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Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 937–951
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous quantification of seven active metabolites of roxifiban in human plasma by LC/MS/MS in the presence of an interfering displacer at millimolar concentrations

G. Shi [a,b,*], T.L. Lloyd [a,b], S.K.B. Sy [a,1], Q. Jiao [a,2], A. Wernicki [a,b], A. Mutlib [a,b], T.A. Emm [a,3], S.E. Unger [a], H.J. Pieniaszek, Jr. [a,4]

[a] *Bristol-Myers Squibb Company, Drug Metabolism and Pharmacokinetics, Stine-Haskell Research Center, 1090 Elkton Road, Newark, DE 19711, USA*

[b] *Discovery Drug Metabolism and Pharmacokinetics, Bristol-Myers Squibb, Experimental Station, Wilmington, DE 19880, USA*

Received 12 February 2002; received in revised form 21 October 2002; accepted 15 November 2002

Abstract

Roxifiban (DMP 754) is a glycoprotein (GP) IIb/IIIa antagonist. Following oral administration to humans, roxifiban is metabolized to its primary active zwitterionic form, XV459, and several minor, active, hydrolyzed and hydroxylated metabolites, namely, M1a (DPC-AD3508), M1b (DPC-AD6128), M2 (SW156), M3 (DPC-AG2185), M8a (DPC-AF5814), and M8b (DPC-AF5818). Quantification of these metabolites in humans was not workable with a previous analytical method due to ion suppression of at least four of the analytes by a competitive displacer, DMP 728. This compound, which is another GP IIb/IIIa antagonist with very high affinity for the platelet receptor, was added to harvested blood samples in millimolar quantity to liberate XV459 from the GP IIb/IIIa receptor. An automated ion exchange solid phase extraction (IX-SPE) procedure was developed to selectively extract the seven metabolites of roxifiban and its deuterated internal standard while specifically excluding DMP 728. Among the six hydroxylation metabolites, there were two pairs of epimeric diastereomers (M1a/M1b and M8a/M8b) and one pair of geometric isomers (M2/M3), corresponding to three critical chromatographic pairs that needed to be base-line resolved because of the lack of specificity of MS/MS detection for these isomers. A new LC/MS/MS assay was developed to simultaneously quantify the seven metabolites in human plasma. The assay method was validated under GLP conditions over the concentration range of 0.5 to 80 nM for each of the analytes and successfully applied to assaying approximately 500 plasma samples from clinical trials.

* Corresponding author. Present address: Palatin Technologies, 4-C Cedar Brook Drive, Cranbury, NJ 08512, USA. Tel.: +1-609-495-2282; fax: +1-609-495-2204.

E-mail address: gshi@palatin.com (G. Shi).

[1] Present address: Center for Nonlinear Dynamics, McIntyre Building, McGill University, Montreal, Que., Canada.

[2] Present address: Innaphase Corporation, Philadelphia, PA, USA.

[3] Present address: Incyte Genomics, Inc., Stine-Haskell Research Center, 1090 Elkton Road, Building 300, Newark, DE 19711, USA.

[4] Present address: HPP Consulting and Services, Inc., 506 Pebble Beach Drive, Elkton, MD 21921, USA.

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(02)00675-1

Keywords: Roxifiban; XV459; Glycoprotein IIb/IIIa antagonists; Metabolite quantification; LC/MS/MS

1. Introduction

Clinical use of intravenous glycoprotein (GP) IIb/IIIa antagonists is associated with reduction of the risk of morbidity and mortality resulting from various cardiovascular occlusive diseases [1,2]. These agents act on the final common step in platelet aggregation through blocking the fibrinogen crosslink to the active site of GP IIb/IIIa receptors [3]. The fibrinogen-binding site on the GP IIb/IIIa receptor recognizes two amino acid sequences, the arginine–glycine–aspartine (RGD) and the lysine–glycine–aspartine (KQAGDV) sequences [4,5]. Analogues of the two amino acid sequences are currently marketed parenteral agents available in the acute-care setting for unstable angina, myocardial infarction, and percutaneous transluminal angioplasty [1,6–8]. Orally administered GP IIb/IIIa inhibitors offer the potential to lower the risk of recurrences of thrombotic events in the long-term setting.

Roxifiban (methyl N3-[2-{3-(4-formamidinophenyl)-isoxazolin-5(R)-yl}-acetyl]-N2-(1-butyloxycarbonyl-2,3-(*S*)-diaminopropionate; acetate salt) is an oral ester prodrug that is primarily hydrolyzed in vivo to the zwitterion, XV459, an active GP IIb/IIIa antagonist [9]. Six other minor, but active metabolites identified in vivo include hydroxylation at the isoxazoline ring and at the ω-1, and ω-2 of the *n*-butyl side chain followed by ester hydrolysis (Fig. 1). These metabolites are presumed to be formed presystemically since in humans intact roxifiban does not reach the systemic circulation and XV459 is metabolically stable (Bristol-Myers Squibb Pharmaceuticals, data on file).

The primary metabolite of roxifiban, XV459, was designed to inhibit platelet aggregation through blocking fibrinogen binding to the GP IIb/IIIa receptor [10]. With promising pharmacodynamic properties for a potent antithrombotic agent, XV459 exhibits high affinity for and a slow dissociation rate from the receptor similar to that of abciximab, a marketed parenteral GP IIb/IIIa inhibitor [11]. In the clinical development of roxifiban, accurate determination of total XV459 plasma concentration requires XV459 to be initially liberated from platelets. Otherwise, the majority of the compound in the blood will be driven into the platelet rich buffy coat at the plasma-packed cell interface during centrifugation. For other oral GP IIb/IIIa antagonists, EDTA was routinely used since those agents do not bind as avidly to the platelet receptor as XV459. XV459 was released in the presence of EDTA anticoagulant and excess quantity of DMP 728, another selective GP IIb/IIIa antagonist (Fig. 1). EDTA releases XV459 through partial disruption of the heterodimeric calcium-dependent GP IIb/

