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Detection and method validation of peginesatide in human serum by means of LC-MS/MS

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Abstract

Erythropoiesis-stimulating agents (ESAs) have frequently been confessed to be illicitly used in elite sports due to their endurance enhancing effects. Recently, peginesatide, the first representative of a new generation of ESAs, referred to as Erythropoietin (EPO)-mimetic peptides, obtained approval in the USA under the trade name Omontys® for the treatment of anemic patients on dialysis. Lacking sequence homology with EPO, it consists of a pegylated homodimeric peptide of approximately 45 kDa, and thus, specific approaches for the determination of peginesatide in blood had to be developed as conventional detection assays for EPO would not succeed in detecting the EPO-mimetic peptides. Therefore, a mass spectrometric detection method in human serum was developed targeting a proteotypic pentapeptide fragment after protein precipitation, subtilisin digestion, and cation-exchange purification. Eventually, the method was validated for qualitative purposes and proved to be specific, sensitive (limit of detection 1 ng/mL), and both precise and linear over a wide range of expected blood concentrations. Thus, the assay not only demonstrated its fitness for purpose for an application in routine doping control analysis but also allows for a detection of therapeutic doses of peginesatide in human serum for approximately one week when considering plasma maximum concentrations of about 1,000 ng/mL after a single intravenous administration of 0.05 mg/kg bodyweight to healthy volunteers and an observed half-live of around one day.

Introduction

Peginesatide (formerly referred to as Hematide™ and marketed under the trade name Omontys®) is a recently approved erythropoiesis-stimulating agent (ESA) 'for the treatment of anemia due to chronic kidney disease in adult patients on dialysis' [1]. Despite its peptidic structure (~5-kDa homodimeric peptide attached to 40-kDa branched polyethylene glycol (PEG), Figure 1) [2], which is completely dissimilar to that of erythropoietin (EPO), peginesatide was found to specifically bind to the EPO-receptor, thus, stimulating erythropoiesis [3].

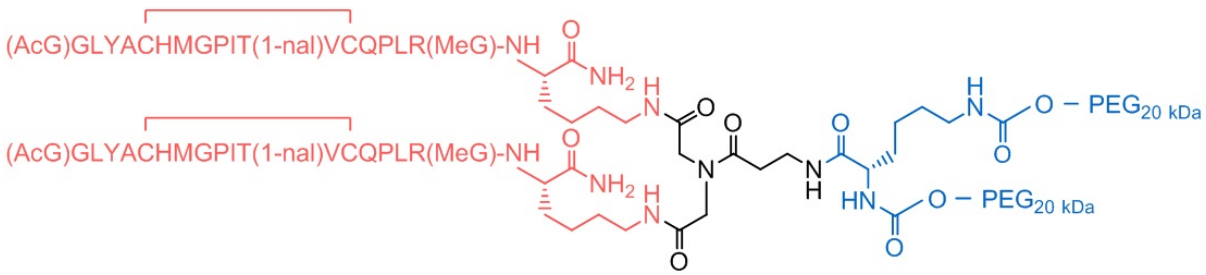

Figure 1: Structure of peginesatide consisting of two peptide monomers (each ~2.4 kDa, red), a trifunctional linker (black), and a branched polyethylene glycol (PEG) derivative of approximately 40 kDa (blue).

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Therefore, it was tested as promising active ingredient for treating anemia and demonstrated its comparability to other EPO products in terms of safety and efficacy while enabling considerably reduced injection intervals [4]. However, not only benefits in the treatment of anemic patients were observed, but also performance enhancing effects and thus, a high misuse potential in elite sports are expected. Consequently, peginesatide was prohibited by the World Anti-Doping Agency [5]. Knowing that conventional detection assays for EPO do not allow for the analysis of EPO-mimetic peptides, specific approaches were developed for the determination of peginesatide in human blood: While one methodology relies on an enzyme-linked immunosorbent assay (ELISA) screening method and a gel electrophoretic separation after immuno-purification with Western double blotting detection for confirmatory purposes [6], the other one is based on mass spectrometry [7]. Here, we report a specific and sensitive mass spectrometric detection method as well as its validation for peginesatide in human serum targeting a proteotypic and xenobiotic pentapeptide.

Experimental

Chemicals and reagents. All reagents, chemicals, and the protease from *Bacillus licheniformis* (type VIII, ~12 units/mg, subtilisin) were obtained from Sigma-Aldrich (Steinheim, Germany) or Merck (Darmstadt, Germany), and solvents (all analytical grade) were purchased from VWR (Leuven, Belgium). The pegylated reference material was synthesised in-house as described elsewhere [7], whereas the internal standard (AcGGLYA**C**HM[$^{13}C_2$,^{15}N]GPIT(1-nal)V**C**QPLR(MeG)K-NH_2, oxidised form) was ordered from BMFZ (Düsseldorf, Germany), the pentapeptide *GPIT(1-nal)* from PANATecs (Tübingen, Germany), and strong cation-exchange cartridges *Strata SCX* (100 mg, 1 mL) from Phenomenex (Aschaffenburg, Germany).

Liquid chromatography-mass spectrometry. Analyses by liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed on an Agilent 1100 series liquid chromatograph (Waldbronn, Germany) coupled to an API4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) as well as on a Thermo Open Accela liquid chromatograph and a Thermo Q Exactive (Bremen, Germany). The most important parameters are summarized in Tables 1 and 2; for further details please refer to [8].

