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ScienceDirect
Journal of Chromatography B, 852 (2007) 631–634
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Quantitative analysis of Tenecteplase in rat plasma samples using LC–MS/MS as an alternative for ELISA

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Received 19 July 2006; accepted 31 December 2006
Available online 13 January 2007

Abstract

An LC–MS/MS method has been developed for the quantitative determination of a protein drug (Tenecteplase; M_W 58,777 Da) in rat plasma. The protein was digested with trypsin without prior clean-up of the plasma sample, without the use of a label nor internal standard. A limited validation was performed to assess the linearity, the sensitivity and the specificity of the method. In addition, the developed method was applied to the quantitative analysis of Tenecteplase in rat plasma samples originating from a single-dose study in rats.

Keywords: Quantitative analysis; Proteins; Plasma; Digestion; Liquid chromatography; Mass spectrometry

1. Introduction

In the discovery of drug candidates, the importance of the biopharmaceuticals is growing. Some biomolecules like peptides, oligonucleotides (recombinant) proteins, and monoclonal antibodies can be used as therapeutic agents. Quantitative bioanalytical methods for these classes of biopharmaceuticals are mandatory for the support of pharmaco- and toxicokinetic studies for drug registration. Until recently, quantitative bioanalysis of proteins was mainly performed using Enzyme Linked Immuno Sorbent Assays (ELISA). Very low detection levels can be reached with this technique but method development for new biologicals is time-consuming.

Liquid chromatography (LC) in combination with mass spectrometry (MS) of proteins [1,2] in biological matrices has been mainly performed for qualitative purposes or for *relative* protein quantification, i.e. in general, in proteomics studies protein levels are compared between samples, i.e. cells, tissues or organisms (relative quantification) [3]. To compare the amount/concentration levels of proteins between groups of samples a variety of labeling techniques can be applied in combination with (capillary) LC–MS/MS, such as Isotope Coded Affinity Tag (ICAT) labeling [4,5], 4-sulfophenyl isothiocyanate (SPITC) labeling [6,7], guanidino labeling [8], derivatization with *N*-acetoxy-succinimide [9] and per-methyl esterification of peptides [10].

Only a few analytical methods have appeared in literature for the *absolute* quantification of proteins in biological matrices using LC–MS/MS. The absolute quantification of a protein in monkey plasma was described by Ji et al. [11]: the intact protein (M_W 10.4 kDa) was isolated from plasma using solid phase extraction after which the intact protein was analysed using LC–MS/MS. Kirkpatrick et al. [12] and Gerber et al. [13] reported the absolute quantification of a protein from cell lysates using LC–MS/MS: a stable isotope-labeled internal standard (AQUA peptide) was introduced at a known concentration to cell lysates. Chelius and Bondarenko [14] analysed different amounts of tryptic digests of myoglobin (M_W 16.9 kDa) in a standard solution with LC–MS. The method was further evaluated by adding two different concentrations of horse myoglobin to human serum.

In this paper, the absolute quantification of a protein drug (Tenecteplase; M_W 58,777 Da) in rat plasma using LC–MS/MS is presented. Without any sample clean-up the rat plasma samples were digested with trypsin and the resulting peptides were analysed using LC–MS/MS without labeling nor internal standard. A limited validation of the method was performed after which rat plasma samples originating from a single-dose study in rats were analysed for pharmacokinetic profiling.

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doi:10.1016/j.jchromb.2006.12.053

2. Experimental

2.1. Chemicals

Guanidine–HCl (G4505), trypsin (T6567) and dithiothreitol (DTT, D9163) were obtained from Sigma–Aldrich (Steinheim, Germany). The protein TNK-tPA (Tenecteplase) was delivered by a local pharmacy (Zeist, The Netherlands). Formic acid was from Merck (Darmstadt, Germany), acetonitrile from Biosolve (Valkenswaard, The Netherlands), iodoacetamide (RPN6302Y) from Amersham (GE Healthcare, UK) and ammonium hydrogen carbonate from Fluka (Germany).

