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Comparison of two RP-HPLC methods for determination of recombinant human thrombin in pharmaceutical formulations. Porovnanie dvoch RP-HPLC metód pre stanovenie rekombinantného ľudského trombínu vo farmaceutických formuláciách

Original research article

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Abstract Two reversed-phase high performance liquid chromatography analytical methods (Method I and Method II) for determination of assay of recombinant human thrombin in pharmaceutical formulations were developed and validated. Analysis was performed on chromatographic system Agilent 1200 series SL with diode array detection and mass selective detection.
Method I was intended for faster determination of thrombin assay. Gradient programme was optimised to achieve sufficient separation and acceptable runtime. Chromatographic analysis was performed on analytical column Grace Vydac, C4 250 × 4.6 mm, 5 μm. Method II is Method I adapted to use the mass selective detector. Chromatographic separation was performed on analytical column Zorbax 300SB-C8 SolvSaver Plus, 150 × 3 mm, 3.5 μm. Both analytical methods were validated with respect to specificity, linearity, precision and accuracy. The response of thrombin was a linear function of concentration over the range 0.1–1.0 mg/ml. Precision and accuracy of thrombin was evaluated at three concentration levels low (0.2 mg/ml), medium (0.4 mg/ml) and high (0.8 mg/ml).
Both validated methods have been successfully applied for determination of assay and thrombin degradation products in pharmaceutical formulations.

Slovak abstract Na stanovenie obsahu rekombinantného ľudského trombínu boli vyvinuté a validované dve analytické metódy (Metóda I a Metóda II). Analýzy boli uskutočnené na chromatografickom systéme Agilent 1200 series SL s DAD a MS detektorom.
Metóda I je určená pre rýchlejšiu analýzu obsahu trombínu. Pre dosiahnutie lepšej separácie a prijateľného času analýzy bol optimalizovaný gradientový program. Chromatografická separácia sa uskutočnila na analytickej kolóne Grace Vydac, C4 250x4.6mm, 5μm. Metóda II bola odvodená od Metódy I tak aby bola použiteľná v kombinácii s hmotnostným detektorom. Chromatografická separácia sa uskutočnila na analytickej kolóne Zorbax 300SB-C8 Solv Saver Plus, 150x3mm, 3.5μm. U oboch metód boli validované nasledujúce parametre: špecificita, linearita, presnosť a správnosť. Odozva trombínu bola lineárnou funkciou koncentrácie v rozsahu od 0,1 do 1,0 mg/ml. Presnosť a správnosť trombínu bola hodnotená pri troch koncentračných hladinách: nízkej (0,2 mg/ml), strednej (0,4 mg/ml) a vysokej (0,8 mg/ml).
Robustné validované metódy boli úspešne použité pri stanovení obsahu a čistoty trombínu počas formulačného vývoja liekovej formy.

Keywords *Thrombin – RP-HPLC – chromatography – mass spectrometry*

Kľúčové slová: *trombín – RP-HPLC – chromatografia – hmotnostná spektrometria*

1. INTRODUCTION

Thrombin is a pluripotent haemostatic factor that promotes coagulation, thrombosis, and local vasoconstriction that makes topical thrombin an ideal agent for the promotion of haemostasis during surgical procedures (Lew & Weaver, 2008, Cheng *et al*., 2009). Until recently, most commercially available thrombin products were isolated from bovine or human plasma, but sourcing thrombin from plasma carries the potential risk of transmitting plasma-borne pathogens such as HIV, hepatitis B and C, Creutzfeldt–Jakob disease or bovine spongiform encephalopathy (Regan & Taylor, 2002). The use of bovine thrombin can also lead to immunological reactions and related post-operative complications (Ortel *et al*., 2001).
During the last decade, advances in molecular biology and recombinant protein technology have led to an increased availability of protein based on biomolecules. Recombinant therapeutics have already found usage also in therapy of haemophilia and bleeding disorders. Recombinant human thrombin is a haemostatic alternative to bovine or human

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thrombin with similar efficiency but with significantly lower risk of immunologic impact (Chapman *et al*., 2007) and without risk of viral or prion transmission. Disadvantage of currently marketed product is need for reconstitution, which makes it inappropriate for immediate administration at serious car accidents or combat operations. Development of stable liquid formulation is therefore still actual. Therapeutic proteins are complex molecules, subject to variety of chemical and physical degradation pathways. Temperature, pH, ionic strength, mechanical stress during formulation and fill-finish operations can easily lead to structural changes and loss of activity (Manning *et al*., 2010, Patro *et al*., 2002). To ensure efficiency and safety of drug product, reliable stability indicating analytical techniques have to be developed and optimised. Reversed-phase high performance liquid chromatography (RP-HPLC) is one of the most widely used methods for determination of assay and purity of recombinant proteins.

