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A double antibody radioimmunoassay for the determination of XV459, the active hydrolysis metabolite of roxifiban, in human plasma

Henry J. Pieniaszek, Jr. [a,*], Anna F. Davidson [a], Harry L. Walton [b], Donald J. Pinto [b], Richard E. Olson [b], Thomas M. Reilly [b], Yu Chen Barrett [c,1]

[a] *Bristol-Myers Squibb Pharma Company, Stine-Haskell Research Center, 1090 Elkton Road, Newark, DE 19711, USA*
[b] *Bristol-Myers Squibb Pharma Company, Experimental Station, P.O. Box 80500, Wilmington, DE 19880, USA*
[c] *Covance Laboratories, Inc., Immunochemistry Department, 9200 Leesburg Pike, Vienna, VA 22182, USA*

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Abstract

A radioimmunoassay (RIA) was developed for the determination of XV459, the active hydrolysis metabolite of the oral prodrug roxifiban (DMP 754), in human plasma. XV459 is a potent antagonist of the glycoprotein IIb/IIIa receptor. The method utilizes a competitive double antibody format employing an ^{125}I-labeled XV459 analogue tracer which competes with XV459 for antibody binding sites and a second antibody precipitation step to separate antibody bound analyte from free analyte. The method has a validated lower quantification limit of 0.35 ng/ml (0.81 nM) using 12.5 μl of plasma and with dilution can accommodate clinical plasma samples ranging up to 48.0 ng/ml (110.7 nM). Cross-validation to an existing quantitative liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method showed good correlation ($r^2 = 0.98$). The RIA has been successfully used to assay over 10 000 clinical samples with sensitivity and specificity comparable to the LC-MS/MS method, but with faster turn around time and at greatly reduced costs.

Keywords: Radioimmunoassay; Roxifiban; XV459; Glycoprotein IIb/IIIa receptor antagonist

1. Introduction

Roxifiban (DMP 754), an oral ester prodrug, undergoes rapid hydrolysis by intestinal esterases to its active hydrolysis metabolite, XV459, a potent inhibitor of glycoprotein (GP) IIb/IIIa receptor-mediated platelet aggregation. XV459 inhibits agonist-induced (adenosine 5′ diphos-

* Corresponding author. Present address: HPP Consulting and Services, Inc., P.O. Box 7714, Newark, DE 19714, USA. Tel.: +1-302-547-7479; fax: +1-410-620-4911
E-mail address: pieniahj@delanet.com (H.J. Pieniaszek, Jr.).
[1] Present address: Bristol-Myers Squibb Company, 311 Pennington-Rocky Hill Road, Pennington, NJ 08534, USA.

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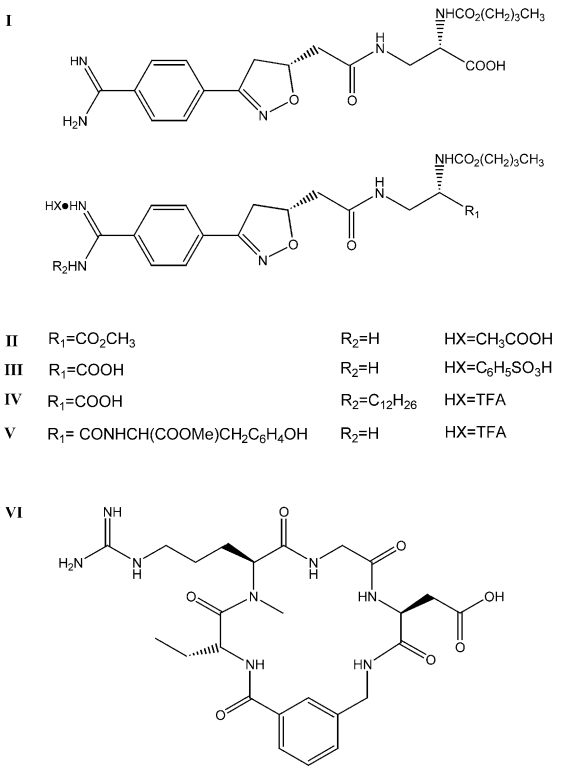

Fig. 1. Chemical structures of XV459 (I), roxifiban (II), XP280 (III, the benzene sulfonate salt of XV459), the immunogen (IV), the radioligand, SJ009 (V), and DMP 728 (VI).

