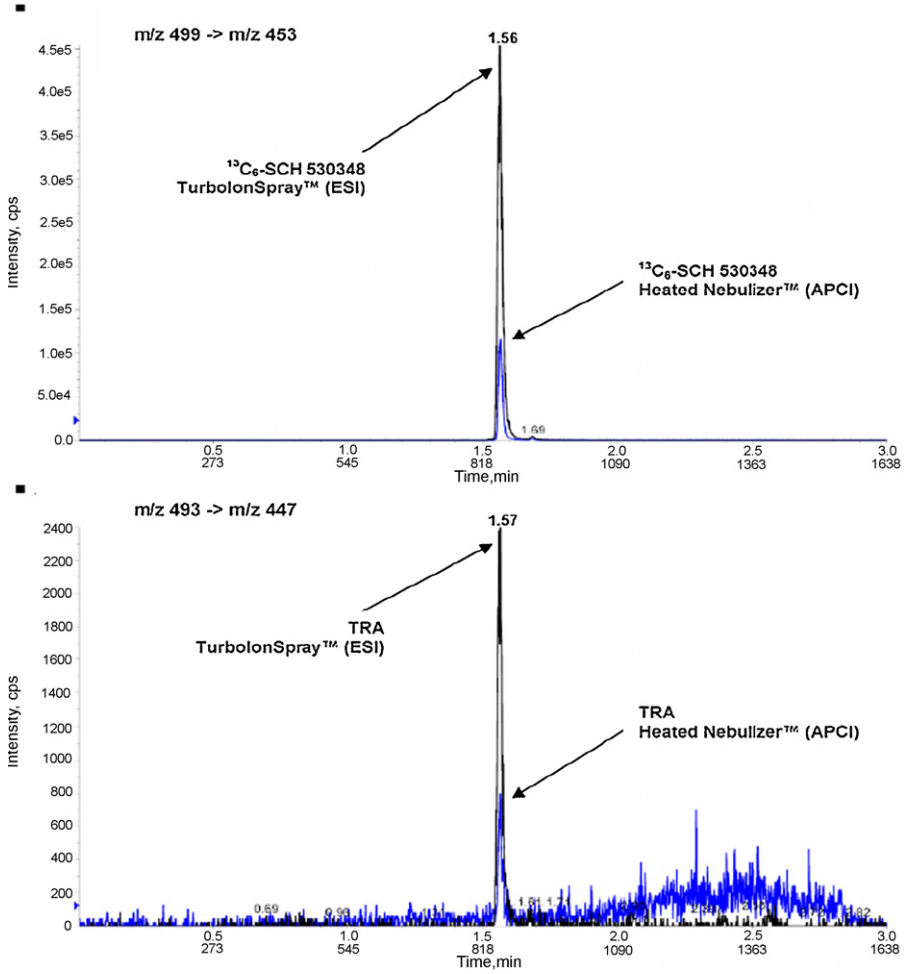

Fig. 4. Comparison between TurboIonSpray™ and Heated Nebulizer™ (APCI) ionization modes following an injection of a 10-μL aliquot of the supernatant extracts at the LLOQ (1.00 ng/mL) of vorapaxar (bottom trace) and its IS, $^{13}C_6$-SCH 530348 (top trace), under described ACQUITY™ UPLC gradient condition.

the analyte and IS. To be acceptable, the response at the expected retention time of vorapaxar must be less than 20% of the mean peak response calculated from the analysis of the LLOQ QC samples. The response at the expected retention time of $^{13}C_6$-SCH 530348 must be less than 5% of the mean peak response of the IS in the LLOQ QC samples.

Integrity of dilution was assessed with six replicates of QC samples prepared at a concentration of 5000 ng/mL and subsequently diluted 10-fold with blank human plasma prior to analysis. To be acceptable, the mean accuracy of the six replicates should be within ±15%, and 50% of the dilution QC samples should have individual accuracy within ±15%.