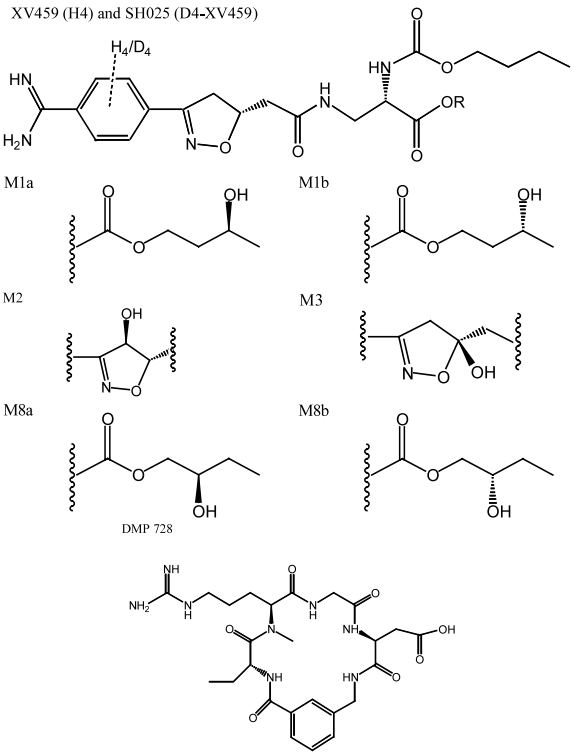

Fig. 1. Chemical structures of roxifiban (R=CH_3), its primary metabolites, XV459 (R=H), its minor metabolites, M1a, M1b, M2, M3, M8a, and M8b, and the displacer compound, DMP728.

Table 1
Reference standards for seven metabolites of roxifiban, SH025 (IS) and DMP 728 (blood platelet displacer)

Analyte	Registration ID	Molecular formula	Formula weight	HPLC purity (%)
XV459	XP280	$C_{20}H_{27}N_5O_6 \cdot 1.00C_6H_6O_3S$	591.65	99.2
M1a	DPC-AD3508-17	$C_{20}H_{27}N_5O_7 \cdot 1.15C_2HF_3O_2$	580.58	>99
M1b	DPC-AD6128-17	$C_{20}H_{27}N_5O_7 \cdot 1.20C_2HF_3O_2 \cdot 1.5H_2O$	613.32	>99
M2	SW156	$C_{20}H_{27}N_5O_7 \cdot 1.00C_2HF_3O_2$	563.48	>95
M3	DPC-AG2185-29	$C_{20}H_{27}N_5O_7 \cdot 0.14C_2HF_3O_2$	465.42	>98
M8a	DPC-AF5814-17	$C_{20}H_{27}N_5O_7 \cdot 1.00C_2HF_3O_2$	563.48	>99
M8b	DPC-AF5818-17	$C_{20}H_{27}N_5O_7 \cdot 1.00C_2HF_3O_2$	563.48	>99
D4-XV459	SH025	$C_{20}H_{23}D_4N_5O_6 \cdot 1.00C_2HF_3O_2$	551.52	>99
DMP 728	XN092	$C_{25}H_{36}N_8O_7 \cdot 1.00CH_4O_3S$	656.72	>99

IIIa receptor. DMP 728 competes with XV459 for the same binding site on the receptor to liberate XV459.

The analytical method development, however, was proven to be exceptionally challenging partly because the displacer DMP 728, used at 2 μmol per sample (approximately 2 mM) in plasma, was readily co-extracted and severely suppressed mass spectrometry ionization. The exceedingly high concentration of DMP 728 caused it to spread out as an extremely broad peak spanning 4 min which easily encompassed and suppressed four early eluting metabolites. XV459 was not affected because it was the last eluting one of all the seven metabolites, but a previous LC/MS/MS method capable of quantifying XV459 only [12] could not be adapted as a multi-component assay. To facilitate the clinical development of roxifiban, a method to extract all the seven metabolites of roxifiban and to specifically exclude DMP 728 was developed. Due to the lack of specificity of the mass spectrometry (MS/MS) detection for isomeric metabolites of roxifiban, the chromatographic method was also developed to distinguish two pairs of epimers and one pair of geographic isomers. The method reported here featured simultaneous quantification of all seven analytes using a LC/MS/MS methodology and was fully validated for a linear analytical range from 0.5 to 80 nM. The method has been successfully applied in clinical studies evaluating the pharmacokinetics (PK) of XV459 and the other six minor metabolites of roxifiban in healthy subjects.

2. Experimental

2.1. Chemicals, reagents and experimental materials

The reference standards for the seven metabolites of roxifiban, SH025 (D4-XV459 internal standard (IS), Fig. 1) and DMP 728 (displacer) were synthesized by the Bristol-Myers Squibb Pharma Company (formerly DuPont Pharmaceuticals, Wilmington, DE) and are listed in Table 1. HPLC grade methanol and acetonitrile and ACS grade formic acid and ammonium hydroxide were purchased from EM Science (Gibbstown, NJ), deionized water from Millipore (Bedford, MA). EDTA control human plasma was supplied by Biological Specialty (Colmar, PA). All 96-well extraction plates (Waters Oasis™ MAX 60 mg) and 96-well reception plates were purchased from Waters (Milford, MA) and Beckman Industrials (Palo Alto, CA), respectively.

2.2. Instrumentation

Chromatographic separations were performed with Shimadzu LC-10ADVP binary pumps and SCL-10AVP controller (Columbia, MD) and LEAP CTC HTS.PAL autosampler with stack cooling option (Carrboro, NC). Mass spectrometric analyses were performed using a Sciex API 3000 triple quadruple mass spectrometer equipped with MassChrom 1.1.2 software bundle (Toronto, Canada). Automated sample preparation was accomplished through the use of Zymark

Table 2
Robotic extraction procedure for roxifiban metabolites in human plasma

Action	Volume (μl)	Tecan pipetting liquid class	Centrifuge speed (rpm)	Centrifuge time (min)
Conditioned with methanol	250	Ethanol	500	2
Loaded sample mixture	550	Serum	750	2
Washed with 0.1% ammonium hydroxide in methanol (pH 10.5)	900	Ethanol	750	2
Exchanged out full deep-well receiving plate at carousel				
Washed with 0.1% ammonium hydroxide in methanol (pH 10.5)	900	Ethanol	750	2
			2000	1
Exchanged out full deep-well receiving plate at carousel				
Eluted with 1% formic acid in methanol	900	Ethanol	500	2
Evaporated partially				
Eluted with 1% formic acid in methanol	900	Ethanol	500	2
			2000	1

XP robot with System V-15 controller (Hopkinton, MA), equipped with Mettler AE200 weighing PyStation balance w/011 data output (Hightstown, NJ) and 7′ TOL-O MATIC linear motion track (Thompson Industries, Pt. Washington, NY).

2.3. Preparation of standard and QC samples

Primary standard solutions of each analyte were prepared by dissolving the analytes in methanol to final concentrations of 1.0 mM for XV459, M1a, M1b, and M3, and 0.2 mM for M2, M8a, and M8b. Working stock solutions consisted of a mixture of the seven metabolites to yield a nominal concentration of 4.0 μM for each analyte. All primary and working standard solutions were stored at −20 °C.