The aim of this study is to establish a simple analytical method for determination of thrombin assay in pharmaceutical formulations. There are several methods, which have been used for thrombin analysis in formulations: size exclusion chromatography (Chang, 1986), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), hydrophobic interaction HPLC (Karlsson, 2003). Presented work has been focused on development of analytical method based on RP-HPLC. RP-HPLC has become well-established tool for the analysis of proteins. RP-HPLC is able to separate polypeptides of nearly identical sequences, not only for small peptides such as those obtained through trypsin digestion, but even for much larger proteins.

The study includes also partial validation of two analytical methods for determination of thrombin assay. One of the methods has been designed to be compatible with mass spectrometer (MS) due to potential qualitative analysis of thrombin degradation products.

EXPERIMENTAL PART

Instruments

Rapid resolution chromatographic system Agilent 1200 Series SL with diode array detection (DAD) was used for development and validation of two analytical methods. Analytical Method II was developed to be compatible with mass spectrometer Agilent TripleQuad 6410.

Chemicals and reagents

A working standard of recombinant human thrombin and active pharmaceutical ingredients (API) for preparation of tested formulations was obtained from Scil Proteins GmbH (Halle, Germany).

Acetonitrile (HPLC grade), water (HPLC grade) and trichloroacetic acid (spectroscopy grade) were purchased from Merck Chemicals (Darmstadt, Germany). Formic acid (HPLC grade) was from Sigma-Aldrich (Steinheim, Germany).

ANALYTICAL METHODS

Analytical method I

Chromatographic separation was carried out on analytical column Grace Vydac, C4 250 × 4.6 mm, 5 mm at column temperature 25°C. The mobile phase components were prepared as follows. Mobile phase A was 0.1% v/v trifluoroacetic acid in deionised water and mobile phase B was 0.085% v/v trifluoroacetic acid in acetonitrile. The chromatographic analysis was carried out at a flow rate of 1.5 ml/min. The gradient programme was an object of optimisation to achieve the best separation and the shortest retention time of thrombin. The autosampler temperature was set at 15°C and injection volume was 10 µL. The wavelength of DAD was set at 280 nm. Analysis time of optimised method was 40 minutes.

Analytical method II

Analytical method II was derived from the previous analytical method I. Retention of analytes was increased for better separation of degradation products of recombinant human thrombin due to shallower gradient compared to method I. Chromatographic column Zorbax 300SB-C8 Solv Saver Plus, 150 × 3 mm, 3.5 mm at temperature 25°C was used for analytical separation.

The mobile phase consisted of two eluents, solvent A (0.1% formic acid in deionised water) and solvent B (0.085% formic acid in acetonitrile) at flow rate 0.5 ml/min. The starting composition of mobile phase was 5% B followed by linear gradient to 60% B over 82 minutes. The composition of mobile phase was changed back to the initial conditions over 1 minute. Column was equilibrated for 9 minutes resulting into 92 minutes long runtime. The injection volume, detector wavelength and the autosampler temperature were identical to Method I. Since the analytical method II is compatible with mass spectrometer due to used mobile phase composition, it was used for qualitative analysis of degradation products of recombinant human thrombin. Mass spectrometric analysis was performed using Agilent 6410 TripleQuad mass spectrometer equipped with electrospray ion source in positive ion mode, heated to 300°C with flow of drying gas set to 12 l/min. Fragmentor was set to 130 V and capillary voltage to 4000 V. Qualitative analysis was performed using MS2Scan mode in range from 50 to 1600 m/z.

Preparation of standards and quality control samples

The set of calibration standard solutions were prepared from stock standard solution of recombinant human thrombin by dilution with succinate buffer to achieve target concentration

ranged from 0.1 to 1 mg/ml. Calibration curve was constructed from 11 calibration points.