The stability of vorapaxar was assessed by analyzing QC samples at low and high concentrations ($n \leq 4$ at each concentration) that were stored under various conditions. To be acceptable the mean accuracy and precision at each level should be within ±15% of the nominal value. The stability of vorapaxar in stock solutions during long-term storage at refrigerated and at room temperature was also assessed. Vorapaxar stock solutions were considered stable if the mean difference of the peak response of the evaluated stock solution was ≤5% from the mean peak response of a freshly prepared vorapaxar stock solution.

The recovery for vorapaxar and $^{13}C_6$-SCH 530348 were assessed by comparing the mean peak responses from extracted QC samples to the mean peak responses from neat samples.

3. Results and discussion

3.1. Mass spectrometry

The positive ion TurboIonSpray™ mass spectrum of vorapaxar is dominated by the protonated molecular ion of *m*/*z* 493. Following collisional activation, the protonated molecular ion of vorapaxar gave rise to a predominant product ion of *m*/*z* 447 (Fig. 2). The internal standard, $^{13}C_6$-SCH 530348, displayed similar fragmentation behavior, and the MRM transition *m*/*z* 499 → *m*/*z* 453 was selected for detection.

3.2. Sample extraction optimization

Protein precipitation is typically the least suited extraction technique for clinical studies because of its susceptibility to ion suppression and matrix effects [14]. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are techniques that offer much cleaner sample extracts, that in turn, may give rise to more robust methods. Indeed, to support the initial clinical safety and tolerance studies a SPE method was developed and validated for a dynamic range of 0.100–50.0 ng/mL. Unfortunately, the method suffered the drawbacks of requiring a large (200 μL) sample aliquot along with the complexity of performing SPE for large sample batch sizes. The opportunity to evaluate a different approach presented itself when,