phate, collagen, and thrombin receptor agonist peptide) platelet aggregation by blocking fibrinogen binding to the platelet GP IIb/IIIa receptor [1]. Roxifiban, currently in late-stage clinical development for the treatment of peripheral arterial disease (PAD), required the development of a suitable assay for population pharmacokinetic plasma sample analysis during Phase II/III investigations. The desire for a rapid, sensitive, robust, yet cost-effective assay suggested a radioimmunoassay (RIA) approach would be an attractive alternative to an existing liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method for routine clinical assays. The presence of known and unknown human metabolites and the zwitterionic nature of XV459 presented unique challenges in both the design and coupling of XV459 derivatives to protein. Four different XV459 analogues were synthesized and evaluated, including coupling via the right-hand side (carboxylic acid function) and three left-hand side (amidine-linked) analogues. It was thought that attachment to protein via the carboxylic acid function would result in stronger immune response, while attachment via the amidine function may allow for more effective discrimination among XV459, roxifiban, and the only known metabolite (in dogs) at the time, SE222, the hydroxylated butyl ester of roxifiban.

2. Experimental

2.1. Chemicals

XP280 (N^3-[2-{3-(4-formamidino-phenyl)-isoxazolin-5(R)-yl}-acetyl]-N^2-(1-butyloxycarbonyl)-2,3-(*S*)-diaminopropionate), the benzene sulfonate salt of XV459; the immunogen; and the radioligand, SJ009, were all synthesized by Bristol-Myers Squibb Pharma Company (formerly DuPont Pharmaceuticals) (Fig. 1). DMP 728 (cyclic[D-2-aminobutyryl-N2-methyl-L-arginyl-glycyl-L-aspartyl-3-aminomethyl-benzoic acid], methanesulfonic acid salt), used to displace XV459 from platelets, was also prepared by Bristol-Myers Squibb Pharma (Fig. 1). Iodination of SJ009 was performed at Covance Laboratories (Vienna, VA). Glacial acetic acid and sodium hydroxide were purchased from Mallinkrodt (Paris, KY) and hydrochloric acid was obtained from Fisher (Pittsburgh, PA). Methanol, dichloromethane, ammonia, ethyl acetate and trifluoroacetic acid (TFA) were acquired from EM Science (Gibbstown, NJ). Goat anti-rabbit gamma globulin (GARGG) was purchased from OEM Concepts (Toms River, NJ). Bovine serum albumin (BSA), carbowax polyethylene glycol (PEG), dimethyl sulfoxide (DMSO), glutaraldehyde, glycine, keyhole limpet hemocyanin (KLH), lithium hydroxide, and 1-*N*-Boc-aminohexanol were acquired from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate buffer saline (DPBS) was purchased from Biofluids (Rockville, MD). AG 1-X8 resin was obtained from Bio-Rad Laboratories (Hercules, CA). Compounds tested for cross-reactivity

were obtained from a variety of sources, including Sigma and Bristol-Myers Squibb Pharma.