The plasma standard solutions at the upper limit of quantification concentration (80 nM) was prepared daily by diluting 80 μl of working stock solution to 4.0 ml with blank plasma or mobile phase. Subsequent serial dilutions were performed using a Zymark robot to prepare standards and quality control samples to yield final concentrations 0.5–40 nM in plasma and mobile phase.

A primary IS stock solution was prepared by dissolving 1.0 mg of SH025 in 10 ml of H_2O. A working IS solution was prepared by diluting 200 μl of primary IS stock solution to 1000 ml with H_2O to yield a nominal concentration of 20 ng/ml. Reconstitution solution (0.01% formic acid) was prepared by adding 50 μl of formic acid to 500 ml of H_2O.

2.4. Sample preparation (automated extraction procedure)

A microplate strong anion exchange extraction (SAX) was performed using a 96-well extraction plate. Centrifugation was applied to elute successive solvents through the sorbent bed, into a Corning Costar Square-Well 2 ml capacity microplate (Cambridge, MA). The robot carried the stacks of plates (extraction plate on top of the deep-well plate) repetitively between the liquid handler for reagent addition and the centrifuge for elution at each step. The system processed two plates at a time, requiring two exchanges of the receiving plate at the carousel and some intermediate evaporation of the first elution fraction during processing. The number of samples being extracted was split evenly between the two plates.

Prior to extraction, the liquid handler distributed 200 μl of working IS solution into a Porvair

Round Well microplate (Shepperton, UK). It then added 250 μl of the matrix sample and 150 μl of an aqueous 2.5% ammonium hydroxide solution. Each well in the 96-well extraction plate was conditioned with 250 μl methanol. A 550 μl of the sample mixture was drawn using vacuum and applied to the extraction plate. The wells were then washed sequentially with 900 μl 0.1% ammonium hydroxide in methanol (pH 10.5). The plate was positioned and then centrifuged to remove retained solvents. A second wash with 900 μl of 0.1% ammonium hydroxide in methanol was then followed by elution of 900 μl 1.0% formic acid in methanol. The remaining elution reagent was evaporated to dryness for 60 min at 15 psi, 40 °C. The residues were reconstituted with 200 μL of reconstitution solution usually within an hour of reaching dryness, and mixed on a Multi-Tube Vortexer for 30 s at speed setting of 6. The above procedure is summarized in Table 2.

Samples were prepared to measure extraction recovery and matrix effect. Using a Tecan™ Genesis 100 liquid handler (Research Triangle, NC), aqueous solutions of the seven metabolites were prepared at concentrations of 0.5, 5 and 80 nM. A correction factor of 0.873 was applied to account for the ratio of plasma volume (250 μl) to reconstitution volume (200 μl), and for the ratio of sample mixture volume (600 μl) to extraction volume (550 μl). The unextracted 200 μl sample solutions were used to reconstitute residues of extracted blank plasma.

2.5. LC/MS/MS conditions

The HPLC column was Phenomenex Luna 3μ C18, 2.0 × 50 mm (Torrance, CA). Mobile phase solvents A and B were 0.01% formic acid in distilled water and methanol, respectively. The mobile phases were delivered at a flow rate of 0.3 ml/min, using a variable linear gradient for B% (time): 5% (0 and 3 min), 9% (4 min), 10.5% (8 min), 12% (12 min), 23% (15 and 18 min), and 5% (18.1 and 23 min). An automated post-column switching valve (Jones Chromatography electronic valve actuator, Jones Chromatography Ltd., Lakewood, CO) was employed to divert the eluent from the analytical column to waste for the first 8 min following injection and then into the MS/MS systems for the rest of the run. Approximate retention times (minutes) of peaks were 10.3 (M1a), 11.2 (M1b), 11.9 (DMP 728), 12.6 (M8a), 13.3 (M8b), 15.7 (M2), 16.1 (M3), 17.8 (XV459 and SH025).

Precursor and product ions for analytes and IS were determined from spectra obtained during the injection of neat solutions into the mass spectrometer using Turbo IonSpray™ source. The probe temperature was set to 500 °C. The collision gas used was argon. The mass spectrometer was operated in multiple reaction monitoring mode by monitoring the following precursor/product ion pairs:

Analyte	Precursor ion	Product ion
M1a	450	212
M1b	450	212
M8a	450	212
M8b	450	212
M2	450	141
XV459	434	212
M3	450	246
D4-XV459	438	216
DMP 728	561	112

The state file of the mass spectrometer was optimized to achieve maximum sensitivity for each detection channel. The general parameters are as follows (with values in parentheses): TEM (500), NEB (12), CUR (8), CAD (12), IS (4500), OR (50), RNG (180), Q0 (−9), IQ1 (−9.5), ST (−16), RO1 (−12), DF (−400), CEM (2000). The specific parameters related to each analyte are listed below:

Analytes	IQ2	RO2	ST3	RO3
M1a, M1b, M8a, M8b, XV459, SH025	−55	−60	−75	−65
M2	−45	−50	−65	−55
M3	−35	−40	−55	−45

2.6. Method validation

The assay for simultaneous determination of seven metabolites of roxifiban was validated over a linear concentration range from 0.5 to 80 nM. A calibration curve for each batch was determined using calibration standards at seven concentration levels (0.5, 1, 5, 10, 20, 40 and 80 nM). The lines of best fit for calibration standards were determined using linear least-squares regression analysis based on the peak area ratios of the analytes to the IS (Watson LIMS software, Version 5.4, Innaphase Corp., Philadelphia, PA). Five concentration levels of QC samples (0.5, 1, 5, 40 and 80 nM) were prepared and analyzed on the same day, along with an independent standard curve for quantification. Six and two QC replicates were prepared at each concentration for intraday and interday validations, respectively. The accuracy was expressed as the absolute percent deviation from the theoretically determined concentration (% difference). The precision was evaluated as the relative standard deviation of the mean expressed as percent (coefficient of variation: CV) for each sample. For intraday validation, CV and a mean of the absolute percentage difference of $<15\%$ ($<20\%$ at the LLOQ) for each concentration level were acceptable provided that at least 5 out of 6 of the replicates at each level met the criteria (except one level may have only 4 acceptable replicates). For interday validation, the mean of the absolute values of percentage difference of $<15\%$ ($<20\%$ at the LLOQ) at each concentration level was acceptable, and the calculated concentrations over the three days must yield a CV at each concentration level of $<15\%$ ($<20\%$ at the LLOQ).