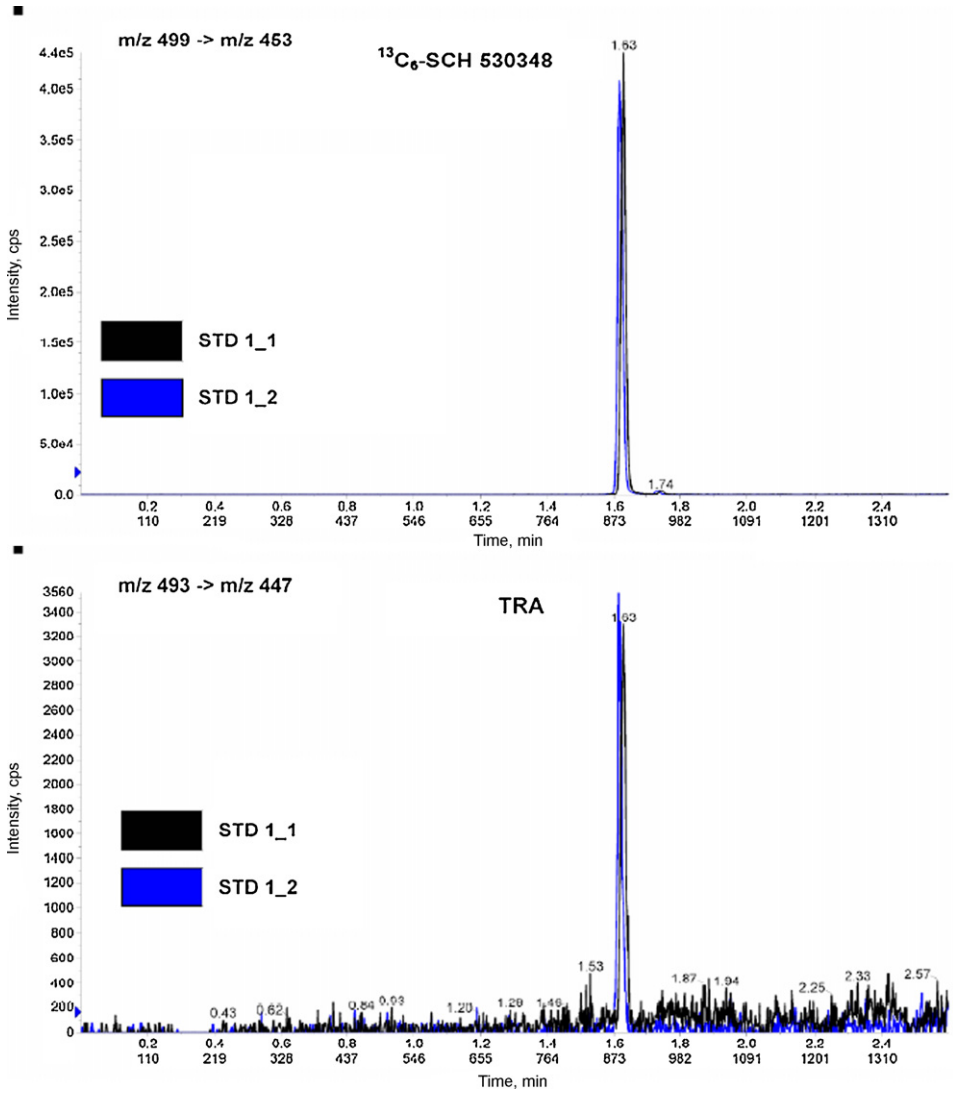

Fig. 5. Overlay of vorapaxar STD1 chromatograms 270 injections apart in a clinical study analytical run.

in subsequent clinical trials with optimized doses, sufficient samples were projected to exceed the dynamic range of the existing method to warrant validation of a new dynamic range. Moreover, these future clinical trials were anticipated to generate substantial increases in sample numbers than was previously supported. In order to meet the future demands for fast-turnaround of data, protein precipitation was ideal for its simplicity and high capacity. The final method used a 100-μL sample aliquot that was diluted with 500-μL of spike of Internal Standard in acetonitrile/acetone (95:5, v:v). Following centrifugation, a 200 μL aliquot of the supernatant was transferred to individual wells of a 96-well plate for analysis.

3.3. Chromatography

To provide further increases in productivity, the Waters ACQUITY™ UPLC was selected as the platform to perform the chromatographic separation of vorapaxar and its IS because of its ability to work more efficiently with higher speed, sensitivity and resolution. Additionally, because of the coarse nature of protein precipitation sample preparation, the quality of the results would improve using the ACQUITY™ UPLC in conjunction with the appropriate ionization mode to ensure that no residual endogenous interferences would potentially interfere with the selectivity of the assay.

A gradient profile was constructed to maintain a high organic concentration following the elution of the analyte and IS to ensure complete elution of any non-polar endogenous material. Elution of vorapaxar and its IS occurred at ~1.5 min, during the gradient ramp from 40% mobile phase B to 98% mobile phase B. At 2.0 min, the 98% mobile phase B composition was held for 1.0 min before rapidly returning to initial conditions. The 1.0 min wash at 98% organic was sufficient to elute endogenous lipids. Fig. 3 illustrates the separation of vorapaxar and its IS from the three most commonly occurring phosphatidylcholine esters present in human plasma.

While the original method validation of vorapaxar at the lower range used TurboIonSpray™ ionization, the Heated Nebulizer™ (APCI) interface was evaluated on the basis that it is less susceptible to ion suppression [15] and results were compared to those of TurboIonSpray™. Fig. 4 represents a comparison between the two ionization modes following injection of a 10-μL aliquot of the supernatant extract at the LLOQ. It illustrates that TurboIonSpray™ ionization is optimal for the detection of vorapaxar in human plasma.

The initial chromatographic system utilized a Varian (formerly MetaChem) MonoChrom MS 2.0 mm × 50 mm, 5 μm HPLC column that was adequate for small sample batch sizes but demonstrated variability in retention time and peak shape for larger sample batch sizes. Sub-2 micron particle chemistries afford the potential of substantial gains in efficiency and several sub-2 micron columns were evaluated. The Waters ACQUITY™ BEH C_{18} 2.1 mm × 50 mm, 1.7 μm column provided the best results for a vorapaxar high-throughput method and imparted the appropriate robustness and reproducibility to support clinical sample batch sizes of 200–300 samples.

