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Development and Validation of Eltrombopag Determination in Human Plasma Blood by HPLC-MS Method

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Abstract

Introduction. "Eltrombopag" is a thrombopoietin receptor agonist (TPO-RA) that is approved for the treatment of immune thrombocytopenic purpura (ITP). According to the literature, very few analytical methods for determining eltrombopag in biological samples have been reported. To study the pharmacokinetics of new formulations of eltrombopag, a sensitive and specific method is required that allows one to accurately determine the concentration of eltrombopag in human blood plasma. Normally, HPLC methods should provide time, accuracy, and sensitivity as a result, it is necessary to develop fast or ultra-fast methods such as LC-MS/MS without any loss in sensitivity or separation efficiency.

Aim. We aimed to develop and validate a method for the quantitative determination of eltrombopag levels in human plasma by using HPLC with mass spectrometric detection for performing the analytical part of pharmacokinetic studies.

Materials and methods. Eltrombopag levels were determined in human plasma by HPLC with mass spectrometric detection. The samples were prepared using protein deposition.

Results and discussion. The method was validated for selectivity, matrix effect, calibration curve, accuracy, precision, the limit of quantification, carry-over effect, and sample stability.

Conclusion. The method for the determination of eltrombopag levels in human plasma has been developed and validated by HPLC-MS. The analytical range of eltrombopag levels in human plasma was 10–6750 ng/ml. This method could be used to determine eltrombopag levels in plasma for PK and BE studies.

Keywords: "Eltrombopag", plasma, HPLC-MS/MS, validation, bioequivalence

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Abdullah M. AL-Dhuraibi, Alexandr L. Kulikov have developed and validated an analytical method and carried out statistical processing of the obtained results. Mikhail V. Pokrovskiy carried out the organization of work in this direction. All the above authors participated in the discussion of the results in the format of scientific discussion.

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Разработка и валидация определения элтромбопага в плазме крови человека методом ВЭЖХ-МС

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Резюме

Введение. «Элтромбопаг» – агонист рецепторов тромбопоэтина (TPO-RAs), одобренный для лечения иммунной тромбоцитопенической пурпуры (ИТП). В соответствии с изученной литературой, было обнаружено, что существует очень мало аналитических методов для определения элтромбопага в биологических образцах. Для изучения фармакокинетики новых составов элтромбопага необходим чувствительный и специфичный метод, позволяющий точно определять концентрации элтромбопага в плазме крови человека. Обычно методы ВЭЖХ должны обеспечивать время, точность и чувствительность. Поэтому необходимо разработать быстрые или сверхбыстрые методы, такие как LC-MS/MS, без какой-либо потери эффективности и чувствительности разделения.

Цель. Наша цель заключалась в разработке и валидации метода, нацеленное на количественное определение элтромбопага в человеческой плазме крови при том, чтобы использовались методы высокоэффективной жидкостной хроматографии с одноквадрупольным масс-спектрометрическим детектированием (ВЭЖХ-МС) для выполнения аналитической части фармакокинетических исследований.

Материалы и методы. Количество элтромбопага в плазме человека определяли с помощью ВЭЖХ-МС. Способ осаждения белков был проведен в качестве пробоподготовки.
Результаты и обсуждение. Разработанную методику валидировали по линейности, переносу пробы, стабильности, селективности, точности, эффекту матрицы, прецизионности, пределу количественного определения препарата.
Заключение. Метод определения элтромбопага в плазме крови человека был разработан и валидирован методом ВЭЖХ-МС. При исследовании аналитического диапазона препарат элтромбопаг в плазме составил 10–6750 нг/мл. Данный аналитический диапазон разработанной методики может быть применен для проведения аналитической части фармакокинетических исследований препаратов, содержащих в составе элтромбопаг.

Ключевые слова: «Элтромбопаг», плазма, ВЭЖХ-МС/МС, валидация, биоэквивалентность

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. А. М. Аль-Дураиби, А. Л. Куликов участвовали в разработке и валидации биоаналитической методики и проводили статистическую обработку данных. М. В. Покровский отвечал за организационную часть исследования. Все вышеуказанные авторы участвовали в обсуждении полученных результатов в форме научной дискуссии.

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INTRODUCTION

Idiopathic thrombocytopenic purpura (ITP) is a disease in which the immune system attacks platelets and causes them to decrease in number, putting the patient at risk of bleeding [1]. Eltrombopag is an orally bioavailable thrombopoietin receptor agonist (TPO-RAs) approved for the treatment of immune thrombocytopenic purpura (ITP). The drug is prescribed for patients who do not have an adequate response to corticosteroids, immunoglobulins, or splenectomy. Current indications for the use of eltrombopag include the treatment of hepatitis C and severe aplastic anemia [2–4] (figure 1). The usual starting dose is 25 mg daily, and the maintenance dose is 75 mg daily [1].

The drug has a high affinity for binding to plasma proteins and is predominantly excreted by metabolism in the liver and gastrointestinal system [5]. According to the literature, very few analytical methods have been reported for the determination of Eltrombopag in biological samples, including liquid chromatography/mass spectrometry methods [6, 7] and high-performance liquid chromatography (HPLC) methods [8]. For example, a simple HPLC method was developed to determine the concentration of eltrombopag in serum using a conventional octadecylsilyl silica gel (ODS) column, isocratic elution, and UV detection [4]. Olamine in pharmaceutical dosage form using RP-

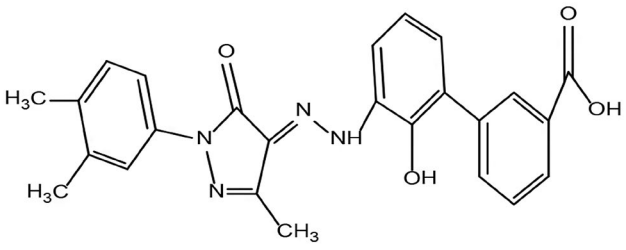

Figure 1. Chemical structure of "Eltrombopag"