The analysed samples were prepared from thrombin stock solution by buffer exchange into respective buffer solution on HiTrap® desalting column. The protein concentration was measured by ultraviolet (UV) absorption at 280 nm, excipients were dissolved and solution was diluted to 0.8, 0.4 and 0.2 mg/ml, respectively, with suitable buffer. pH was adjusted, samples were filtered through 0.2 µm low-protein binding filter (polyethersulfone membrane, Pall, USA) and aseptically filled into sterile cryotubes. We tested two formulations as follows: 25 mM succinate buffer, pH 6.2, 150 mM NaCl; 10% w/v propylene glycol and 25 mM succinate buffer, pH 6.2, 150 mM NaCl, 5% w/v trehalose.

RESULTS AND DISCUSSION

Method development (Method I)

Interactions of polypeptides with stationary phase during separation processes in the column are completely different in comparison to interactions of small molecules. The separation of small molecules involves continuous partitioning of the molecules between the mobile phase and the hydrophobic surface. Polypeptides remain adsorbed on the stationary phase surface after loading on the column until the critical concentration of organic modifier cause polypeptide desorption (Geng & Regnier, 1984). Polypeptides desorb from the surface in very narrow window of organic modifier concentration. Gradient elution is preferred for separation of polypeptides using very shallow gradient.

The emphasis has been put on the optimisation of the gradient programme since it has been found that the retention time of thrombin as well as the column efficiency and resolution due to peaks of major degradation products of thrombin were very sensitive to slight changes of the mobile phase composition as it is shown in Figure 1. Changes of the organic modifier at starting conditions at constant gradient slope also has significant effect on thrombin retention time, but practically, no influence on resolution between thrombin and main degradation products (Figure 2). Optimal gradient programme for thrombin assay analysis has started from 34% of the organic modifier followed by the linear gradient to concentration of organic modifier of 49% over 70 minutes. For the sake of analysis time and based on the findings during the method development, the time of analysis of Method I has been shortened to 40 minutes with the same gradient slope.