An example of column performance can be seen in Fig. 5, which shows an overlay of the 1.00 ng/mL vorapaxar STD 1 chromatograms. The first replicate LLOQ is essentially identical to a second replicate LLOQ assayed after 270 injections of calibration standards, quality control samples, blanks and clinical study samples. While some columns cannot withstand more than a few hundred injections from human extracts via protein precipitation, the BEH C_{18} 2.1 mm × 50 mm, 1.7 μm displayed remarkable column longevity of >2500 injections for a 1900 sample clinical study and >1600 injections for a 2000 sample clinical study.

To further demonstrate the column's superior sensitivity, Fig. 6 represents a comparison between the low-range method HPLC chromatography and this method's ACQUITY™ UPLC chromatography at 0.100 ng/mL vorapaxar. While the left panel illustrates an on-column injection of 0.356 pg using the initial method, the right panel depicts a 0.287 pg on-column injection using the current method. This comparison clearly illustrates the advantage of using sub-2 micron chromatography; *viz*., about 80% amount on-column achieved more than 5 times the sensitivity.

3.4. Linearity

The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 1.00–1000 ng/mL. The calibration curves appeared quadratic and were well described by least-squares regression lines. A weighting factor of 1/concentration^2 was chosen to achieve homogeneity of variance. The slopes, intercepts, and coefficients of determination (r^2) from the validation core runs are presented in Table 2.

3.5. Accuracy and precision

For calibration standards, the inter-run precision and accuracy results from the 3 analytical core runs are listed in Table 2. The inter-run accuracy (%DIFF) ranged from −6.3% to 3.0%. The inter-run precision (%CV) ranged from 2.0% to 8.8%.

For QC samples, the intra-run precision and accuracy from each of the 3 analytical core runs, as well as, the inter-run precision and accuracy results from 3 analytical core runs are summarized in Table 3. The inter-run accuracy was better than 5.7% (−14.8% at the LLOQ). The inter-run precision (%CV) was better than 8.9% (12.7% at the LLOQ). The variance (ANOVA) of the intra-run and inter-run precision is presented in Table 4.

3.6. Selectivity and matrix effect

Blank plasma samples from 6 sources were screened and were found to be free of interference from endogenous components or other sources at the same mass transitions and retention times as vorapaxar and its IS. Fig. 7 shows typical MRM chromatograms for an extracted blank human plasma sample of vorapaxar (bottom trace) and its IS, $^{13}C_6$-SCH 530348 (top trace).

Table 2
Back-calculated vorapaxar concentrations in human plasma, calibration standards and calibration curve parameters.[a]

Conc. (ng/mL)	STD1 1.00 ng/mL	STD2 2.00 ng/mL	STD3 4.00 ng/mL	STD4 10.0 ng/mL	STD5 20.0 ng/mL	STD6 50.0 ng/mL	STD7 100 ng/mL	STD8 400 ng/mL	STD9 850 ng/mL	STD10 1000 ng/mL	A	B	C	r^2
Core run 1	1.02	1.85	4.25	11.2	21.5	53.4	100	369	873	1030	−4.14E−07	0.005	0.00147	0.995
	1.01	1.94	3.94	9.13	21	49.9	88.9	367	834	1040				
Core run 2	1.02	2.06	3.79	9.4	20.3	51.1	105	379	832	1020	−3.45E−07	0.0049	0.0008	0.998
	0.967	2.02	3.97	10.6	21.4	48.2	97	371	911	984				
Core run 3	0.911	2.05	4.03	9.79	22.4	50.8	105	380	856	999	−8.22E−08	0.0046	0.00154	0.994
	1.09	1.92	3.95	10.5	17.2	44.1	111	386	876	992				
n	6	6	6	6	6	6	6	6	6	6	3	3	3	3
Overall mean	1	1.97	3.99	10.1	20.6	49.6	101	375	864	1010	−0.00000028	0.0048	0.00127	0.995
S.D.	0.0599	0.0833	0.151	0.793	1.82	3.18	7.68	7.45	29.7	22.4	0.000000175	0.0002	0.00041	0.002
%CV	6	4.2	3.8	7.9	8.8	6.4	7.6	2	3.4	2.2	−62.5	3.7	32.1	0.2
%DIFF	0	-1.5	-0.3	1	3	-0.8	1	-6.3	1.6	1				

%CV indicates %coefficient of variation (inter-run precision); %DIFF indicates %difference (inter-run accuracy); S.D. indicates standard deviation.
[a] $y = Ax^2 + Bx + C$, weighted 1/concentration^2, where y is the peak area ration of SCH 530348 to IS, x is the concentration of SCH 530348 and A–C are calibration curve parameters.