2.2. Sample pre-treatment

The calibration samples were prepared by spiking blank rat plasma (anticoagulant heparin; obtained from Harlan, Horst, The Netherlands) with Tenecteplase at a concentration range from approximately 2 to 125 μg/ml (0.034–2.125 μmol/l). The plasma samples were pre-treated as follows: 20 mg of guanidine–HCl was added to each rat plasma sample (25 μl). Subsequently, 10 μl dithiothreitol (DTT) (100 mM) was added and the mixture was incubated at 56 °C for 1 h. After cooling at room temperature, 5 μl iodoacetamide (0.5 μM) was added and the mixture was kept at room temperature in the dark for 30–60 min. Subsequently, 150 μl ammonium bicarbonate (NH_4HCO_3, 100 mM, pH 8.5) was added. Then, 10 μl trypsine (0.25 mg/ml) in hydrochloric acid (1 mM) was added. Incubation was performed at 37 °C for 1 night. A volume of 50 μl was analysed using LC–MS/MS.

2.3. LC–MS/MS conditions

The LC–MS/MS analysis of the digested protein was performed with a Surveyor LC system (LC pump and autosampler, ThermoElectron) coupled to a TSQ Quantum mass spectrometer (ThermoElectron). Chromatographic separation of the peptides was carried out using a Vydac LC column (250 mm × 4.6 mm, C18, art. 218TP54). Gradient elution (1 ml/min) was performed with mobile phase A consisting of formic acid (0.1%, v/v) and mobile phase B consisting of formic acid (0.1%, v/v) in acetonitrile/water (90/10, v/v). The injection volume was 50 μl. The flow to the mass spectrometer was split (ratio 9/1; waste/mass spectrometer). A divert valve was used. The LC gradient was as follows: 100% mobile phase A from 0 to 5 min, 100–44.5% mobile phase A from 5 to 30 min, 44.5–0% mobile phase A from 30 to 40 min, 0–100% from 40 to 40.1 min, 100% mobile phase A to 45 min. Mass spectrometric experiments were performed in the MRM mode. In the MRM mode, the three most abundant peptides were quantified. Electrospray ionization was carried out in the positive ionization mode. The electrospray voltage was 4.5 kV. The source CID was 10 V. The collision energy was 30 V.

3. Results

3.1. Method development Tenecteplase in plasma using LC–MS/MS

To set up a quantitative bioanalytical method, Tenecteplase in a standard solution was digested with trypsin: 50 μl of the resulting solution containing a mixture of peptides was analysed using LC–MS (full scan). The three most abundant peptides obtained after digestion were selected to perform MS/MS and to set up an MRM method. In Table 1, the retention time, the *m/z* values of the selected peptides (parent ions), the charge state, the identity of the peptides and the resulting peptides after fragmentation (product ions) are given. The next step was to analyse Tenecteplase in a biological matrix: blank rat plasma was spiked with Tenecteplase at a concentration of approximately 25 μg/ml. Without any purification, 25 μl of rat plasma sample was digested and analysed using LC–MS/MS. Fig. 1a shows the ion chromatogram of the three selected peptides from Tenecteplase in rat plasma monitored after tryptic digestion. Subsequently, a calibration line was constructed by spiking blank rat plasma samples with Tenecteplase at concentrations ranging from ca. 2 to 125 μg/ml. The calibration samples were digested and analysed using LC–MS/MS. The method was linear in the range of ca. 5–125 μg/ml.