2.2. Hapten synthesis

Four different haptens were derivatized to accommodate preparation of conjugates via both ends of the molecule enabling comparison of antisera specificities. Hapten synthesis of the amidine-linked precursor ultimately chosen (i.e. 3-{[((5R)-3-{4-[[(6-aminohexyl)amino] (imino)methyl]phenyl}-4,5-dihydro-5-isoxazolyl)acetyl]amino}-*N*-(butoxycarbonyl)-L-alanine trifluoroacetate) is described as follows in detail. HCl gas was bubbled into an ice cold methanol (50 ml) solution of methyl (R)—3-[[[3-(4-cyanophenyl]-4,5-dihydro-5-isoxazolyl]acetyl]amino]-*N*-(butoxycarbonyl)-L-alanine (0.189 g, 0.587 mmol) for 15 min. The mixture was stirred at 0 °C for 4 h then allowed to warm-up to room temperature overnight. The reaction mixture was concentrated to an oily residue. The oily residue was titrated with anhydrous ether to afford the resulting imidate as a semi-solid mass. The imidate was then re-dissolved in methanol (50 ml) and to this solution was added 1-*N*-Boc-aminohexanol (0.250 g, 1.17 mmol). The reaction mixture was stirred at room temperature for 48 h and concentrated. The crude mixture was purified via silica gel column chromatography (dichloromethane:methanol:1%ammonia) to afford the desired *N*-Boc-hexylamidine isoxazoline intermediate (0.21 g). ESI mass spectrum 647 (M+H, 100). The above isoxazoline compound was then treated with lithium hydroxide (0.014 g, 0.325 mmol) in an aqueous methanol solution (20 ml). The reaction mixture was stirred at room temperature overnight, concentrated and quenched with 1 N HCl. The organics were extracted several times with ethyl acetate (5 × 100 ml), concentrated, re-dissolved in dichloromethane (20 ml) followed by treatment with TFA (0.5 ml). The reaction was complete in a few hours and concentrated and purified via preparative reverse phase HPLC (C18 Dynamax column). Lyophilization then afforded the desired product as a colorless oil (0.09 g). ^{1}H-NMR (DMSO d_6): 8.19–8.04 (m, 1H), 7.98 (m, 2H), 7.80–7.45 (m, 3H), 7.30 (d, 1H), 5.04 (m, 1H), 4.17 (m, 1H), 3.98 (m, 2H), 3.61–3.46 (m, 3H), 3.30–3.15 (m, 2H), 2.82–2.69 (m, 2H), 2.62–2.40 (m, 2H), 1.62–1.44 (m, 8H), 1.40–1.32 (m, 8H), 0.99 (m, 3H) ppm. ESI mass spectrum 534 (M+H, 100). High resolution mass spectrum calculated 533.308759; found 533.309529.

2.3. Preparation of the immunogen

A single-step glutaraldehyde coupling approach was employed [2]. The hapten precursor was diluted with PBS (DMSO was initially added at one tenth the final volume to assist solubilization) and a carrier protein (KLH) was added (~ 1 mol of hapten per 50 amino acids of carrier). An equal volume of 0.2% glutaraldehyde in PBS was slowly added and the solution was incubated at room temperature for 1 h. Glycine was added at a final concentration of 200 mM and incubation was continued for another hour with constant stirring. The conjugated immunogen was separated from unbound immunogen by dialysis against PBS.

2.4. Antiserum production in rabbits

Three New Zealand white rabbits were immunized with immunogen (500 μg/animal) in saline solution by intradermal injection. The animals received subsequent booster immunizations (250 μg subcutaneous injections) at 3 week intervals. Test bleeds were obtained 10 days after each booster and evaluated for acceptable titre levels with final production bleeds performed ~4 months after initial immunization. Antisera were stored at −20 °C until use.

2.5. Preparation of the radioligand

Radioiodination of SJ009 (Fig. 1), an XV459 analogue, was performed using a modified Chloramine-T procedure [3]. The radioligand was purified by reverse-phase HPLC using a C-18 column and a gradient mobile phase system consisting of 10% acetonitrile in 0.1% TFA which increased to 90% acetonitrile over 60 min. Purified ^{125}I-SJ009 was collected with a fraction collector and tested with anti-XV459 antibody before use. Expiration

of the ^{125}I-SJ009 tracer stock was 3 weeks from date of iodination.

2.6. Preparation of radioimmunoassay reagents and buffers

^{125}I-SJ009 radioligand (1.5 ml) was repurified each day before use with 10 mg of AG 1-X8 resin in a 12 × 75 mm glass tube followed by centrifugation for 5 min at 2200 × g. The supernatant was subsequently diluted to 25 ml with assay buffer (1% BSA and 0.005% normal rabbit serum in DPBS) resulting in a radioligand working solution containing ~20 000 cpm/200 μl.

The XV459 antiserum was diluted 1:2500 in the assay buffer and used within 1 week. GARGG solution was diluted 1:10 in the assay buffer just prior to use.

DMP 728, a GP IIb/IIIa receptor antagonist, was used to displace XV459 from platelet receptors. For patient samples, the displacer (2.0 μmol) was immediately added to 2.0 ml EDTA whole blood samples which then underwent a 120–180 min incubation period prior to centrifugation and plasma harvest. For assay calibration standards, a 2.0 mM DMP 728 displacer solution was prepared in pH 4.0 acetic acid and then further diluted to 0.25 mM using assay buffer at the time of assay to mimic the DMP 728 plasma concentration in subject samples which were assayed as a 1:8 dilution. Both solutions were stored at 2–8 °C. For quality control (QC) samples, DMP 728 (2.0 mM final concentration) was added to plasma prior to the addition of XV459.