Table 3
Analytical performance of vorapaxar quality control samples in human plasma.

Run Parameter	Core Run	LLOQ 1.00 ng/mL	QCL 3.00 ng/mL	QCM 80.0 ng/mL	QCH 800 ng/mL
	1	0.727[a]	2.32[b]	73.4	736
		0.892	2.83	93.7[b]	793
		0.827	2.99	74.1	783
		0.963	2.71	86.9	784
		0.846	3.08	86.7	791
		0.813	2.68	83.4	808
Intra-run mean		0.845	2.77	83	783
Intra-run S.D.		0.0793	0.269	7.94	24.5
Intra-run %CV		9.4	9.7	9.6	3.1
Intra-run %DIFF		−15.5	−7.7	3.8	−2.1
n		6	6	6	6
	2	0.875	2.94	74	723
		0.883	2.8	85.6	782
		1.05	3.08	80.2	774
		0.828	2.92	79.3	795
		0.905	2.91	91.7	824
		1.03	3.23	82.1	814
Intra-run mean		0.929	2.98	82.2	785
Intra-run S.D.		0.0902	0.152	6.02	35.9
Intra-run %CV		9.7	5.1	7.3	4.6
Intra-run %DIFF		−7.1	−0.7	2.8	−1.9
n		6	6	6	6
	3	0.839	2.22[b]	86.3	727
		0.854	2.66	91.6	817
		0.785[a]	2.87	84.4	904
		0.885	3.01	81.4	815
		0.757[a]	2.94	85.1	712
		0.576[a]	2.73	73.1	795
Intra-run mean		0.783	2.74	83.7	795
Intra-run S.D.		0.111	0.285	6.15	69.7
Intra-run %CV		14.2	10.4	7.3	8.8
Intra-run %DIFF		−21.7	−8.7	4.6	−0.6
n		6	6	6	6
Mean concentration found (ng/mL)		0.852	2.83	82.9	788
Inter-run S.D.		0.108	0.253	6.38	44.9
Inter-run %CV		12.7	8.9	7.7	5.7
Inter-run %DIFF		−14.8	−5.7	3.6	−1.5
n		18	18	18	18

LLOQ indication the lower limit of quantitation; %CV indicates %coefficient of variation (inter-run precision); %DIFF indicates %difference (inter-run accuracy); QCL indicates quality control of low concentration; QCM indicates quality control of medium concentration; QCH indicates quality control of high concentration; S.D. indicates standard deviation.

[a] Beyond ±20% limit from nominal concentration and thus not included in calculation.
[b] Beyond ±15% limit from nominal concentration and thus not included in calculation.

The method is also sufficiently selective between analyte and IS.

The method also proved to be free of suppression or enhancement of vorapaxar from the presence of endogenous matrix components when evaluated at the QCL concentration prepared from 6 different sources ($n = 3$ replicates). Data for the matrix effect evaluation of vorapaxar are presented in Table 5.

3.7. Integrity of dilution

Integrity of dilution was acceptable using dilution quality control (QCD) samples prepared at a concentration of 5000 ng/mL and diluted 10-fold ($n = 6$ replicates). Data for vorapaxar integrity of dilution are presented in Table 6.

Table 4
Vorapaxar quality control ANOVA summary.

Nominal concentration	LLOQ 1.00 ng/mL	QCL 3.00 ng/mL	QCM 80.0 ng/mL	QCH 800 ng/mL
Mean observed conc.	0.966	2.97	79.4	800
%DIFF	−3.4	−1	−0.8	0
Between run precision (%CV)	12.2	5.6	7.5	3.3
Within run precision (%CV)	10.2	8.1	7.4	7.1
Total variation (%CV)	15.9	9.9	10.5	7.8
n	48	48	48	48
Number of runs	8	8	8	8

%CV indicates %coefficient of variation; %DIFF indicates %difference (inter-run accuracy); QCL indicates quality control of low concentration; QCM indicates quality control of medium concentration; QCH indicates quality control of high concentration.