3.2. Limited method validation

A limited validation of the method was performed. The parameters of investigation were the specificity, the linearity and the sensitivity of the method. On 3 days, calibration samples (rat plasma spiked with Tenecteplase) were prepared freshly (5–125 μg/ml), digested and analysed. In addition, a blank rat plasma sample was digested and analysed in each run. The results are shown in Table 2. The correlation coefficient *r* of the calibration lines of the three peptides ranged from 0.993 to 0.999. The ion chromatogram obtained from a blank rat plasma sample did not show a significant signal at the retention time of Peptide II. However, at the retention times of Peptides I and III signals corresponding to Tenecteplase concentrations ranging from 2.7 to 10.9 μg/ml (Peptide I) and 1.4 to 2.9 μg/ml (Peptide III) were observed. An example of an ion chromatogram obtained from blank rat plasma is shown in Fig. 1b. By the injection of blank solvent the signal at the retention time of Peptide III was gone

Table 1
Peptides selected for the quantification of Tenecteplase in rat plasma after tryptic digestion

Peptide code	Retention time (min)	Parent ion *m/z*	Charge state	Peptide sequence	Product ion *m/z*
Peptide I	19.7	729.16	$[M+3H]^{3+}$	TQMIYQQHQSWLRPVLR	856.11; 978.66
Peptide II	23.0	821.03	$[M+3H]^{3+}$	GTHSLTESGASCLPWNSMILIGK	203.88; 1159.87
Peptide III	26.2	861.08	$[M+3H]^{3+}$	MTLVGIISWGLGCGQKDVPGVYTK	926.75; 1068.27