HPLC, Reversed Phase Ultra High Performance Liquid Chromatography (RP-UPLC), or a new flexible, mass-compatible isocratic stability method developed using Ultra High Performance Liquid Chromatography (UHPLC) to evaluate eltrombopag together with its impurities in the composition of tablets [9–11]. Most of the analytical methods reported so far have only been designed to investigate the pharmacokinetic properties of a drug. The quantification of eltrombopag in human plasma remains difficult due to its physicochemical properties and polarity. Studying the pharmacokinetics of new formulations of eltrombopag, a sensitive and specific method is required that allows one to accurately determine the concentration of eltrombopag in human blood plasma. Typically, HPLC methods must sacrifice time, resolution, or sensitivity. Therefore, it is necessary to develop fast or ultra-fast methods such as LC-MS or MS without any loss in separation efficiency or sensitivity.

Table 1. **Bioanalytical methods of quantitative determination of Eltrombopag levels**

Analytical method	Sample preparation	Object	Analytical range, ng/ml	Reference
HPLC-MS/MS	Protein precipitation by acetonitrile	Human blood plasma	10–2500	[12]
HPLC-MS/MS	Protein precipitation by acetonitrile	Human blood plasma	50–10007	[8]

Thus, this study aimed to develop a simple, fast, and sensitive LC-MS/MS method for the determination of eltrombopag in human plasma (tables 1, 2).

Table 2. **The concentrations of analiytes at each calibration level**

Level	Analityte concentration "Eltrombopag", ng/ml	IS concentration, ng/m
1	10	748
2	30	748
3	337	748
4	1012	748
5	1350	748
6	1687	748
7	5061	748
8	6748	748
LLOQ	10	748
L	30	748
M	4049	748
H	6750	748

MATERIALS AND METHODS

Equipment

- Laboratory centrifuge, multichannel Centrifuge 5430R (Eppendorf, Germany, certified until 25.02.2020).
- Laboratory shaker Thermo Mixer C (Eppendorf, Germany).
- Electronic scales PA 213C (OHAUS Corporation, США, verification certificate is valid until 25.02.2021).
- Liquid chromatograph Thermo Fisher Scientific UltiMate 3000 RS LC (Thermo Fisher Scientific, USA) with mass-selective detector Thermo Fisher Scientific Velos Pro, verification certificate is valid until 06.02.2020.
- Chromatographic column 2.1 x 150 mm, ZORBAX Eclipse Plus, 2.1 × 50 mm, 1.8 µm (cat. No. 959757-902).
- Ultrasonic bath with a radiator power of 3 W.

Reagents and solutions

- Ammonia (25 %) (PanReac, Spain, catalogue number 141129).
- Acetic (glacial) acid, for HPLC (PanReac, Spain, catalogue no. 361008.1611).
- Acetonitrile (Gradient Grade, Merck KGaA, Germany, catalogue number 100030.2500).
- Methanol (hyper grade for LC-MS LiChrosolv® MerckMillipore, USA, catalog no. 106035).

1. Standard samples

- Standard sample of eltrombopag mono ethanol amine E10619 (valid until 06.21).
- Active pharmaceutical substance used to prepare quality control solutions, lot 010719.
- Internal standard USP RS lot G0K135 (valid in use day) glimepiride: 1-[[4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl]phenyl]sulfonyl]-3-trans-(4-methylcyclohexyl)urine-on, molecular formula: C24H34N4O5S and molecular weight: 490.6 g/mol CAS: 93479-97-1.

2. Biological matrix

- Blood plasma from 6 individual sources.

Conditions for carrying out and preparation of solutions

Preparation of solutions

During sampling and subsequent measurement of parameters, all available solutions were 18–20 °C (by the State Pharmacopoeia XIII-XIVed). The measurement determinations of each parameter were carried out under constant conditions. All solutions were prepared by the bioanalytical method (Tables 3, 4). Reagents used for preparation:

Internal standard solution m = 12.63 mg.

- Initial solution 1 for the preparation of calibration solutions: weighed portion of eltrombopag monoethanolamine (exact weighed portion) *m* = 14.40 mg (in terms of eltrombopag *m* = 12.65 mg);

Table 3. **An aliquot of stock solutions was added to C**

№. of original solution	№ C							
	1	2	3	4	5	6	7	8
Aliquot of stock solution 3, ml	0,015	0,045	–	–	–	–	–	–
Aliquot of stock solution 2, ml	–	–	0,01	0,03	0,04	0,05	0,15	0,2
[1]C_{eltr}, in solution, ng/ml	3	9	101	303	405	506	1518	2024
[2]C_{eltr}, in terms of 1 ml of plasma, ng/ml	10	30	337	1012	1350	1687	5061	6750

Note. [1] Quantity of ELTR in the final solution.
[2] The amount of ELTR in terms of 1 ml of blood plasma.

- Solutions for quality standards (C) $m = 14.40$ mg (in terms of eltrombopag m = 12.65 mg) The equipment used is presented in section 2.4. Preparation procedures and chromatographic conditions were consistent with bioanalytical procedures (Tables 3, 4).

Table 4. **An aliquot of stock solutions was added to QC**

№. of original solution	QC			
	LLOQ	L	M	H
Aliquot of stock solution 3, ml	0,015	0,045	–	–
Aliquot of stock solution 2, ml	–	–	0,12	0,2
[1]C eltr, in solution, ng/ml	3	9	1215	2024
[2]Celtr, in terms of 1 ml of plasma, ng/ml	10	30	4049	6750

Note. [1] Quantity of ELTR in the final solution.
[2] The amount of ELTR in terms of 1 ml of blood plasma.