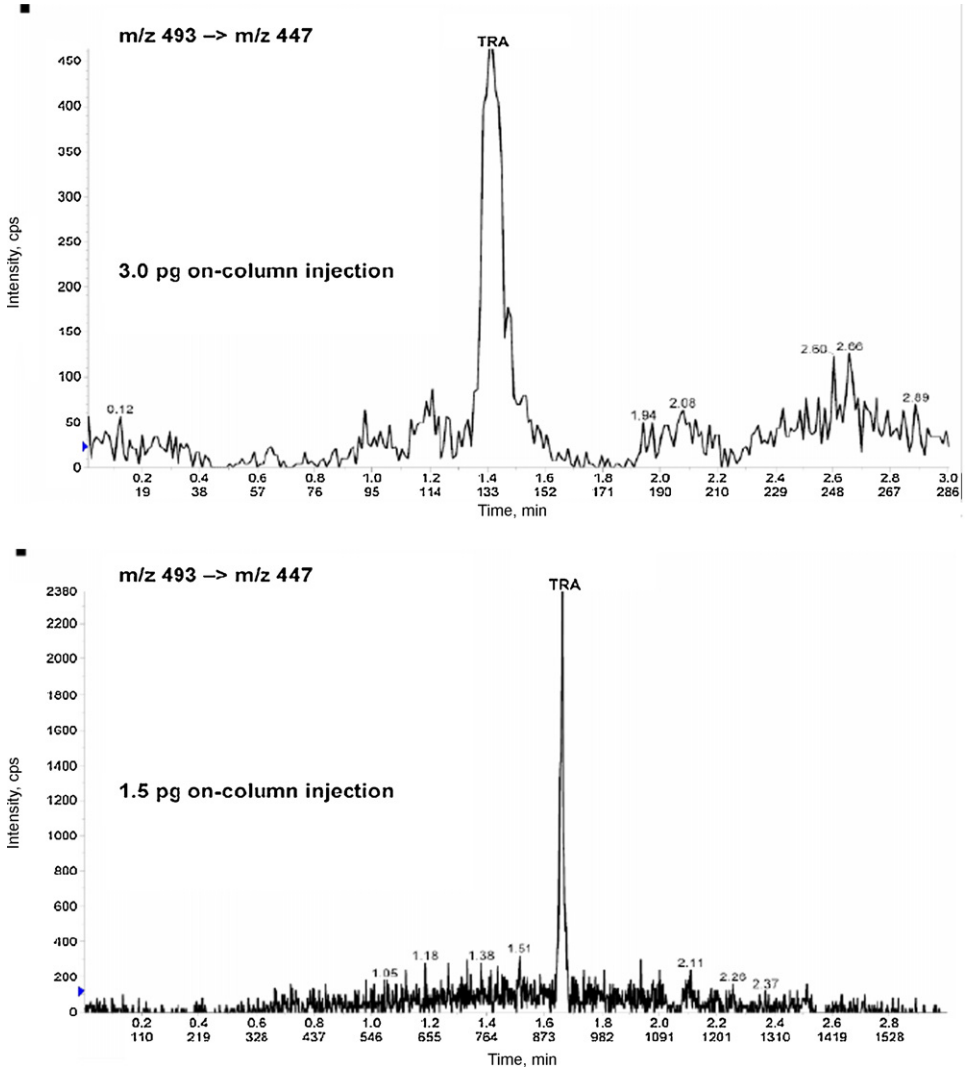

Fig. 6. Comparison between low-range method HPLC chromatography and current method UPLC chromatography at 0.100 ng/mL vorapaxar.

3.8. Stability

The stability of vorapaxar in human plasma was acceptable under various storage conditions. A summary of these stability experiments is reported in Table 7. Vorapaxar is stable in DMSO for at least 428 days when refrigerated and for at least 120 h at room temperature stability.