2.7. XV459 stock preparations

XV459 primary stock solutions for standards and QC samples were prepared from XP280 (the benzene sulfonate salt of XV459) in pH 4 acetic acid and stored at 2–8 °C for up to 6 months. Working stock solutions were prepared by diluting the primary stock with the assay buffer. Calibration standards were prepared daily by serial dilution (1:2 with assay buffer) to yield seven standards over a free form concentration range of 0.014–0.905 ng/ml (0.032–2.09 nM). XV459 QC samples at concentrations of 0.35, 0.70, 1.0, 2.0, 3.5, and 6.0 ng/ml (0.81, 1.61, 2.31, 4.61, 8.07, 13.8 nM) were prepared in advance in EDTA human plasma containing 2.0 mM DMP 728 and stored at $\leqslant -15$ °C along with patient samples. QC samples were assayed as a 1:8 dilution in assay buffer unless otherwise noted.

2.8. Radioimmunoassay procedure

Duplicate standards (100 μl) and QC samples (12.5 μl) were aliquoted into individual 12 × 75 mm glass culture tubes followed by the addition of 300 or 587.5 μl assay buffer for standards and QC samples, respectively. Standards also received 100 μl of EDTA plasma (diluted 1:8 in assay buffer) and 100 μl 0.25 mM DMP 728 buffer solution. To all tubes, 200 μl of tracer and 200 μl of antiserum were added. After 1 min of vortexing, tubes were incubated in a 37 °C waterbath for 1 h then at room temperature for an additional 3 h. At the end of the incubation period, 200 μl of GARGG were added and tubes were vortexed and incubated again at room temperature for 30 min. Following incubation, 500 μl of 6% PEG were added, tubes were vortexed then centrifuged for 20 min at 2200 × g, and the supernatant was immediately decanted to waste. Radioactivity in the pellet was measured by counting in a micromedic gamma spectrometer for 2 min. Unknown total XV459 (unbound and GP IIb/IIIa displaced) plasma concentrations were determined from a sigmoid regression equation (weighted 4-parameter curve) used to fit the standards.

2.9. RIA validation

Intraday assay accuracy and precision were assessed using six replicates of each QC sample. Interday assay performance was evaluated using duplicate QC samples at each concentration level measured over 13 separate assays, except for the highest QC (6.0 ng/ml) which was only evaluated over the last 4 days. Assay specificity was evaluated with respect to both non-specific (matrix effects) and specific (cross-reactivity) sources of interference. For non-specific interference, 12 different blank EDTA human plasma pools were screened for background XV459 readings to

Table 1
XV459 intra- and inter day assay accuracy and precision

	Neat plasma	Nominal XV459 concentration (ng/ml)					
		0.35	0.70	1.00	2.00	3.50	6.00
Intraday ($n=6$)							
Mean	<0.173	0.33	0.62	0.92	1.87	3.35	5.40
SD	–	0.03	0.03	0.04	0.06	0.09	0.53
% RSD	–	9.1	4.8	4.3	3.2	2.7	9.8
% diff [a]	–	7.8	10.8	8.2	6.6	4.3	11.5
Interday ($n=26$)							
Mean		0.35	0.68	1.01	1.99	3.56	5.84[b]
SD		0.06	0.08	0.08	0.16	0.33	0.46
% RSD		17.1	11.8	7.9	8.0	9.3	7.9
% diff[a]		14.6	9.5	6.4	6.6	7.1	5.8

[a] Mean % difference calculated without regard to sign.
[b] $n=8$.

evaluate potential interference by endogenous plasma components. Specific interference was investigated by evaluating 20 compounds, including a putative roxifiban metabolite (SE222), for antibody selectivity. Compounds were selected based on structural similarity or probability as a concomitant medication used in anti-platelet therapy or other cardiovascular treatment. Finally, definitive demonstration of the immunoassay's specificity was accomplished by its cross-validation to an existing LC-MS/MS method. Thirty blinded test samples (a combination of pooled and individual dosed subject samples) were prepared and assayed by both methods. LC-MS/MS was performed on a Sciex (Ontario, Canada) Model API III+triple quadrupole mass spectrometer using positive ion Turbo IonSpray with multiple reaction monitoring ion detection. Prior to detection, XV459 and a deuterium (d_4) labeled internal standard were isolated from plasma by solid phase extraction. The LC-MS/MS method was validated over a 0.1–40 ng/ml (0.2–92 nM) concentration range using a 0.2 ml sample aliquot. Details of the LC-MS/MS method have been described elsewhere [4]. For comparative purposes, the LC-MS/MS results were defined as the reference method results due to presumed specificity superiority of this technique. Plasma concentrations determined from both methods were statistically compared using ratio analysis, paired t-test, and regression analysis [5].