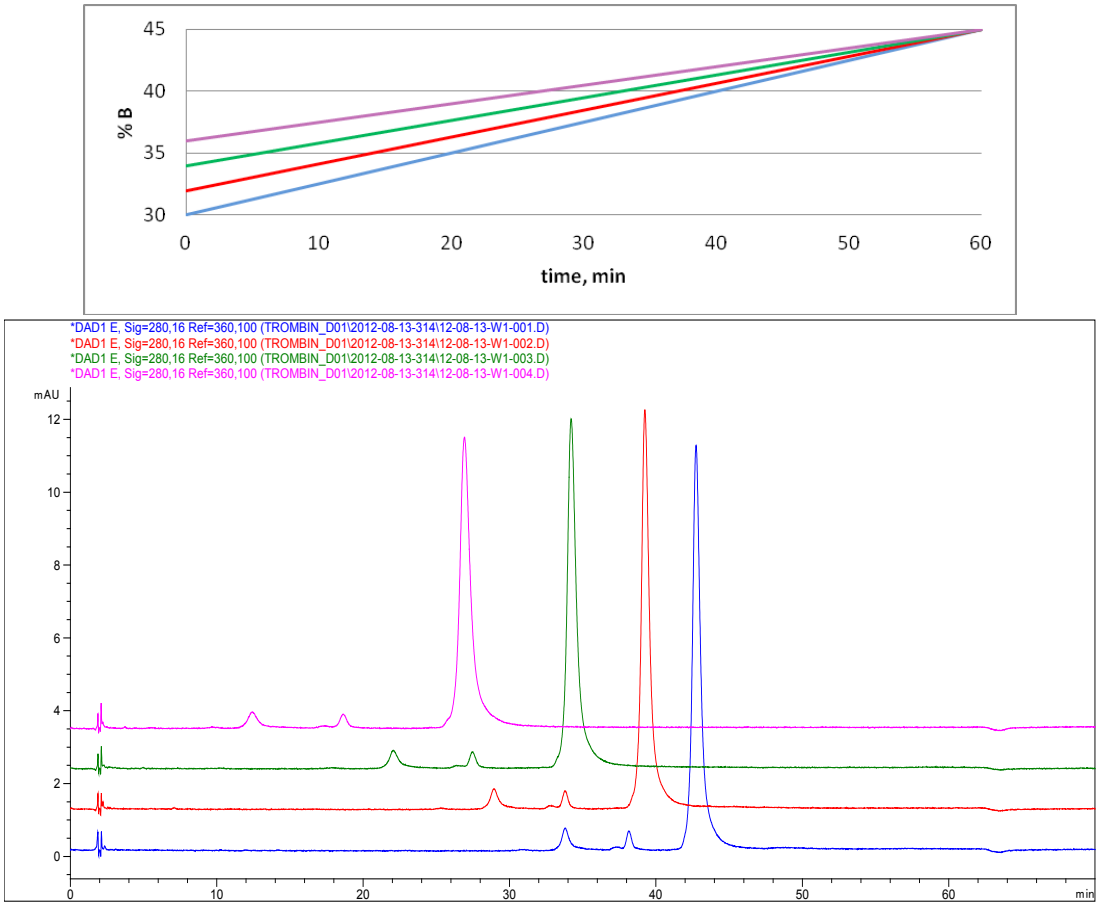

Figure 1. Influence of the gradient slope on the retention time of thrombin starting from different initial concentration of the organic modifier