Conditions for chromatographic separation and detection

Equipment preparation. At the beginning of the work, the liquid chromatography was switched on, and then an exposure was carried out for 30 minutes for the operating parameters to stabilize. Next, a steel chromatographic column with a size of 2.1 × 50 mm was connected, which was filled with a reversed-phase sorbent octadecyl silica gel with a particle size of 1.8 µm (for example, ZORBAX Eclipse Plus, 2.1 × 50 mm, 1.8 µm cat. No. 959757-902). Next, the temperature of the column thermostat is set to 30 ± 5 °C, and the flow rate of 0.4 ml/min is set to the pumping system. In this case, the separation mode is gradient, consisting of two stages (main elution and washing) according to the following scheme (Tables 5).

Table 5. **Gradient elution**

Time, min	Mobile phase flow rate, ml/min	0.03 % acetic acid, % vol	Acetonitrile, % vol	Stage name
0	0,4	40	60	Main elution
3,0	0,4	25	75	
3,1	0,6	10	90	Flushing, equilibration
4,5	0,6	10	90	
4,6	0,4	40	60	
5,5	0,4	40	60	

5.0 µL of an automatic sample introduction system was indicated. Chromatography time for one cycle: 5.5 min.

Approximate retention times:

- internal standard: about 1.0 min;
- eltrombopag: about 2.8 min.

All work with the mass selective detector was carried out in strict accordance with the instruction manual and SOP. Then the "Stand by" mode was turned off, holding for 15 minutes to stabilize the baseline, and the following parameters were set in Table 6:

RESULTS AND DISCUSSION

Method development

This work is devoted to the study of the validation characteristics of the developed bioanalytical method for the quantitative determination of the concentration of eltrombopag in a biological matrix (human blood plasma).

Table 6. **Parameters**

Ionization type	ESI, SRM «+», CID ionization mode for eltrombopag ESI, SRM «+», CID ionization mode for internal standard
Mass transition	Eltrombopag 443.22 → 322.7; IS 491.60 → 452.1
Source temperature	380 °C
Capillary temperature	400 °C
Source voltage	3000 V (Eltr), 3000 V (IS)
Impact energy	Eltr – 30 IS – 35
Collision gas	Helium
Flow Sheath газа	60
Aux gas flow	20
Sweep gas flow	1
Other parameters according to the automatic optimization of the device.	

In modern clinical research practice, there are a huge number of bioanalytical methods for determining 95 % of publicly published methods are based on high-performance liquid chromatography (HPLC) with various detections. High-level studies associated with clinical trials typically use tandem mass spectrometric (MS/MS) detection. This is the gold standard for reliable and accurate results. HPLC MS/MS as a method is highly dependent on many factors. For example, the type of ionization used, the conditions and temperature regimes, the composition and quality of mobile and stationary phases, and other key characteristics. The complexity of all the main processes, and especially the processes of MS/MS detection, is so high that it is extremely difficult to achieve an acceptable reproducibility of conditions on devices of the same types but from different manufacturers. This is due to the difference in the implementation of the fundamental physicochemical law underlying the definition.

In this regard, the need to develop bioanalytical methods concerning a specific set of equipment, a specific matrix, and a specific range of definitions is an urgent task. When viewed through a regulatory lens, it becomes clear that validation is an integral part of any development.

Thus, the purpose of the work was to confirm, based on laboratory studies, the reliability of the method for determining the concentration of Eltrombopag in a biological matrix to obtain a reliable result, and the subject of validation was a bioanalytical method for determining the concentration of Eltrombopag in a biological matrix.

Method validation

The validation of the research method aimed at the drug eltrombopag was carried out following the GLP standards, and the recommendations of the guidelines for the examination of medicines (FGBU "NCESMP" of the Ministry of Health of Russia) (2013) [13]. Bioanalytical Method Validation Guidance for Industry, FDA 05.2018[1]. Guideline on validation of bioanalytical methods. European Medicines Agency. Committee for medicinal products for human use[2]. Method validation includes indicators:

- selectivity;
- matrix effect;
- linearity/calibration curve;
- accuracy (at the inter-day and intra-day levels);
- accuracy and precision (at inter-day and intra-day levels);
- the lower limit of quantification;
- carryover;
- stability of eltrombopag stock solution and internal standard;
- stability of the drug eltrombopag in plasma under processing conditions and during the entire storage period (post-preparative stability; short-term temperature stability; stability after freezing or thawing; long-term temperature stability);
- recovery.

Selectivity

The selectivity of the bioanalytical technique was studied using six blank matrix samples from six individual sources. For this, the test solutions of the blank matrix were prepared and analyzed by the bioanalytical technique. The results are shown in Figures 2 and 3. The results obtained show that no interference is observed in any of the sources for any of the peaks under study; the technique has acceptable selectivity. The selectivity of the method is considered

[1] Bioanalytical Method Validation Guidance for Industry. Food and Drug Administration. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry. Accessed: 25.02.2021.

[2] Bioanalytical method validation. European Medicines Agency. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation. Accessed: 25.02.2021.