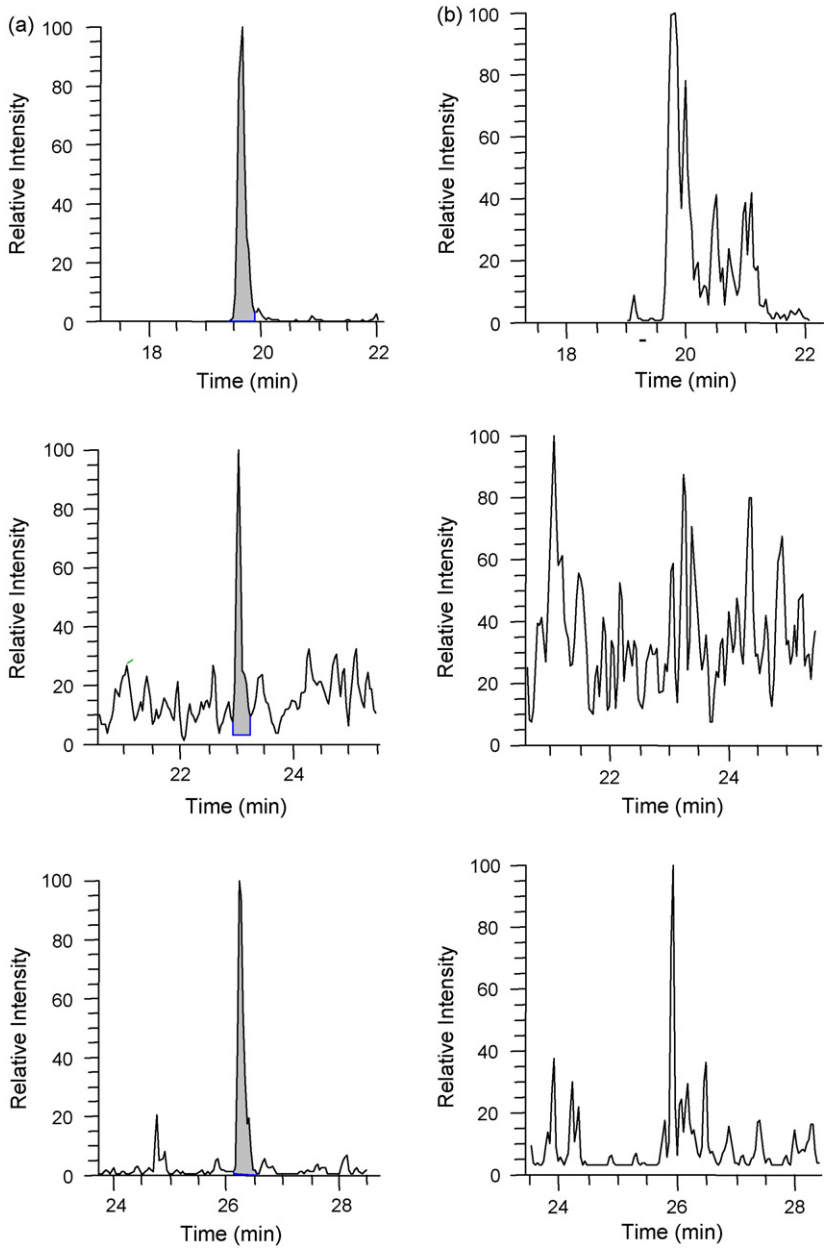

Fig. 1. (a) LC–MS/MS analysis of three peptides from digested Tenecteplase (concentration approximately 25 μg/ml) in rat plasma (a) and in blank rat plasma (b) (Peptide I at 19.7 min, Peptide II at 23.0 min, Peptide III at 26.2 min).

Table 2
Results limited method validation Tenecteplase in rat plasma using digestion and LC–MS/MS on 3 days

Peptide	Validation day	Correlation coefficient *r*	Signal in blank plasma (μg/ml)
Peptide I	1	0.999	2.7
	2	0.998	10.9
	3	0.999	8.4
Peptide II	1	0.995	–
	2	0.999	–
	3	0.997	–
Peptide III	1	0.999	2.9
	2	0.993	1.4
	3	0.993	–

whereas the signal at the retention time of Peptide I was reduced but carry-over was still observed. Therefore, the limit of quantification of the method was raised to approximately 25 μg/ml rat plasma.

3.3. Application to plasma samples from a single-dose study in rats

The developed method was applied to the analysis of rat plasma samples obtained from a single-dose study in rats (dosing level 10 mg/kg). The determined Tenecteplase concentrations were used to construct a pharmacokinetic profile of Tenecteplase in rats directly after intravenous dosing. The results are shown in Fig. 2. As an alternative, the rat plasma samples were mea-

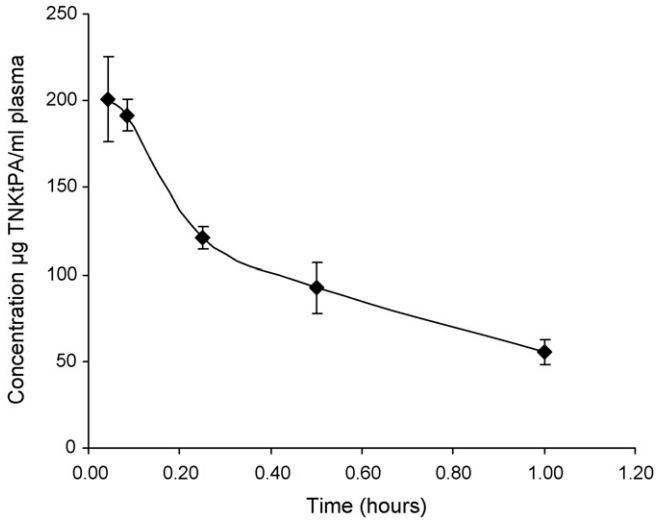

Fig. 2. Pharmacokinetic curve of Tenecteplase in rat plasma after a single-dose study in rats (dosing level 10 mg/kg).

sured with ELISA (data not shown). The LOQ obtained with ELISA was much lower (pg/ml) than the LOQ obtained with LC–MS/MS (μg/ml). However, LC–MS/MS can be set up much more quickly and cross-reactivity of structurally related compounds, as can happen with ELISA, does not occur. Quantitative LC–MS/MS analysis of large proteins in very complex matrices, such as plasma, is therefore an interesting alternative to ELISA.

4. Conclusions

A method has been developed for the quantitative analysis of the protein drug Tenecteplase in rat plasma samples. The method was based on direct tryptic digestion in plasma samples followed by LC–MS/MS analysis of three selected peptides. A limited method validation was performed on three days. The developed method has been used for pharmacokinetic profiling of Tenecteplase in rat plasma samples after an intravenous single-dose study in rats. Quantitative LC–MS/MS analysis of large proteins in very complex matrices, such as plasma, has proven to be an interesting alternative to ELISA.

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