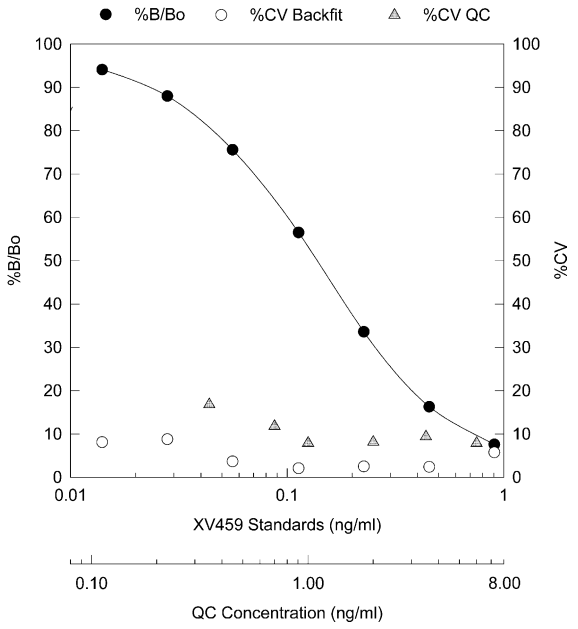

Fig. 2. Calibration curve and precision profiles for the determination of XV459 in human plasma.

Both long-term and freeze/thaw stability of XV459 were also evaluated using the RIA method. QC samples were prepared fresh and assayed immediately and then at periodic intervals for up to 16 months.

Table 2
XV459 dilutional parallelism

Dilution factor	Replicate controls of 6.00 ng/ml XV459 ($n = 4$)			
	Mean	SD	% RSD	Mean % diff[a]
1:8	5.97	0.47	7.9	6.3
1:16	5.86	0.21	3.6	2.5
1:32	5.35	0.48	9.0	10.8
1:64	5.16	0.55	10.7	14.0

[a] Mean % difference calculated without regard to sign.

3. Results

3.1. Assay accuracy, precision, and range

Intra- and inter day accuracy and precision estimates are summarized in Table 1 and a representative calibration curve and interday precision profiles for standards and QC samples are presented in Fig. 2. The concentration range of the calibration standards (0.014–0.905 ng/ml) correlated to 94.1–7.6% binding, respectively. The assay was judged sufficiently accurate as the QC samples intra- and inter day absolute mean % differences between measured and nominal values ranged from 4.3 to 11.5% and 5.8 to 14.6%, respectively. The assay precision, as assessed by relative standard deviation (RSD) of QC samples, was $< 18\%$. Assay range was defined as the lowest (0.35 ng/ml; 0.81 nM) and highest (6.0 ng/ml; 13.8 nM) plasma QC concentrations that could be reproducibly measured with an interday RSD of $\leqslant 20\%$. In order to extend the assay range, appropriate dilutional parallelism was examined by measuring selected QC samples at further dilutions of 1:16, 1:32, and 1:64 (Table 2). The assay was shown to be dilutionally parallel up to a dilution factor of 1:64, thus the assay can accommodate plasma samples up to 48.0 ng/ml (110.7 nM).

3.2. Assay specificity

Twelve different blank EDTA human plasma pools were screened for background XV459 readings to evaluate potential interference by endogenous plasma components. All blank samples read below the lower limit of quantification, suggesting that matrix interference was minimal.