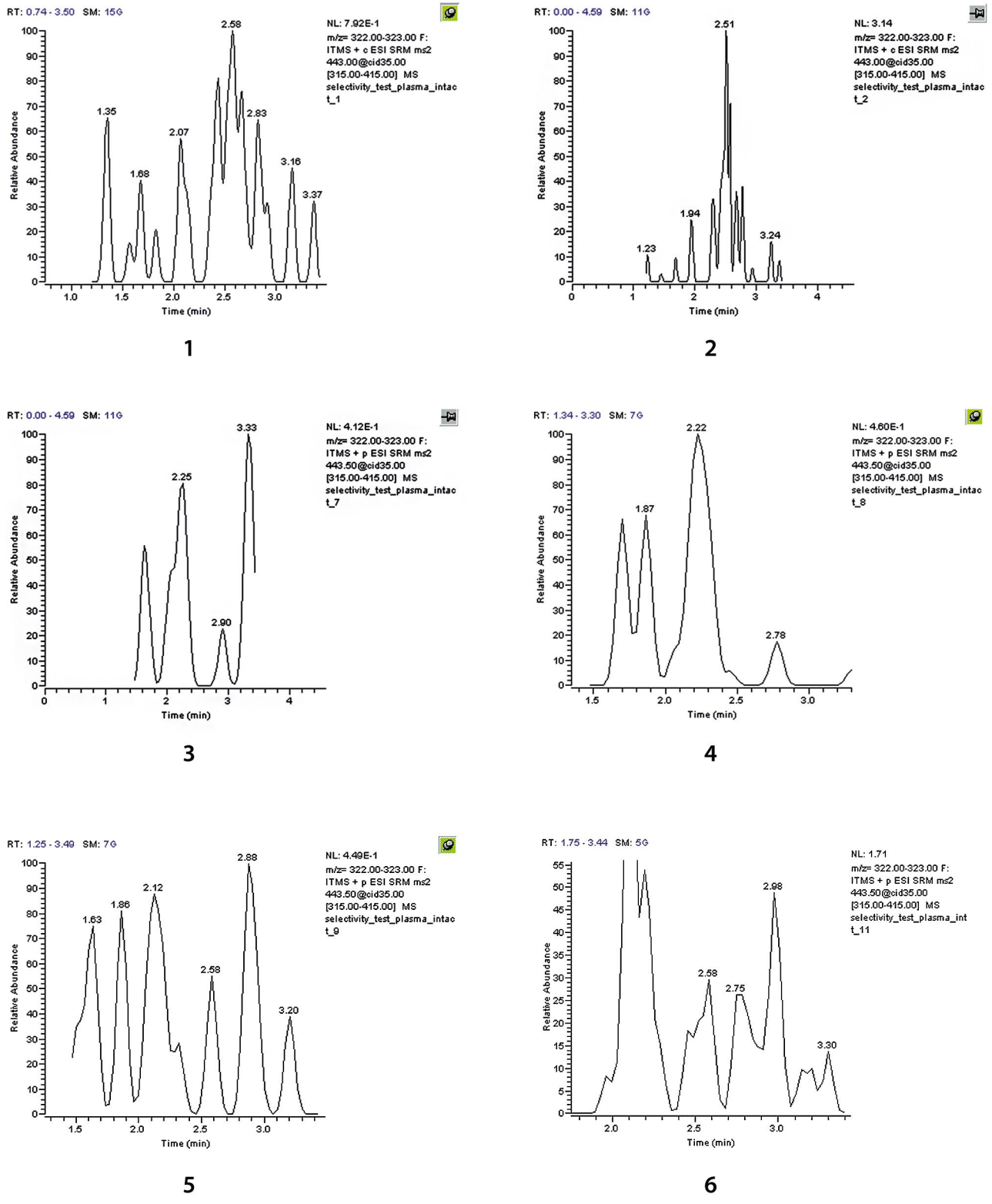

Figure 2. **Zero blood plasma from six different sources (1-6) in the scan range of Eltrombopag**

satisfactory, provided that the indicators are not more than 20% of the lower concentration of the determined substances and not more than 5 % of the content of the internal standard.

Matrix effect

The effect of the matrix on the results obtained was studied under the conditions of a bioanalytical method. For this, solutions of the blank matrix were