To evaluate assay specificity, six independent lots of human control plasma were extracted without IS and analyzed for endogenous co-eluting interference. Triplicates of extracted and unextracted QC samples and a set of post-extracted spiked QC samples were analyzed in the same assay run to determine the extraction recovery and matrix effect. The extraction efficiency (or recovery) was determined by measuring an extracted sample against a post-extraction spiked sample:

$$\%\ \text{Recovery} = \left(\frac{\text{Response of extracted}}{\text{Response of postextracted spike}}\right)100\%$$

The matrix effect was measured by referring the post-extracted spiked sample to the unextracted sample:

$$\text{Matrix Effect} = \frac{\text{Response of postextracted spike}}{\text{Response of unextracted}}$$

The absence of a matrix effect was indicated by a ratio of 1.0. No response due to total matrix suppression would give a value of 0.

The stability for each analyte was evaluated for stock solutions stored at -20 °C in a freezer, undergone three separate freeze/thaw cycles, unextracted compounds in human plasma at room temperature, extracted samples placed in an autosampler at 4 °C, and long-term frozen samples. A batch of samples were prepared in two concentrations, 2 and 40 nM, at time zero. One set (in triplicate) was immediately analyzed for their time zero concentrations. Another set was analyzed at a predetermined time point and results compared to the time zero reading. A compound was considered to be unstable if the calculated concentration from two consecutive determinations was less than the time zero concentration by more than 15%. Due to their limited quantity, fresh primary stock solutions of analytical reference standards could not be prepared. The stock solution stability of each analyte was examined by comparing the

Table 3
Extraction recovery and matrix effect for the determination of roxifiban metabolites in human plasma

Metabolite	Extraction recovery (%)	Matrix effect
XV459	52.0	1.10
M1a	41.0	1.05
M1b	41.0	0.94
M2	47.5	1.01
M3	58.9	1.00
M8a	42.9	1.06
M8b	44.2	1.06

Calculated values were based on the mean of three independent determinations.

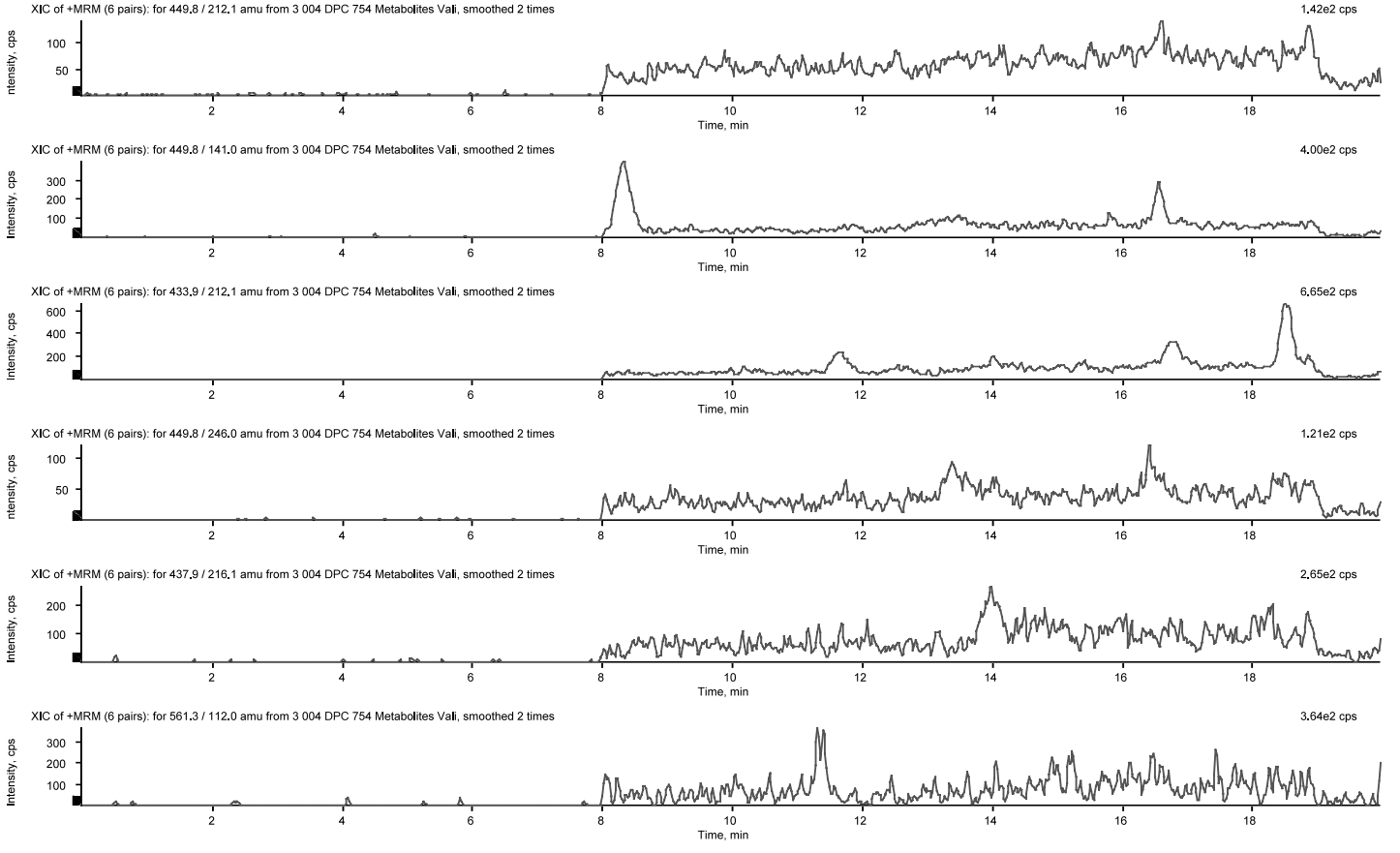

Fig. 2. Representative LC/MS/MS chromatograms of the seven metabolites in extracted human plasma from the control blank.