3.9. Recovery

Recovery of vorapaxar was determined by comparing the peak area responses of extracted QC samples ($n = 6$ replicates) to the peak area responses of neat solutions at the same concentrations. Mean absolute recovery was better than 95.7% (see Table 8).

Table 5
Matrix effect evaluation for vorapaxar in human plasma.

	QCL 1 3.00 ng/mL	QCL 2 3.00 ng/mL	QCL 3 3.00 ng/mL	QCL 4 3.00 ng/mL	QCL 5 3.00 ng/mL	QCL 6 3.00 ng/mL
	2.76	2.68	2.97	2.95	2.77	3.06
	2.93	2.83	2.85	2.84	2.85	3.06
	2.61	2.98	3.1	2.74	2.81	2.82
Mean	2.77	2.83	2.97	2.84	2.81	2.98
S.D.	0.16	0.15	0.125	0.105	0.04	0.139
%CV	5.8	5.3	4.2	3.7	1.4	4.7
%Theoretical	92.3	94.3	99	94.7	93.7	99.3
%DIFF	−7.7	−5.7	−1	−5.3	−6.3	−0.7
n	3	3	3	3	3	3

QCL 1–6 indicate quality control of low concentration in six individual lots of human plasma; %CV indicates %coefficient of variation (intra-run precision); %DIFF indicates %difference (intra-run accuracy); S.D. indicates standard deviation.