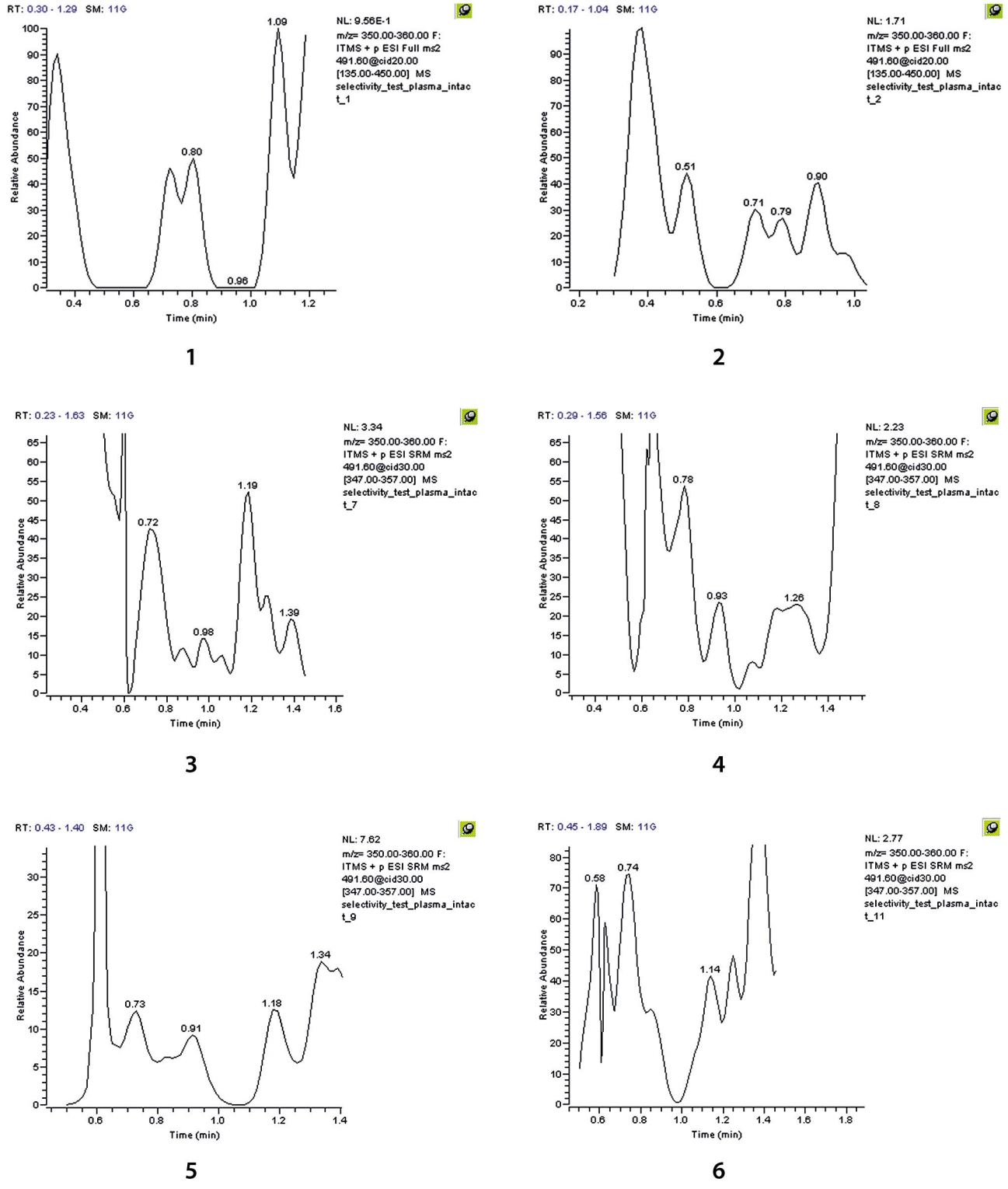

Figure 3. Zero blood plasma from six different sources (1–6) in the scan range of the internal standard

prepared and analyzed; the analyte was added to them at the level of L and H by the bioanalytical method (in tables 3–4). In addition, solutions were prepared in which the blank matrix was replaced with water (for calculating the absolute matrix factor). The results of the work performed are presented in tables 7–8. For the normalized matrix factor, CV does not exceed 15 %. For the absolute matrix factor, neither suppression nor amplification of the signal is observed. The influence of the matrix is stable.

Table 7. **Calculation of the normalized matrix factor**

Solution	S_{r_0}	S_{is_0}	Normalized ELTR area without matrix	S_r	S_{is}	Normalized ELTR area with matrix	MF	CV, %
LQC	51	652	0	49	654	0	1	4
	49	654	0	51	656	0	1	
	47	612	0	48	658	0	1	
	48	627	0	47	653	0	1	
	50	668	0	46	651	0	1	
	49	647	0	44	621	0	1	
HQC	9366	662	141	8990	661	136	1	4
	9288	639	145	8755	639	137	1	
	8999	622	145	8749	635	138	1	
	9211	637	145	8762	697	126	1	
	9087	644	141	8788	655	134	1	
	9120	672	136	9122	644	142	1	

Calibration Curve

Working solutions of internal standard glimepiride and standard mixed working solutions of eltrombopag were added to 7 samples of intact blood plasma at certain concentrations (Table 2). As a result of the data obtained after the analysis of the described samples, calibration graphs were built (Figure 3), including the equation of the calibration curve.

Following the bioanalytical methodology (Tables 3–4), solution C was prepared. There must be at least 7 concentration levels. Then they were analyzed, and a calibration curve was drawn. The equation of the curve and the correlation coefficient were also calculated. The amount found was recalculated according to the calibration curve. The technique has a linear response in the concentration range from 10.12 ng to 6748.29 ng in 1 ml of human blood plasma. Described by the equation $y = cx + b$. The correlation coefficient approaches 1 (well above 0.98). None of the errors in the recalculation of concentrations exceeded the permissible limits.

The results are presented in Table 9 and Figure 4.

Table 8. **Calculation of the absolute matrix factor**

Solution name	S	Average S ratio	CV, %	Solution name	S	Average S ratio	CV, %	Solution name	S	Average S ratio	CV, %
LQC without matrix addition	51	1,03	4,25	HQC without matrix addition	9366	1,04	2,41	Internal standard without matrix addition	662	0,986	3,10
	49				9288				639		
	47				8999				622		
	48				9211				637		
	50				9087				644		
	49				9120				672		
Average	49			Average	9179			Average	646		
CV,%	2,9			CV,%	1,5			CV,%	2,8		
LQC with matrix addition	49			HQC with matrix addition	8990			Internal standard with matrix addition	661		
	51				8755				639		
	48				8749				635		
	47				8762				697		
	46				8788				655		
	44				9122				644		
Average	48			Average	8861			Average	655		
CV,%	5,1			CV,%	1,8			CV,%	3,5		

Table 9. **Calculation of the linearity of the method for determining Eltrombopag**

Injected concentration, ng/ml	S_{ELTR}	S_{is}	S_{ELTR}/S_{is}	C_{ELTR}/C_{is}	C_{found}, ng/ml	*E*, %	Acceptance criterion
10	20	675	0	0	9	–7	No more than 20 %
10	19	635	0	0	10	–5	No more than 20 %
30	46	694	0	0	28	–8	No more than 15 %
30	47	682	0	0	29	–4	No more than 15 %
337	452	625	1	0	357	6	No more than 15 %
337	445	620	1	0	354	5	No more than 15 %
1012	1256	590	2	1	1060	5	No more than 15 %
1012	1198	598	2	1	997	–1	No more than 15 %
1350	1455	589	2	1	1231	–9	No more than 15 %
1350	1488	593	3	1	1250	–7	No more than 15 %
1687	2156	584	4	1	1842	9	No more than 15 %
1687	2355	660	4	1	1780	6	No more than 15 %
5061	6388	667	10	4	4787	–5	No more than 15 %
5061	6847	676	10	4	5063	0	No more than 15 %
6750	9201	677	14	5	6796	1	No more than 15%
6750	9301	676	14	5	6880	2	No more than 15 %