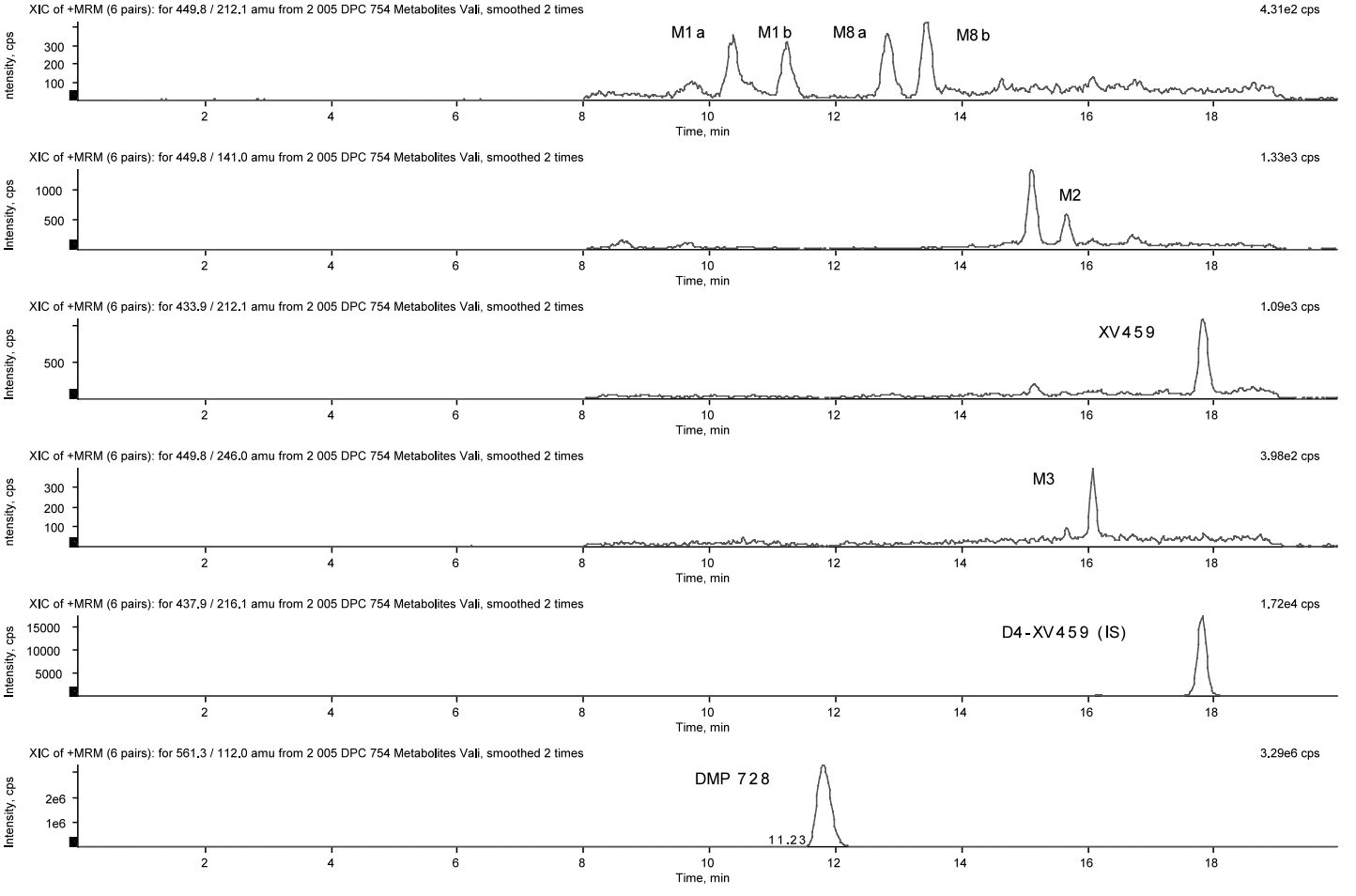

Fig. 3. Representative LC/MS/MS chromatograms of the seven metabolites in extracted human plasma from the LLOQ (0.5 nM).

Table 4
Intraday precision and accuracy for the determination of the seven metabolites of roxifiban in human plasma

		Nominal concentration (nM)				
		0.5	1	5	40	80
XV459	Calculated	0.449	0.981	4.847	38.37	76.80
	% Difference	−10.2	−1.90	−3.06	−4.07	−4.00
	% CV	9.80	3.16	2.50	1.41	0.95
M1a	Calculated	0.491	0.948	4.826	40.11	78.24
	% Difference	−1.80	−5.20	−3.48	0.29	−2.20
	% CV	12.63	9.60	6.98	3.08	5.49
M1b	Calculated	0.542	1.021	4.835	39.96	77.50
	% Difference	8.40	2.10	−3.30	−0.11	−3.12
	% CV	10.2	3.82	6.47	5.41	3.97
M2	Calculated	0.448	0.973	4.902	39.93	79.75
	% Difference	−10.4	−2.70	−1.96	−0.19	−0.31
	% CV	11.2	9.97	4.71	3.29	2.75
M3	Calculated	0.511	1.093	5.011	40.47	81.82
	% Difference	2.20	9.30	0.22	1.18	2.28
	% CV	11.9	5.40	7.20	3.10	3.74
M8a	Calculated	0.494	0.934	4.801	39.25	77.23
	% Difference	−1.20	−6.60	−3.98	−1.883	−3.47
	% CV	4.05	6.53	4.603	3.52	3.56
M8b	Calculated	0.541	1.009	4.793	39.10	76.59
	% Difference	8.20	0.90	−4.14	−2.24	−4.26
	% CV	7.02	5.25	2.96	3.25	3.09

average peak area of triplicate injections of a 4 μM solution stored separately at −20 and −80 °C for a specific period of time. An analyte stock solution was judged unstable at −20 °C if the calculated mean peak area is less than 90% of that stored at −80 °C (13).

2.7. Clinical samples

A representative individual PK profile was randomly selected from a clinical study that evaluates twice-daily dosing of roxifiban (0.75 and 1.0 mg) versus once-daily dose of 1.5 mg. The protocol was in full compliance with the declaration of Helsinki (amended), the International Conference on Harmonization, good clinical practices, and FDA regulations (21 CFR parts 50 and 56) and was approved by the institutional board of Quintiles (Lenexa, KS). Plasma samples were collected from a healthy volunteer who received multiple oral roxifiban doses on three separate occasions (1.5 mg q.d., 0.75 mg b.i.d. and 1.0 mg b.i.d.) for 7 days. There was at least a 2-week washout period between dosing regimens. The samples were stored frozen at −80 °C until analysis.