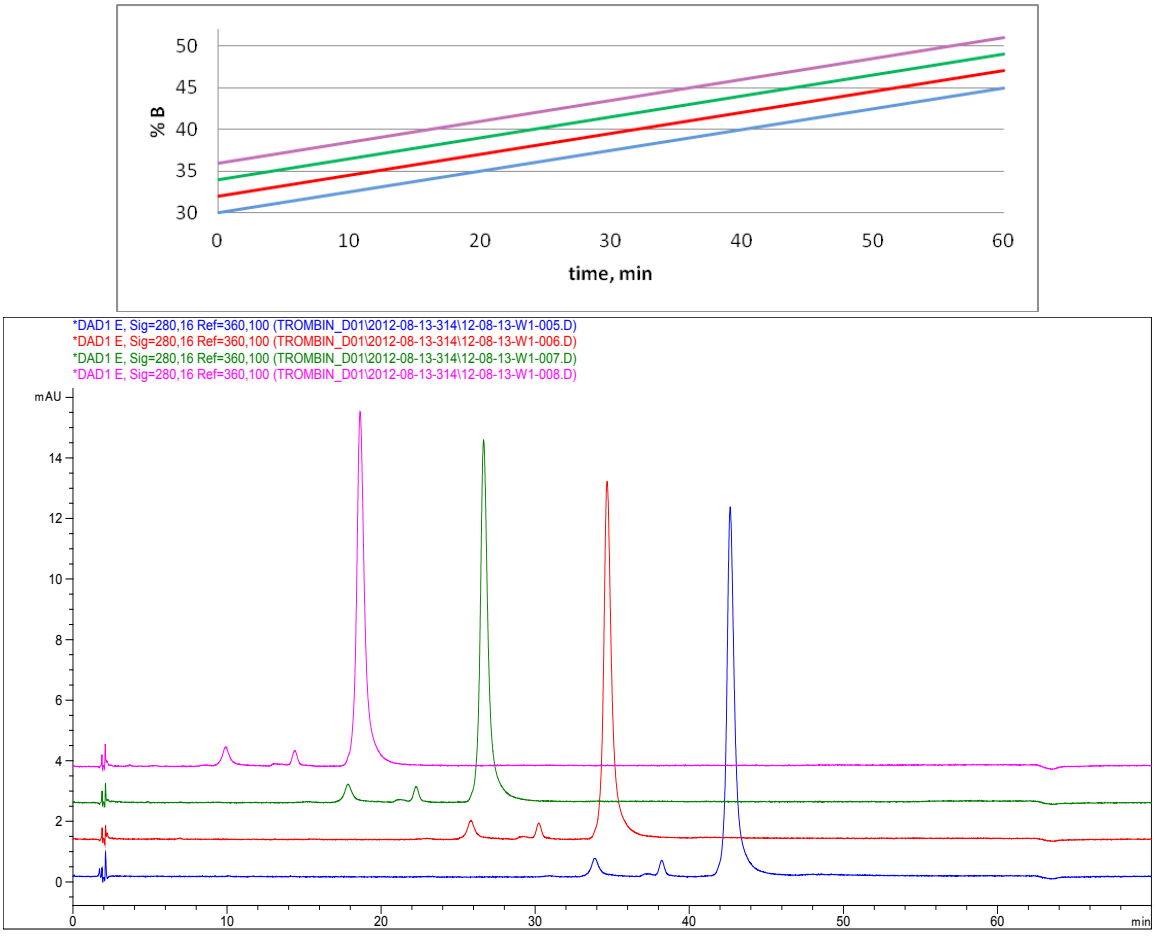

Figure 2. Influence of initial concentration of organic modifier on the retention time of thrombin at constant slope of the gradient programme

Method development (Method II)

Since Method I seemed to be not suitable for separation of degradation products of recombinant human thrombin with lower molecular weight, it was necessary to develop a method with very shallow gradient and wider gradient range. Liquid chromatography/mass spectrometry (LC/MS) compatible mobile phase composition was used due to potential usage of the method in combination with mass spectrometer. The method has been found to be suitable also for qualitative analysis that allows determining molecular weight of thrombin and its degradation products by deconvolution of mass spectra (Figure 3 and Table 1).

Method validation

Partial validation of developed methods has been performed to confirm presented analytical methods are suitable and reliable for the determination of thrombin assay. Method validation includes following validation parameters: specificity, linearity, precision and accuracy, intermedial precision and accuracy.

Evaluation of degradation products of thrombin by LC/MS method is not included in the validation.

Method validation (Method I)

Specificity

The ability of the analytical method to differentiate and quantify thrombin in the presence of other excipients in pharmaceutical formulation (placebo) and sample diluent was confirmed. There were not observed interferences of other peaks with the peak of thrombin.

Linearity

Calibration curve of thrombin was plotted as peak area versus thrombin concentration and constructed from 11 calibration samples, singly in the three calibration curves, including blank sample. Each blank sample in the calibration curve was examined for interference using the proposed chromatographic conditions. The standard concentration ranged from 0.1 to 1 mg/ml. Average correlation coefficient was calculated to be 0.9983 (Figure 4).

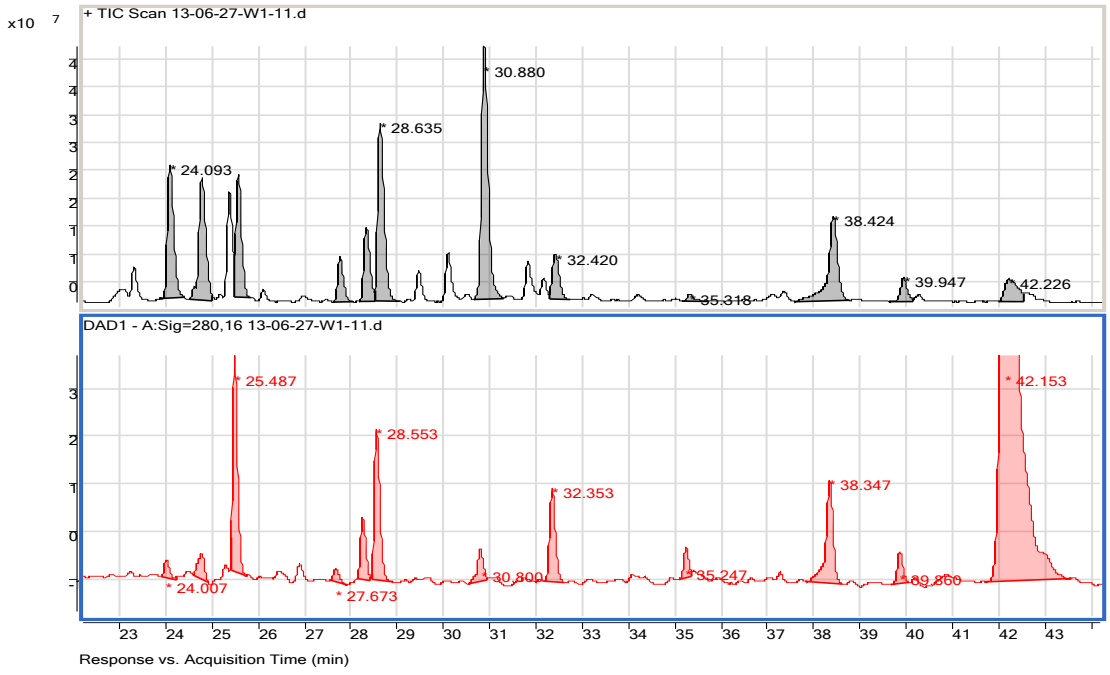

Figure 3. LC/UV/MS2Scan chromatogram of thrombin and its degradation products for 14 days at 37°C