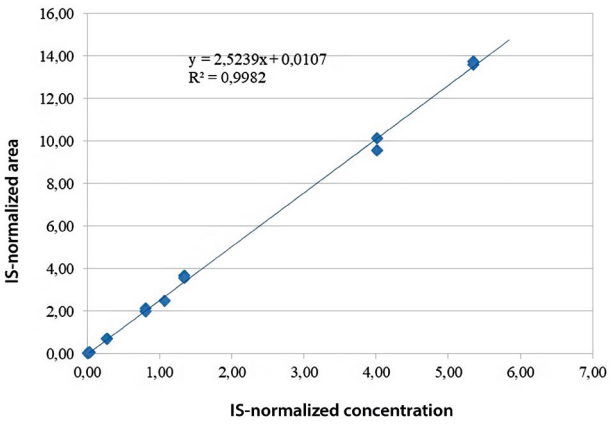

Figure 4. **Graphical display of the linear range of the peak area of Eltrombopag (normalized to internal standard) versus concentration (normalized to internal standard)**

Accuracy

Accuracy within a cycle

The Accuracy of the bioanalytical technique was studied within one analytical cycle. For this, solutions of C and QC were prepared and analyzed by the bioanalytical technique (in Tables 3–4). The results were processed. Acceptance criteria were used in evaluating the results. The results are presented in Table 2. As a result, the error of the found concentration relative to the introduced one does not exceed 20 % for the minimum concentration (LLOQ) for all other levels, no more than 15 %. The correctness of the technique within the cycle is satisfactory (Table 10).

Accuracy and precision

Table 2 shows the analysis of the calibration blood plasma samples that correspond to the levels LLOQ, L, M, and H. The analysis of the validation samples was carried out in three sequences of five samples for each level. Accuracy and precision were assessed within a cycle (sequence 1), between two cycles (sequences 1 and 2), and between three cycles (sequences 1, 2, and 3). As a result, the CV between the average results of the found concentration of eltrombopag, calculated from 4 analytical cycles performed on 4 different days using 4 different calibration curves, does not exceed 20 % for the minimum concentration (LLOQ) and no more than 15 % for all other levels. The precision of the technique between cycles is satisfactory (Table 11).

Table 10. **Accuracy of determination of Eltrombopag in human blood plasma (within the cycle, day 1)**

Solution	Injected C, ng/ml	C_{ELTR}/C_{is}	S_{ELTR}	S_{is}	f	C_{found}, ng/ml	C_{found}, ng/ml	s^2	S	P	t	Δ_x, ng	CV, %	e_r, %	Accuracy, %
НПК			19	646		9								–8	92
О			21	667		10								3	103
LLOQ	10	0	20	656	4	10	10	0	1	1	3	2	7	–2	98
			19	661		9								–11	89
			21	655		11								5	106
НКК			49	646		33								7	107
L			44	651		28								–6	94
	30	0	46	655	4	30	31	5	2	1	3	7	8	–2	98
			45	663		29								–6	94
			50	644		33								10	110
СКК			4988	656		3800								–6	94
M			4931	652		3779								–7	93
	4049	3	5188	657	4	3946	3977	51000	230	1	3	630	6	–3	98
			5325	662		4020								–1	99
			5764	664		4339								7	107
ВКК			9130	646		7067								5	105
H			8788	650		6760								0	100
	6748	5	9143	654	4	6991	6990	26000	160	1	3	450	2	4	104
			9041	652		6934								3	103
			9244	642		7200								7	107
$e_{r\ cpav.}$, %														0	
s														6	
Δe_r, %														3	

Table 11. **Calculation of the precision of the determination of Eltrombopag between cycles of quality control solutions**

Level	$C_{found\ cp}$, ng/ml	$C_{found\ cp}$, ng/ml	$C_{found\ cp}$, ng/ml	$C_{found\ cp}$, ng/ml	CV,%	Acceptance criterion
	Day 1	Day 2	Day 3	Day 4		
LLOQ	10	10	10	10	3	No more than 20 %
L	31	30	31	31	1	No more than 15 %
M	3977	3941	4019	4020	1	No more than 15 %
H	6990	6718	6608	7115	3	No more than 15 %

Lower Limit of Quantification (LLOQ)

From the linearity, accuracy, and precision data, the lower limit for the quantification of the methods was determined. Methods LLOQ: the minimum concentration of eltrombopag in blood plasma in the analytical range for which it is possible to quantify eltrombopag with RSD and E values less than 20 %. The lower detection limit of the method was 10.12 ng/ml. The signal-to-noise ratio was 19 at a concentration of 10.12 ng/ml (in terms of 1 ml of human blood plasma). Solution (LLOQ) is a less stringent calibration standard. The detection limit for eltrombopag is about 3.3 ng in 1 ml of plasma using this method. The result is shown in Figure 5.

Stability

The stability of the samples used at all stages of the bioanalytical method has been studied. Short-term stability: samples are stable after thawing for 4 hours; final solutions are stable in an auto-sampler for eight hours; samples withstand three thawing and freezing cycles. Long-term stability: The stability of the test samples was confirmed for 120 days at a storage temperature of –70 °C and for 45 days at –20 °C. All stock solutions are stable during their time of use (Tables 12–14).