Table 3
Summary of compounds tested for cross-reactivity

Compound	Therapeutic class	% cross-reactivity
Acetaminophen	Analgesic	< 0.001
Amiodarone	Coronary vasodilator	< 0.001
Aspirin	Analgesic, anti-platelet	< 0.001
Atenolol	Beta adrenergic blocker	0.001
Captopril	Ace inhibitor	< 0.001
Clopidogrel	Anti-platelet	< 0.001
Digoxin	Cardiotonic	0.002
Diltiazem	Calcium channel blocker	< 0.001
DMP 728	Anti-platelet	< 0.001
Roxifiban	Anti-platelet	111
Gemfibrozil	Anti-hyperlipidemic	< 0.001
Isosorbide dinitrate	Nitrate anti-anginal	< 0.001
Metoprolol	Beta adrenergic blocker	< 0.001
Nifedipine	Calcium channel blocker	< 0.001
Procainamide	Anti-arrhythmic	< 0.001
Quinidine	Anti-arrhythmic	< 0.001
Quinine	Anti-arrhythmic	< 0.001
Salicylic acid	Analgesic	< 0.001
SE222	Putative roxifiban metabolite	0.26
Warfarin	Anti-coagulant	< 0.001

Table 3 provides a summary of the antibody cross-reactivity. Except for roxifiban, which had a cross-reactivity of 111%, all other compounds had cross-reactivities of 0.26% or less. In vivo studies have shown that no detectable roxifiban plasma concentrations are evident after oral administration to humans (Pieniaszek, unpublished data).

For the cross-validation portion of the study, both pooled and individual roxifiban-dosed subject samples were evaluated at concentrations that covered the validated RIA assay range. Comparison of RIA to LC-MS/MS results produced individual ratios (RIA/LC-MS/MS) ranging from 0.80 to 1.18 with a mean ratio of 1.00 and RSD of 8.0%. The majority of the ratios (82%) fell between 0.90 and 1.10 with an unbiased distribution of results above and below unity (Table 4). Results of the paired t-test showed no significant differences with a P value of 0.91. Regression analysis, plotted in Fig. 3, showed good correlation between the two methods ($r^2 = 0.98$).

Table 4
Comparison of individual XV459 assay results[a] from RIA and LC-MS/MS methods

LC-MS/MS ng/ml	RIA ng/ml	% difference	Ratio (RIA/LC-MS/MS)
0.47	0.43	−8.5	0.91
1.10	1.04	−5.5	0.95
4.11	3.96	−3.6	0.96
4.80	4.39	−8.5	0.91
5.92	6.54	10.5	1.10
6.62	6.77	2.3	1.02
6.78	6.12	−9.7	0.90
7.14	6.63	−7.1	0.93
7.77	8.06	3.7	1.04
8.02	8.06	0.5	1.00
8.89	8.94	0.6	1.01
9.36	7.47	−20.2	0.80
9.75	10.7	9.7	1.10
10.3	10.1	−1.9	0.98
11.4	12.7	11.4	1.11
12.1	12.2	0.8	1.01
13.1	14.2	8.4	1.08
13.9	14.2	2.2	1.02
15.5	16.3	5.2	1.05
16.7	16.8	0.6	1.01
17.5	20.6	17.7	1.18
18.5	20.5	10.8	1.11
21.2	21.8	2.8	1.03
22.8	23.7	3.9	1.04
27.0	26.6	−1.5	0.99
27.1	25.7	−5.2	0.95
28.4	27.2	−4.2	0.96
36.7	32.0	−12.8	0.87
Mean[b]		6.4	1.00
SD			0.08
% RSD			8.0

[a] Pooled and individual roxifiban-dosed subject samples.
[b] Mean % difference calculated without regard to sign.

3.3. Analyte stability in plasma

Stability studies showed that XV459 was stable in human plasma frozen at $\leqslant -15$ °C for at least 16 months and through at least 3 freeze/thaw cycles (Table 5).

3.4. Application to clinical trials

This RIA method has been successfully used to quantify XV459 plasma concentrations in over 10 000 plasma samples from healthy subjects and from coronary artery disease and PAD patients. Fig. 4 contains representative XV459 plasma concentration–time profiles from two healthy subjects following the last two doses of a 1.0 mg bid dosing regimen of roxifiban. The profiles are characteristic of XV459 steady-state pharmacokinetics: minimal concentration flux during the dosing interval, a long terminal $t_{1/2}$ associated with the decline in concentration following cessation of roxifiban administration, and large inter-patient variability in plasma concentrations presumably due to low and variable absorption [6]. XV459 plasma concentrations were determined using both the LC-MS/MS assay and the RIA. These profiles depict a slight negative bias in the RIA method; although previous cross-validation