Table 1. Retention characteristics and molecular weight of thrombin degradation products

Compound	Relative retention time (RRT)	Molecular weight [Da]
Imp A	0.58	2689
Imp B	0.60	2686
Imp C	0.62	1860
Imp D	0.67	2154
Imp E	0.69	1732
Imp F	0.69	1937
Imp G	0.74	5056
Imp H	0.78	7734
A-chain	0.83	4090
Imp I	0.84	8064
Imp J	0.91	4830
Imp K	0.95	2265
Imp L	0.97	21890
Thrombin	1.00	33820
β-Thrombin	1.02	29723
Imp M	1.03	8445
Imp N	1.04	7995

Precision and accuracy

To assess precision and accuracy, three distinct concentration levels of thrombin in the range of expected dosage form concentration were evaluated using six determinations per concentration. Precision and accuracy were assessed at within-day basis, which defines those parameters during a single analytical run; and at between-day basis, which measure the between-day variability. Quality control samples concentrations were finally chosen as defined in Table 2. Precision for thrombin ranged from 5.3% to 8.8% for intra-assay and from 4.9% to 8.5% for inter-assay. Accuracy for thrombin ranged from 90.1% to 102.4% for intra-assay and from 92.1% to 100.8% for inter-assay (Tables 3–5).

Method validation (Method II)

Specificity

The ability of the analytical method to differentiate and quantify thrombin in the presence of other excipients of

Table 2. Analysed samples definition

QCs Code	Defined Value mg/mL
QCA	0.2
QCB	0.4
QCC	0.8

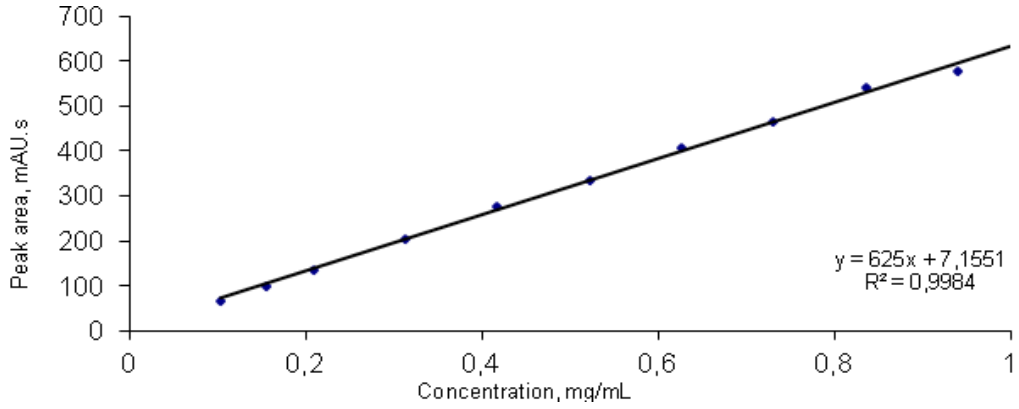

Figure 4. Calibration curve of thrombin (data obtained by Method I)

Table 3. Thrombin intra-assay (ID), precision and accuracy (batch I) (data obtained by Method I)

Sample Code	ID-QCA		ID-QCB		ID-QCC	
	Nominal Concentration, mg/mL					
	0.21		0.42		0.84	
Replicate #	Peak Area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL
1	147.11	0.22	223.81	0.35	518.68	0.82
2	147.14	0.22	233.87	0.36	552.58	0.87
3	149.84	0.23	235.39	0.37	554.25	0.87
4	120.06	0.18	248.70	0.39	556.02	0.88
5	131.86	0.20	248.51	0.39	496.35	0.78
6	146.15	0.22	264.39	0.41	501.33	0.79
Mean		**0.21**		**0.38**		**0.83**
CV, %		**8.8**		**6.1**		**5.3**
Recovery, %		**102.4**		**90.1**		**99.9**