3. Results and discussion

3.1. Method development

A major challenge in the current method development was the presence of an excessive amount of DMP 728, which suppressed the ionization of most of the analytes when co-extracted. Since DMP 728 co-eluted with several metabolites on a reverse or normal phase column, attempts to develop an extraction method based on polarity difference were bound to be unsuccessful. We noticed that the guanidine group ($pKa \approx 12$) of DMP 728 was much more basic than the benza-

Table 5
Interday precision and accuracy for the determination of the seven metabolites of roxifiban in human plasma

		Nominal concentration (nM)				
		0.5	1	5	40	80
XV459	Calculated	0.526	1.041	5.056	39.06	78.98
	% Difference	5.27	4.13	1.12	−2.35	−1.28
	% CV	15.3	5.59	2.81	1.93	2.57
M1a	Calculated	0.452	0.973	5.152	40.58	83.45
	% Difference	−9.57	−2.70	3.04	1.45	4.31
	% CV	5.59	9.26	8.64	2.93	5.02
M1b	Calculated	0.516	1.003	5.111	41.05	81.62
	% Difference	3.10	0.32	2.22	2.63	2.03
	% CV	0.73	0.68	11.31	1.94	5.97
M2	Calculated	0.480	0.984	4.941	39.29	80.06
	% Difference	−3.97	−1.60	−1.19	−1.77	0.08
	% CV	17.3	4.36	3.72	4.44	3.06
M3	Calculated	0.487	1.044	5.132	39.40	80.30
	% Difference	−2.63	4.43	2.63	−1.50	0.38
	% CV	19.2	4.97	5.37	3.30	4.84
M8a	Calculated	0.499	0.946	5.027	40.32	81.33
	% Difference	−0.20	−5.43	0.54	0.79	1.66
	% CV	4.93	4.74	7.06	2.14	3.65
M8b	Calculated	0.500	1.000	5.134	40.23	81.59
	% Difference	−0.10	0.03	2.68	0.57	1.98
	% CV	11.1	2.35	6.26	2.02	3.91

midine group ($pKa \approx 10$) of the metabolites, suggesting that the former molecule could be readily differentiated by ion exchange chromatography based on its distinctive charge state at certain pH values. At a pH of approximately 11, the metabolites should be negatively charged but DMP 728 remains as a neutral zwitterionic species. A selective extraction of analytes was thus possible using SAX SPE, which, under the conditions described, removed more than 99.9% of DMP 728. It was necessary to adjust the pH of plasma to approximately 10.5 prior to loading onto the SPE extraction plate. A pH value below 10 resulted in an inadequate retention of analytes on the SPE cartridge. On the other hand, a pH value higher than 11 caused chemical degradation of the metabolites and coextraction of DMP 728 due to the deprotonation of its guanidine group. For the same reason, adjusting the pH of the wash solution to 10.5 was important.

The extraction procedure was about 30% less efficient than that normally seen for routine SPE procedures (Table 3). The lower recovery was related to loss of analytes at the initial plasma loading step due to analyte breakthrough and at the final elution step due to strong adsorption of the analytes by the sorbent bed. Experiments were carried out to maximize the recovery. The optimum pH of plasma prior to loading was approximately 10.5 and that of elution solvent was below 2.5. A large volume of elution solvent was found to be necessary to achieve maximum extraction recovery. Reagent retention in the extraction cartridge could contribute to lower recovery, especially at the end of washing cycle, and hence, additional steps of higher speed centrifugation (2000 rpm) were added in the robotic program. No apparent advantage was observed for using an aqueous elution solvent containing a high concentration of salt. In addition, an increase in absor-

Table 6
Freeze and thaw, short-term, long-term and post-extraction stability of the roxifiban metabolites

Metabolite	Stability test	Nominal conc. (2 nM)	% Remaining	Nominal conc. (40 nM)	% Remaining
XV459	Time 0/Cycle 0	1.966	100	39.53	100
	Freeze/thaw cycle 1	1.845	93.8	39.08	98.9
	Freeze/thaw cycle 2	1.829	99.2	39.47	101
	Freeze/thaw cycle 3	1.912	105	38.97	98.7
	In plasma—30 h at room temperature	1.995	101	38.72	101
	Post-extraction—48 h at 4 °C	1.737	88.4	39.86	101
	Long-term—8 days at −20 °C	1.968	100	42.11	107
	Long-term—64 days at −20 °C	1.943	99	40.06	101
M1a	Time 0/cycle 0	1.919	100	41.11	100
	Freeze/thaw cycle 1	1.850	96.4	39.30	95.6
	Freeze/thaw cycle 2	1.862	101	40.19	102
	Freeze/thaw cycle 3	1.895	102	40.12	99.8
	In plasma—30 h at room temperature	2.046	107	43.06	109
	Post-extraction—48 h at 4 °C	1.920	100	44.98	109
	Long-term—8 days at −20 °C	2.094	109	43.31	105
	Long-term—64 days at −20 °C	2.526	132	47.26	115
M1b	Time 0/cycle 0	1.991	100	41.76	100
	Freeze/thaw cycle 1	1.838	92.3	40.01	95.8
	Freeze/thaw cycle 2	1.902	103	40.57	101
	Freeze/thaw cycle 3	2.002	105	40.71	100
	In plasma—30 h at room temperature	2.025	102	42.78	107
	Post-extraction—48 h at 4 °C	1.941	97.5	44.87	107
	Long-term—8 days at −20 °C	2.083	105	41.94	100
	Long-term—64 days at −20 °C	2.262	114	44.68	107
M2	Time 0/cycle 0	1.846	100	38.42	100
	Freeze/thaw cycle 1	1.756	95.1	38.12	99.2
	Freeze/thaw cycle 2	1.877	107	38.22	100
	Freeze/thaw cycle 3	1.831	97.6	37.74	98.7
	In plasma—30 h at room temperature	1.954	106	38.67	101
	Post-extraction—48 h at 4 °C	1.738	94.1	38.79	101
	Long-term—8 days at −20 °C	2.058	111	43.86	114
	Long-term—64 days at −20 °C	2.252	122	43.13	112
M3	Time 0/cycle 0	1.822	100	38.00	100
	Freeze/thaw cycle 1	1.864	102	39.34	104
	Freeze/thaw cycle 2	1.852	99.4	39.29	100
	Freeze/thaw cycle 3	1.913	103	37.89	96.4
	In plasma—30 h at room temperature	1.755	96.3	38.44	103
	Post-extraction—48 h at 4 °C	1.759	96.5	38.99	103
	Long-term – 8 days at −20 °C	2.018	111	43.82	115
	Long-term—64 days at −20 °C	2.119	116	44.79	118
M8a	Time 0/cycle 0	1.860	100	39.93	100
	Freeze/thaw cycle 1	1.908	103	38.45	96.3
	Freeze/thaw cycle 2	1.846	96.8	39.07	102
	Freeze/thaw cycle 3	1.895	103	38.93	99.6
	In plasma—30 h at room temperature	1.880	101	40.62	108
	Post-extraction—48 h at 4 °C	1.800	96.8	43.32	108
	Long-term—8 days at −20 °C	2.225	120	43.45	109
	Long-term—64 days at −20 °C	1.944	104	39.78	100
M8b	Time 0/cycle 0	1.997	100	40.74	100
	Freeze/thaw cycle 1	1.855	92.9	39.27	96.4

Table 6 (*Continued*)

Metabolite	Stability test	Nominal conc. (2 nM)	% Remaining	Nominal conc. (40 nM)	% Remaining
	Freeze/thaw cycle 2	1.898	102	39.79	101
	Freeze/thaw cycle 3	1.943	102	39.75	99.9
	In plasma—30 h at room temperature	1.914	95.8	41.13	106
	Post-extraction—48 h at 4 °C	1.923	96.3	43.24	106
	Long-term—8 days at −20 °C	2.225	111	43.45	107
	Long-term—64 days at −20 °C	2.053	103	38.33	94

Calculated values were based on the mean of three independent determinations.

bent mass from 30 to 60 mg resulted in greater recovery of the analytes in pure solvents.