When studying the stability of the samples, no errors were found that exceeded the permissible limits, these samples were stable after thawing for four hours. Further study of more than four hours is not rational, since it takes no more than 1.0 hours to prepare.one batch of samples (in extreme cases).

Table 12. Short-term stability during the period – from the moment of thawing to the final sample preparation

Solution	$C_{found\ cp}$, ng/ml	4 hours storage after stage (MO ELTR 05-20 Step 1)		Acceptance criterion
		$C_{found\ cp}$, ng / ml	E, %	
LQC	31	32	3	No more than 15 %
HQC	6990	7115	2	No more than 15 %

Carry-over effect

Analysis of the calibration samples and the intact blood plasma sample on the chromatogram of the intact blood plasma sample demonstrates the absence of peaks that corresponded in retention times to the peaks

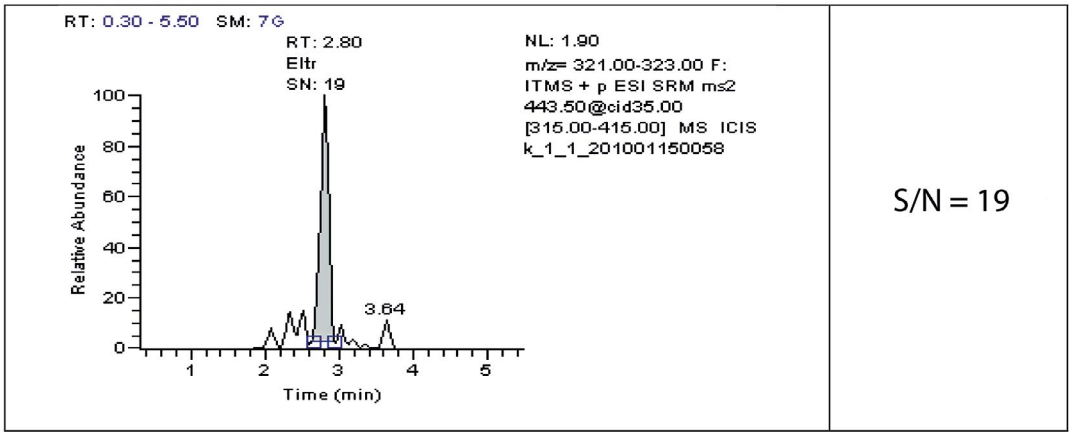

Figure 5. Calculation of the quantification limit for Eltrombopag

Table 13. Stability of the tested solutions after the final sample preparation when stored in an auto sampler +4 °C

Solution	0 hours storage in auto sampler	4 hours storage in auto sampler		8 hours storage in auto sampler		CV,%	Acceptance criterion
	$C_{found\ cp}$, ng/ml	$C_{found\ cp}$, ng/ml	*E*, %	$C_{found\ cp}$, ng/ml	E, %		
LQC	31	30	–2	30	–1	1	No more than 15%
HQC	6990	7032	1	6984	0	0	No more than 15%

Table 14. **Long-term stability under natural storage conditions**

Solution	Injected C, ng/ml	Point 0		Storage 30 days		Storage 45 days		Storage 120 days		CV, %	Acceptance criterion
		$C_{found\ cp}$, ng/ml	E, %	$C_{found\ cp}$, ng/ml	E, %	$C_{found\ cp}$, ng/ml	E, %	$C_{found\ cp}$, ng/ml	E, %		
LQC (storage at –20 °C)	30	30	0	30	–2,7	28	–7	–	–	3	No more than 15 %
LQC (storage at –70 °C)	30	29	–6	30	–1	29	–3	–	–	2	No more than 15 %
HQC (storage at –20 °C)	6748	6954	3	6712	–1	6796	1	6724	0	2	No more than 15 %
HQC (storage at –70 °C)	6748	6947	3	6839	1	6839	1	6755	0	1	No more than 15 %

of the studied substances and the internal standard with an area of more than 20 % of the LLOQ level. On the chromatograms of the blank matrix after the injection of the solution with the maximum concentration (C8), there are no peaks of the determined substances. There is also no carryover effect. The results are shown in Figure 6.

CONCLUSION

For the first time, a highly selective HPLC MS/MS method was successfully developed and tested for the determination of eltrombopag in human blood plasma. The analytical time for this method was 5.5 minutes, which was faster than the conventional HPLC method, and high sensitivity was observed in this method (the LLOQ for eltrombopag was 10 ng in 1 ml of blood plasma). In the study of the analytical range, the drug eltrombopag in plasma was 10–6750 ng/ml. The effect of the matrix was normalized: LQC: 3.6 %, HQC: 4.1 %. Eltrombopag is stable in plasma for 4 hours at room temperature, 8 hours after sample preparation, three freeze/thaw cycles, and 120 days at –70 °C storage. The accuracy range is within and between analytical cycles according to the maximum deviations for intra-serial (89.1–106.7 %) and inter-serial (98.3–102.6 %). The precision range was for intra-serial: 2.3–7.0 %, and for inter-serial: 2.8–3.4 %. Simple and fast protein precipitation was used to extract samples prior to HPLC MS/MS analysis. The method showed good accuracy, precision, and a wide linear concentration range. Therefore, this method is the "gold standard" for obtaining reliable, precise, and accurate results of clinical research in the field of pharmacokinetics.