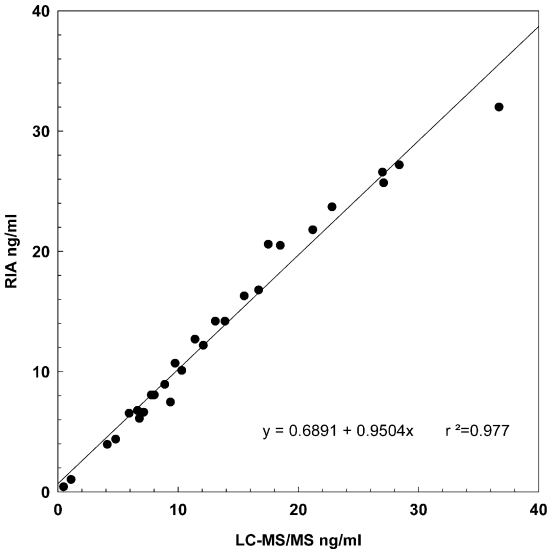

Fig. 3. Linear regression of XV459 plasma concentrations determined after RIA and LC-MS/MS analyses.

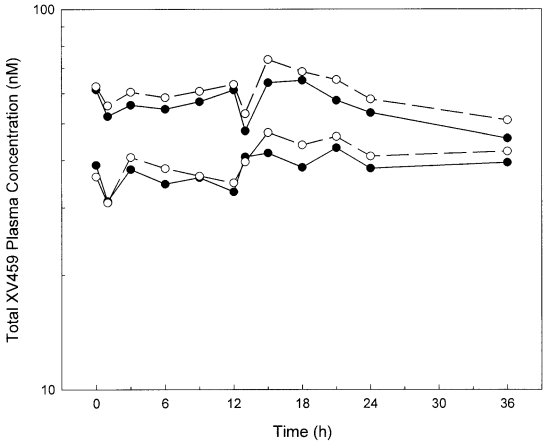

Fig. 4. Representative XV459 plasma concentration–time profiles from two healthy subjects receiving 1.0 mg bid roxifiban: measured by LC-MS/MS (open circles) and RIA (closed circles).

results were not indicative of assay bias (Table 4). Nevertheless, the difference between subject concentrations determined via both methods was $<$ 15% with most values $<10\%$. Additionally, the negative bias observed in these data would suggest that the apparent difference is not the result of metabolite interference in the RIA.

Table 5
Stability of XV459 in human plasma samples

Storage condition	XV459 added concentration (ng/ml)	% recovery
16 months at −20 °C	0.35	131, 118
	0.70	102, 99.3
	2.00	98.0, 90.5
	6.00	98.8, 87.2
Three freeze-thaw cycles	0.35	103
	0.70	100
	2.00	106
	6.00	90.2

Subsequent to the development and validation of the RIA, six minor hydroxylated human metabolites of roxifiban were identified (Pieniaszek and Mutlib, unpublished data). These metabolites exist in systemic human plasma only as the hydroxylated derivations of XV459. The metabolites were tested for antibody selectivity in the RIA and cross-reactivity ranged from 0.22 to 1.6%. SE222, previously believed to be a potential human metabolite, was not confirmed in human plasma or urine.

4. Discussion

Bioanalytical methods typically undergo an evolutionary development process over the lifespan of a drug. Not only does an individual method undergo adaptation and improvement over time, but the need for more than one method format to efficiently advance compounds frequently arises. In today's fast-paced pharmaceutical R&D environment, RIA methods are not generally employed for early discovery/pre-clinical compound characterization due to their longer development process (>4 months) and potential inability to recognize structurally similar compounds/metabolites. As a compound matures into clinical development, the utility of an RIA has distinct advantages. Although, the present RIA method took approximately 6 months to develop, it offers several benefits over the original

LC-MS/MS method also utilized for this compound, including comparable sensitivity using less sample volume, increased sample throughput, and an appreciable reduction in assay costs. Cross-validation of the RIA back to the LC-MS/MS method confirmed RIA assay specificity and demonstrated assay equivalence of the two methods.

In conclusion, a simple, rapid, and robust RIA for XV459 was successfully developed and validated and shown to be suitable in quantifying plasma concentrations in clinical trials evaluating the pharmacokinetics of XV459.

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