Table 4. Thrombin inter-assay (OD), precision and accuracy (batch II) (data obtained by Method I)

Sample Code	OD-QCA		OD-QCB		OD-QCC	
	Nominal Concentration, mg/mL					
	0.21		0.42		0.84	
Replicate #	Peak Area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL
1	131.57	0.20	258.57	0.40	513.37	0.81
2	145.38	0.22	261.56	0.41	545.73	0.86
3	152.60	0.23	232.26	0.36	550.46	0.87
4	120.98	0.18	246.64	0.38	547.16	0.87
5	132.35	0.20	247.15	0.39	493.05	0.78
6	133.25	0.20	264.03	0.41	501.25	0.79
Mean		**0.21**		**0.39**		**0.83**
CV, %		**8.7**		**4.9**		**4.9**
Recovery, %		**99.1**		**94.0**		**99.5**

Table 5. Thrombin inter-batch validation (batch I-II) (data obtained by Method I)

Inter Batch Precision and Accuracy			
	Nominal Concentration, mg/mL		
	0.21	**0.42**	**0.84**
	Mean Concentration of Batches I and II**, mg/mL**		
	Found conc. mg/mL	**Found conc. mg/mL**	**Found conc. mg/mL**
Batch I	0.21	0.38	0.83
Batch II	0.21	0.39	0.83
Mean	**0.21**	**0.38**	**0.83**
CV, %	**8.5**	**5.7**	**4.9**
Recovery, %	**100.8**	**92.1**	**99.7**

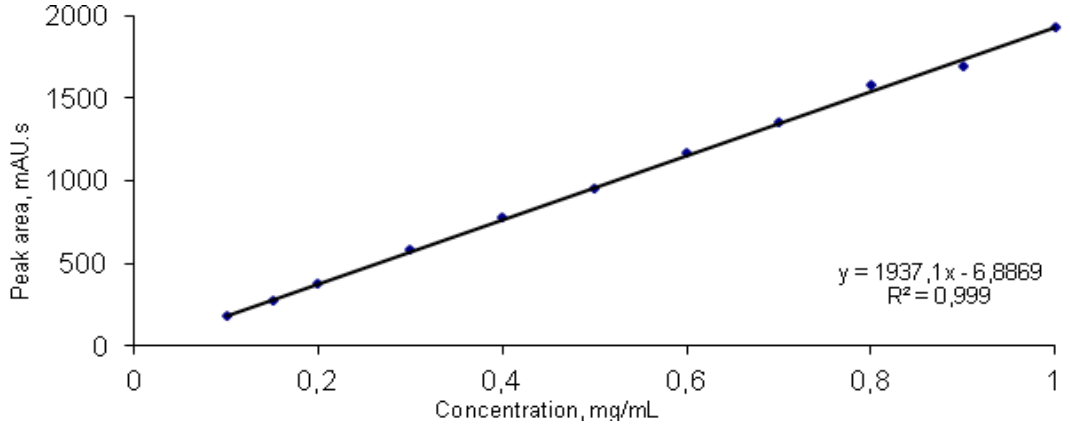

Figure 5. Calibration curve of thrombin (data obtained by Method II)

pharmaceutical formulation or sample diluent was confirmed. There were not observed interferences in retention times of thrombin.

Linearity

Calibration curves of thrombin were plotted as peak area versus thrombin concentration and constructed from 11 calibration samples, singly in the three calibration curves, including blank sample. Each blank sample in the calibration curve was examined for interference using the proposed chromatographic conditions. The standard concentration ranged from 0.1 to 1 mg/ml. Average correlation coefficient was calculated to be 0.9991 (Figure 5).

Precision and accuracy

To assess precision and accuracy, three distinct concentration levels of thrombin in the range of expected dosage form concentration were evaluated using six determinations per concentration. Precision and accuracy were assessed at within-day basis, which defines those parameters during a single analytical run; and at between-day basis, which measure the between-day variability. Precision for thrombin ranged from 4.7% to 9.9% for intra-assay and from 6.1% to 9.3% for inter-assay. Accuracy for thrombin ranged from 95.0% to 102.2% for intra-assay and from 93.2% to 100.1% for inter-assay (Tables 6–8).