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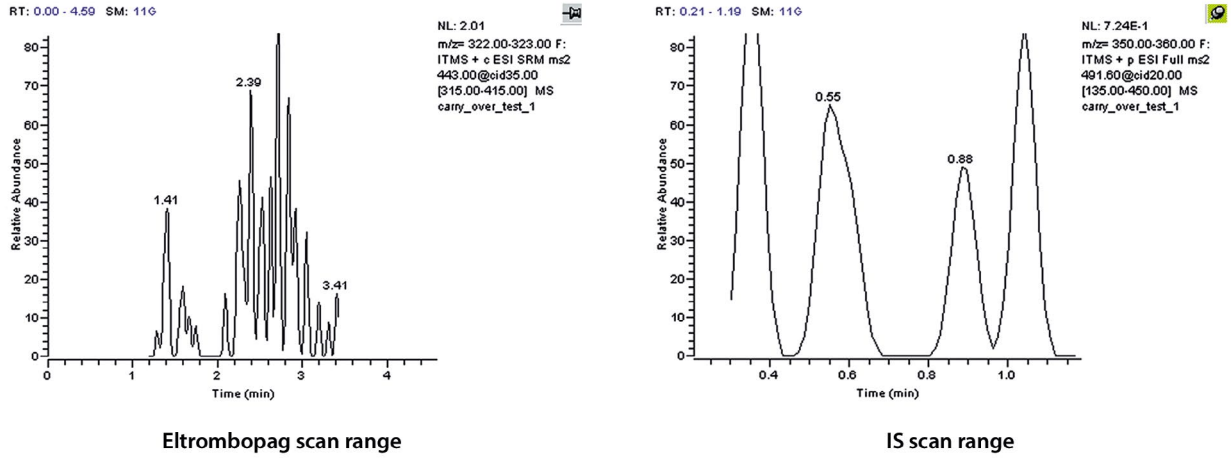
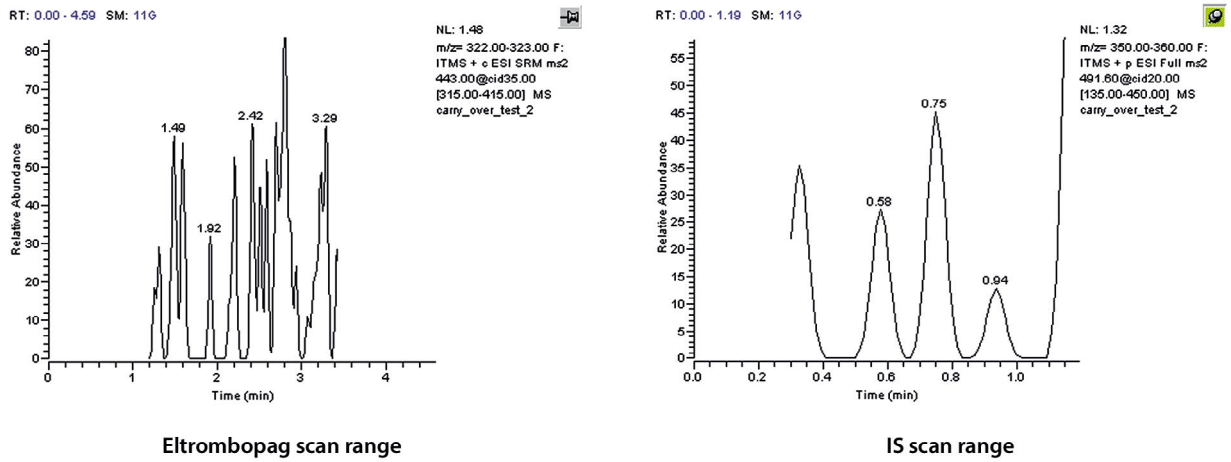


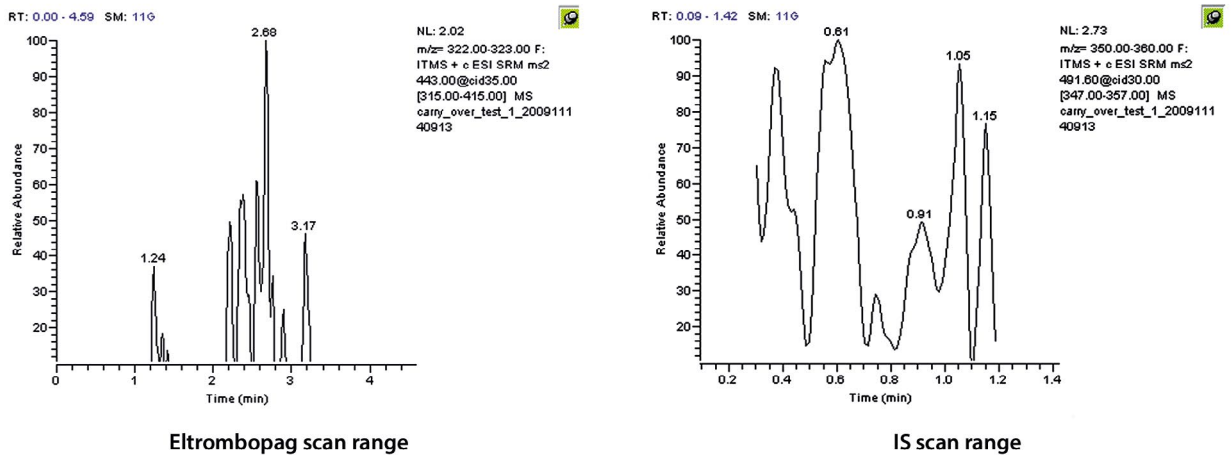
Eltrombopag scan range **IS scan range**

The first series of experiments (blank matrix)



Eltrombopag scan range **IS scan range**

The second series of experiments (blank matrix)



Eltrombopag scan range **IS scan range**

The third series of experiments (blank matrix)

Figure 6. Results of analysis of calibration samples and intact blood plasma sample

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