The other major difficulty was the lack of specificity in detecting these isomeric metabolites by MS/MS. Because all hydroxylated metabolites shared some common MS/MS product ions, no SRM (Single Reaction Monitoring) transition could be found for any metabolite that was totally specific and adequately sensitive. An HPLC separation of all metabolites prior to MS/MS detection became necessary. Numerous combinations of different types of stationary and mobile phases were tested before an optimal chromatographic separation was achieved (Figs. 2 and 3). Some findings during the method development were intriguing. For example, in order for M2 and M3 to be separated on a C18 column, the mobile phase pH must be either basic in an ACN/H_2O system or acidic in a MeOH/H_2O system. The elution sequence of M2/M3 would reverse upon changing from basic to acidic condition or vice versa. The latter system was chosen for its selectivity as well as the fact that the analytes were not stable at pH greater than 11. However, the mobile phases must be made moderately acidic by adding only a small amount of formic acid. When the formic acid strength was increased to a moderate 0.1%, the separation for the other critical pairs (M1a/M1b and M8a/M8b) deteriorated along with a noticeable drop of detection sensitivity, presumably due to ion suppression. To avoid evaporation of formic acid, it was necessary to prepare mobile phases within 48 h of use.

To address the issue of a relatively long analytical cycle time (23 min), two approaches were attempted with little success. Column heating is a commonly used technique to reduce plate height and system back pressure, often resulting in improved separation and shorter analytical time. In this case, even a moderate increase of temperature caused significant loss in selectivity for all three critical pairs, suggesting that conformational differentiation induced by intramolecular hydrogen bonding was important in separating these isomers in the reverse phase chromatography. Another attempted approach to reduce analytical cycle time was to increase flow rate without sacrificing resolution and relative retention. Because the van Deemter curve for a 3 μ particle column is virtually flat starting from 1 mm/s in linear velocity (or $\approx$0.2 ml/min for a 2 mm i.d. column), an increased flow rate from 0.3 to 0.5 ml/min should reduce the cycle time by 40% while maintaining the same quality of separation. Indeed, all the peaks were baseline separated at a flow rate of 0.5 ml/min, as predicted, with the cycle time reduced to 14 min. The maximum column back pressure increased from 110 to 200 bar, which was still considered tolerable in practice. However, the faster method failed in validation trials because the separation was not adequately robust to resolve some analytes, especially M2, from endogenous interference peaks that were closely eluted.

3.2. Method validation

The extraction recovery and matrix effects are listed in Table 3. Interday and intraday assay accuracy and precision for the seven metabolites are presented in Table 4 and Table 5. All metabolites appeared stable after three cycles of freeze and thaw, for at least 30 h in plasma at room temperature and, following extraction, for