Method comparison

Method I has been developed for determination of thrombin assay in pharmaceutical formulations. Analytical method employs shallow gradient with relatively high initial concentration of organic modifier (34% of acetonitrile). The starting composition presents almost critical concentration (about 40% of acetonitrile) needed to desorb thrombin from the surface of stationary phase of chromatographic column. These parameters provide shorter retention time of thrombin with satisfactory separation of thrombin from its degradation products and very closely related polypeptides. Due to high initial organic concentration, separation of the degradation products with lower molecular weight was found insufficient. This drawback of Method I has been resolved by Method II. Although the runtime of method is significantly longer in comparison with Method I, the shallow and long gradient ensures sufficient separation of thrombin and most of its

Table 6. Thrombin intra-assay (ID), precision and accuracy (batch I) (data obtained by Method II)

Sample Code	ID-QCA		ID-QCB		ID-QCC	
	Nominal Concentration, mg/mL					
	0.21		0.42		0.84	
Replicate #	Peak Area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL
1	388.79	0.23	659.67	0.37	1539.83	0.85
2	392.91	0.23	686.42	0.39	1586.04	0.87
3	407.23	0.24	686.30	0.39	1570.67	0.86
4	324.94	0.19	720.21	0.41	1575.52	0.87
5	363.93	0.21	716.62	0.40	1458.74	0.80
6	311.37	0.18	757.44	0.43	1202.91	0.67
Mean		**0.21**		**0.40**		**0.82**
CV, %		**9.9**		**4.7**		**9.7**
Recovery, %		**102.2**		**95.0**		**98.2**

Table 7. Thrombin inter-assay (OD), precision and accuracy (batch II) (data obtained by Method II)

Sample Code	OD-QCA		OD-QCB		OD-QCC	
	Nominal Concentration, mg/mL					
	0.21		0.42		0.84	
Replicate #	Peak Area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL	Peak area mAU.s	Found conc. mg/mL
1	405.36	0.22	643.25	0.35	1504.52	0.81
2	417.79	0.23	672.17	0.36	1588.21	0.86
3	401.23	0.22	669.25	0.36	1592.04	0.86
4	342.57	0.19	742.26	0.40	1596.36	0.86
5	370.71	0.20	768.23	0.41	1431.35	0.77
6	335.64	0.18	750.18	0.40	1492.35	0.81
Mean		**0.21**		**0.38**		**0.83**
CV, %		**9.1**		**7.4**		**4.5**
Recovery, %		**98.0**		**91.5**		**99.1**

Table 8. Thrombin inter-batch validation (batch I-II) (data obtained by Method II)

Inter Batch Precision and Accuracy			
	Nominal Concentration, mg/mL		
	0.21	0.42	0.84
	Mean Concentration of Batches I and II, mg/**mL**		
	Found conc. mg/mL	Found conc. mg/mL	Found conc. mg/mL
Batch I	0.21	0.40	0.82
Batch II	0.21	0.38	0.80
Mean	**0.21**	**0.39**	**0.83**
CV, %	**9.3**	**6.1**	**7.2**
Recovery, %	**100.1**	**93.2**	**98.6**

degradation products. Method II has been found to be a stability-indicating method and suitable for determination of purity of thrombin in pharmaceutical formulations. Since trifluoroacetic acid from Method I was replaced by formic acid, Method II has become LC/MS compatible and suitable for qualitative analysis of thrombin degradation products.
Validation parameters of both methods are comparable. Both methods provide sufficient precision, accuracy and they were found to be linear in tested concentration range.

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CONCLUSIONS

Two simple, specific, precise and accurate analytical methods for the determination of stability-indicating parameters of recombinant human thrombin has been developed and validated. Method I has been found to be suitable for faster analysis of thrombin assay. LC/MS compatible Method II has shown its additional potential for qualitative analysis of thrombin degradation products. Molecular weight of main degradation products was determined after mass spectra deconvolution. Validated analytical methods have been successfully applied for routine analysis of thrombin assay during development of pharmaceutical formulations.

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