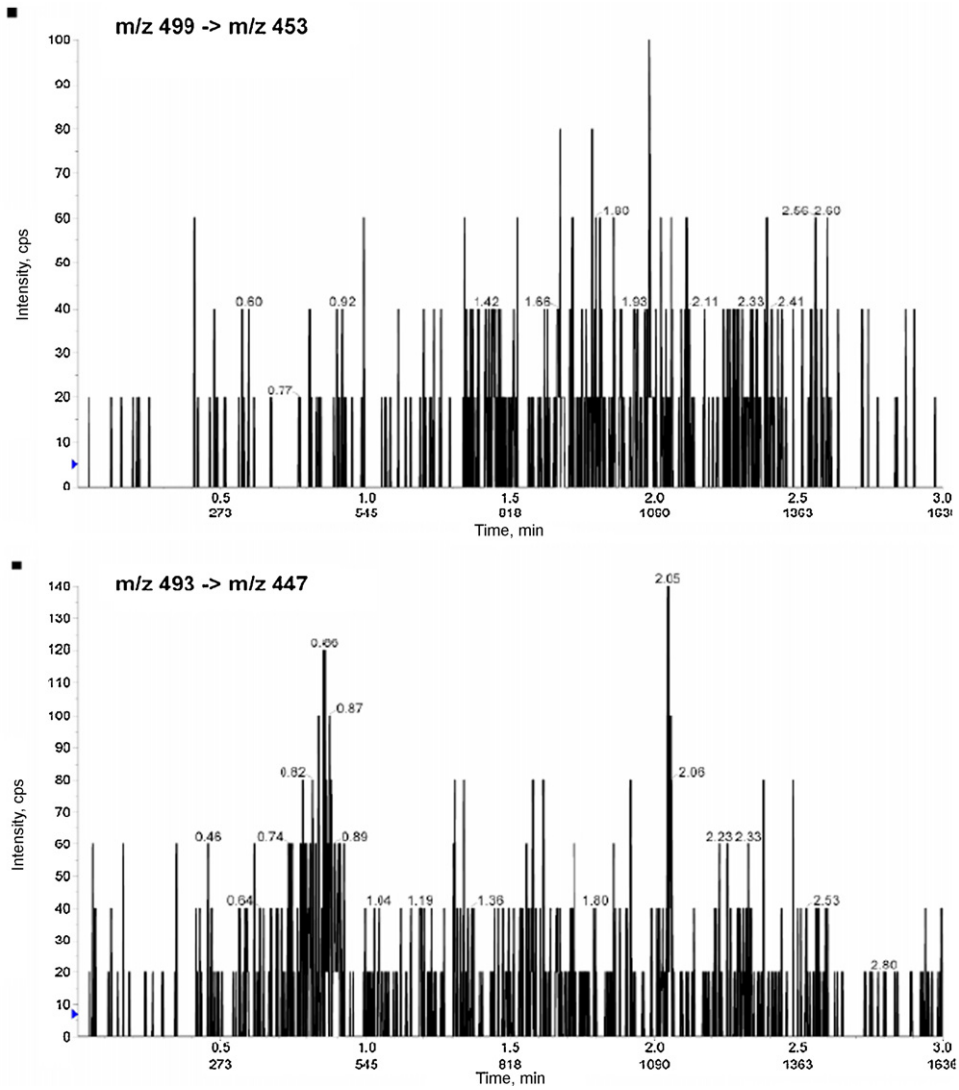

Fig. 7. Typical MRM chromatograms showing elution of 10-μL injection of an extracted blank human plasma sample of vorapaxar (bottom trace) and its IS, $^{13}C_6$-SCH 530348 (top trace), under described UPLC gradient condition.

4. Applicability of method

The method described herein was used to support a number of vorapaxar clinical studies conducted by Schering-Plough Research Institute. To date, phase 1 and 2 clinical trials have indicated that vorapaxar is well tolerated and did not increase adverse events related to the drug or dosing in patients with cardiovascular disease. Ongoing phase 3 trials will further evaluate the efficacy of vorapaxar in combination with anti-platelet therapies in patients with acute coronary syndrome, as well as, assess the compound for secondary prevention in patients with a history of thromboembolic disease.

Table 6
10-Fold dilution integrity for vorapaxar in human plasma.

Replicate	QCD 5000 ng/mL
1	5230
2	5130
3	5140
4	5090
5	5010
6	5140
Mean	5120
S.D.	72
%CV	1.4
%Theoretical	102.4
%DIFF	2.4
n	6

QCD indicates the diluted quality control sample; %CV indicates %coefficient of variation (intra-run precision); %DIFF indicates %difference (intra-run accuracy); S.D. indicates standard deviation.

Table 7
Stability data for vorapaxar.

Conditions	Minimum stability
Short-term room temperature stability in human plasma	7 h
Frozen stability in human plasma stored in a −20 °C freezer	300 days
Freeze–thaw stability in human plasma	3 cycles
Autosampler stability at 5 °C	5 days
Stock solution refrigerated stability in dimethyl sulfoxide	428 days
Stock solution room temperature stability in dimethyl sulfoxide	120 h

Table 8
Mean absolute recovery data for vorapaxar.

	QCs *vs.* Neat			
ID	QCL_Neat	QCH_Neat	QCL	QCH
Concentration	3.00 ng/mL	800 ng/mL	3.00 ng/mL	800 ng/mL
Peak area	6.01E+03	1.34E+06	6.44E+03	1.34E+06
	5.88E+03	1.37E+06	6.09E+03	1.35E+06
	6.06E+03	1.37E+06	6.11E+03	1.36E+06
	6.63E+03	1.41E+06	5.63E+03	1.30E+06
	6.13E+03	1.33E+06	5.78E+03	1.29E+06
	6.43E+03	1.47E+06	5.48E+03	1.31E+06
Mean	6.19E+03	1.38E+06	5.92E+03	1.33E+06
n	6	6	6	6
Precision (%CV)	4.57%	3.73%	6.01%	2.17%
Recovery	N/A	N/A	95.7	95.9

QCL indicates quality control of low concentration; QCH indicates quality control of high concentration; %CV indicates %coefficient of variation (intra-run precision).

5. Conclusion

LC–MS/MS is the preferred technique for bioanalysis because of its sensitivity, selectivity and speed. When coupled with UPLC, these benefits are even further enhanced. The use of sub-2 micron particle chromatography delivered significant efficiency improvements when compared to the initial method that utilized conventional 5 μm particle chromatography. The increased efficiency of the smaller particles permitted a simplified approach to sample extraction with no detriment in assay robustness. Herein, we presented a UPLC–MS/MS method that was validated over a dynamic range of 1.00–1000 ng/mL using a 100 μL aliquot of plasma. The results indicate the method to be sensitive, selective, accurate, and reproducible. SCH 530348 is stable during storage, processing and analysis in human plasma samples.

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