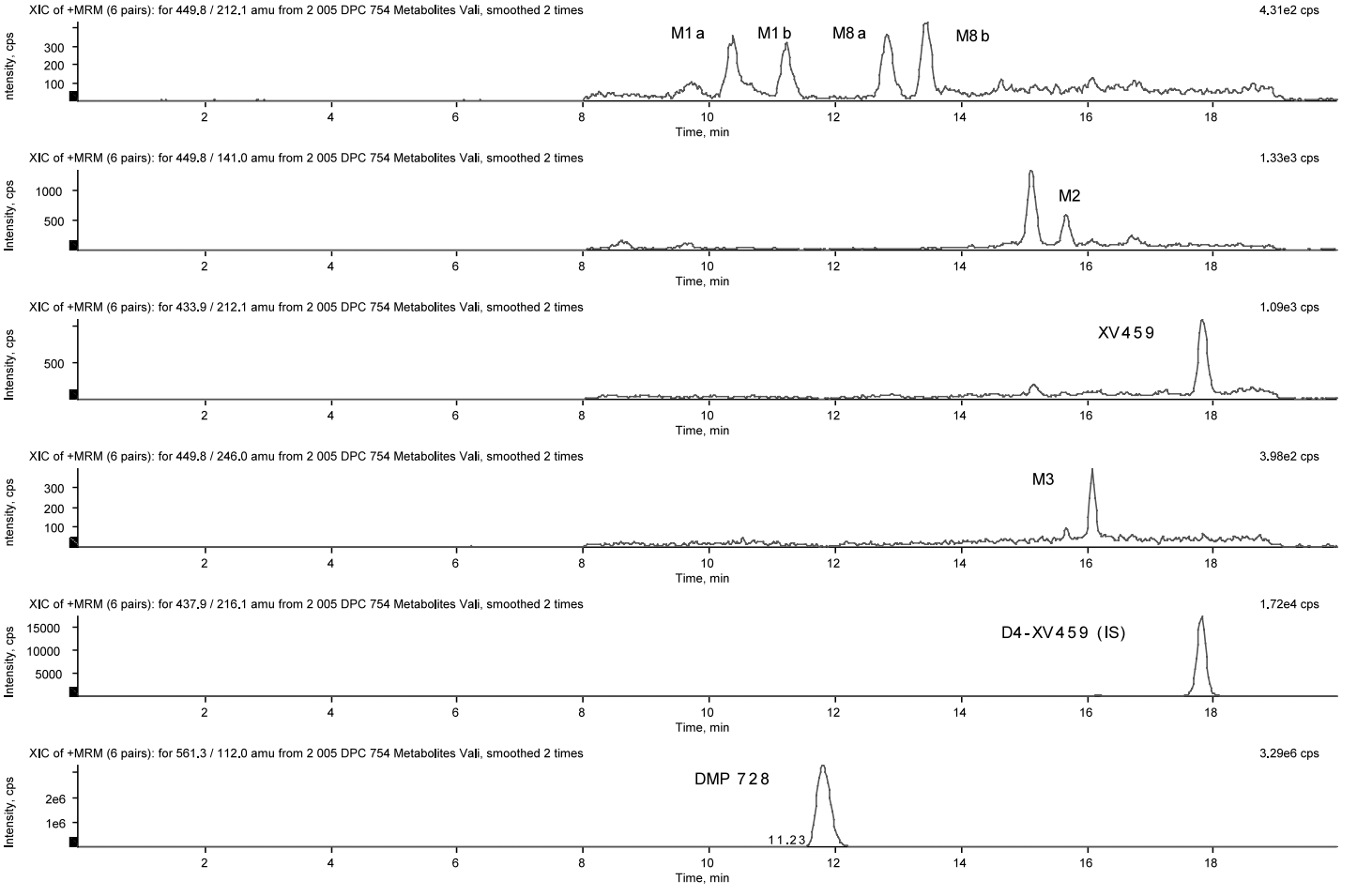

Fig. 4. Representative LC/MS/MS chromatograms of the seven metabolites in extracted human plasma from a sample taken 3 h following dosing at 1.5 mg once daily.

48 h stored at 4 °C (the temperature of cooled autosampler tray stacks). In addition, these metabolites appeared to be stable for at least 2 months when stored in a −20 °C freezer. The stability data were collectively presented in Table 6. Since the lack of reference materials for some analytes prevented a second preparation of stock solutions directly from powders, all primary stock solutions were stored at −80 °C, the condition under which their chemical stability was assumed [13]. In addition, experimental results showed that after 26 days, a solution stored at −20 °C maintained from 98 to 101% of that stored at −80 °C for the seven metabolites, suggesting these molecules were stable when stored at −20 °C for at least 26 days.

3.3. Clinical application

Our quantitative LC/MS/MS method was used to simultaneously determine the concentrations of seven metabolites in plasma following sequential oral roxifiban administrations of 1.5 mg roxifiban once daily (q.d.), 0.75 mg twice-daily (b.i.d.), and 1.0 mg b.i.d. for 7 days in one subject. The washout period between dosing regimens was at least 2 weeks. There was no carryover effect since the predose plasma concentrations of the second and third dosing regimens were below the limit of quantification. Chromatograms at 3 h after oral administration of 1.5 mg roxifiban are shown in Fig. 4. The plasma concentration–time profiles for the metabolites are shown in Fig. 5. The rank order of systemic exposure of roxifiban metabolites was XV459 ≫ M2 > M1a > M8a > M1b = M3 = M8b.

The primary metabolite, XV459, represents approximately 76–82% of the total cumulative exposure of the seven metabolites. Steady state concentration of XV459 at 1.0 mg b.i.d. was higher than the two other dosing regimens which were relatively similar. Relative bioavailability of XV459 at 0.75 b.i.d. versus 1.5 mg q.d. and 1.0 mg b.i.d. versus 1.5 mg q.d. were 1.14 and 0.92, respectively. The PK parameters of XV459 for 0.5 to 1.5 mg q.d. were previously reported in healthy volunteers [12]. Other roxifiban metabolite PK and XV459 PK after twice-a-day administration of

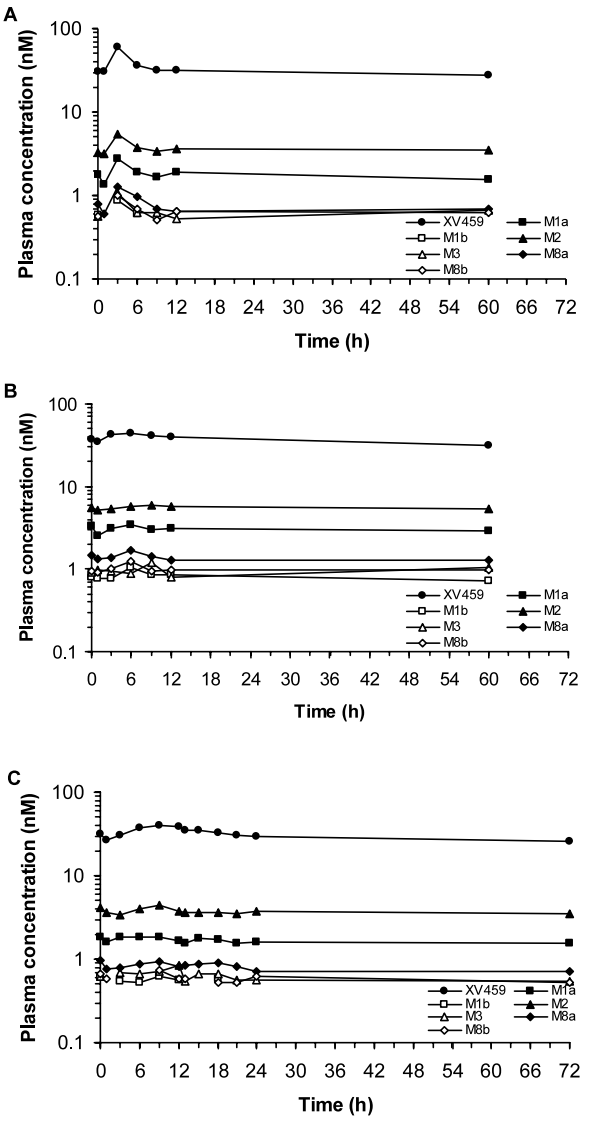

Fig. 5. Plasma concentration–time curves of the seven metabolites following the last oral roxifiban dose of (A) 0.75 mg twice-daily, (B) 1.0 mg twice-daily, and (C) 1.5 mg once daily in a healthy subject.

roxifiban in humans have not been previously reported in the literature.

The six minor metabolites of roxifiban represent approximately 20% of the total cumulative exposure of all the metabolites. It appeared that the six minor metabolites do not contribute significantly to the overall exposure of the total metabolites. A recent study has shown that the six metabolites

were considerably less potent than the primary hydrolysis metabolite, XV459 (Bristol-Myers Squibb, data on file).

4. Conclusion

An exquisite and robust analytical method was developed for selectively extracting and simultaneously quantifying seven active metabolites of roxifiban from human plasma containing an excessive level of displacer DMP 728. By setting the pH to 10.5, DMP 728 remains a neutral zwitterion, and SAX chromatography readily extracts the seven metabolites of roxifiban which are negatively charged. The method was useful for screening these metabolites at nanomolar levels in plasma. Subsequently, simultaneous determination of the seven metabolites, using highly selective and sensitive LC/MS/MS detection, was validated with a linear analytical range from 0.5 to 80 nM. This assay has been applied successfully to the analysis of human plasma samples in a clinical study evaluating different dosing regimens.

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