Sample preparation. Briefly, the sample preparation procedure in human serum is based on a protein precipitation, a proteolytic digestion, and a strong cation-exchange purification as described in detail in [7].

Method validation. Characterisation of important method parameters for the qualitative determination of peginesatide in human serum was performed according to the guidelines of the International Conference on Harmonisation [9] on the API4000 and furthermore, for specificity and limit of detection (LOD) measurements, on the Q Exactive.

Specificity and LOD. Ten blank serum specimens were analyzed on the API4000 and additionally on the Q Exactive to check for interfering signals. For estimating the LOD, six samples each were prepared at a peginesatide concentration of 1 ng/mL for Q Exactive measurements and of 10 ng/mL for analysis on the API4000 QTrap mass spectrometer, respectively.

Recovery. Five serum samples were fortified with 100 ng/mL of peginesatide before sample preparation, while to further five samples, the corresponding amount of the target peptide after proteolytic digestion, *GPIT(1-nal)*, was added at the last resuspension step.

Linearity. Linearity of the assay was checked over a wide concentration range from 10-1,000 ng/mL of peginesatide and furthermore, also for the lower concentration range between 1 and 50 ng/mL (both n = 7).

Precision. Relative standard deviations for intra- and interday precision were calculated by analysing six aliquots each of five fortified serum samples (10, 25, 50, 200, and 500 ng/mL of peginesatide) on three consecutive days.

Robustness. To probe for the influence of variations in incubation time, a serum specimen was fortified with 50 ng/mL of peginesatide and two aliquots each were incubated for 3, 6, and 20 h.

Stability. Stability of the analyte in serum was tested by storing aliquots of a serum specimen spiked with 25 ng/mL of peginesatide at 4 as well as at -18 °C over a period of four weeks.

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Ion suppression/enhancement effects. Six blank serum samples were analyzed while a solution of the pentapeptide *GPIT(1-nal)* (c = 50 ng/mL, flow rate 5 µL/min), being the target analyte after proteolytic digestion, was constantly co-infused using a post-column split.

	Parameter	API4000 QTrap	Q Exactive
LC	Column	Nucleodur C18 Pyramid (2 x 50 mm, 3 µm; Macherey-Nagel)	Thermo Hypersil Gold C18 (2.1 x 50 mm, 1.9 µm)
	Eluents	0.2 % formic acid (A), acetonitrile (B)	0.2% formic acid (A), acetonitrile (B)
	Flow rate	250 µL/min	200 µL/min
	Gradient	90% A → 40% A (9 min), 1-min hold, to 0% A (10.25 min), 0.75-min hold; re-equilibration (5 min)	95% A → 60% A (9 min), 1-min hold, to 0% A (10.5 min), 0.5-min hold; re-equilibration (5 min)
	Injection volume	10 µL	10 µL
MS	MS-mode	Multiple reaction monitoring (MRM), positive electrospray ionization (ESI(+))	Full scan (*m/z* 400-600) + 2 targeted MS^2 experiments (*m/z* 584.31 and 587.31, CE 30 eV); resolution: 35,000; heated ESI(+), 350 °C
	Ionisation voltage	5.5 kV	4 kV
	Capillary temperature	450 °C	320 °C
	Gas	Nitrogen	Nitrogen

Table 1: Summary of LC-MS parameters.

Compound	Declustering potential (V)	Ion transition (*m/z*)	Collision energy (eV)
Peginesatide	60	584.3-127.1	60
		584.3-155.1	40
		584.3-317.1	30
		584.3-369.2	25
Internal Standard	60	587.3-130.1	60
		587.3-158.1	40
		587.3-317.1	30
		587.3-372.2	25

Table 2: Selected ion transitions and their corresponding mass spectrometric parameters for validation measurements performed on the API4000 QTrap mass spectrometer in MRM mode with a dwell time of 50 ms.

Results and Discussion

Due to its effects analogous to EPO, peginesatide is expected to have a great potential to be (mis-)used in elite sports. Thus, a specific and sensitive mass spectrometric detection method in human serum should be developed. As the commonly employed mass spectrometers in doping control laboratories do not allow for a sensitive and selective detection of the intact molecule, several proteolytic enzymes were tested enabling a specific detection of a characteristic peptide fragment. Best results were obtained with the serine protease subtilisin yielding the characteristic pentapeptide fragment *GPIT(1-nal)*, which fulfils the recommended criteria for mass spectrometric detection of peptide hormones in sports drug testing (sequence coverage of at least 10%) [10] and whose xenobiotic composition is furthermore supported by the non-natural amino acid *1-naphthylalanine* (1-nal) present in the fragment.
As internal standard, allowing for an efficient control of all steps of the method including the digestion as well as matrix effects during LC-MS/MS analysis, the stable isotope-labeled peptide monomer was implemented, which contains a [$^{13}C_2$,^{15}N]-labeled glycine, still present in the target pentapeptide after proteolytic digestion. So eventually, the same pentapeptides only differing by three mass units were obtained for analyte and internal standard, respectively, as illustrated by the corresponding product ion spectra (Figure 2).

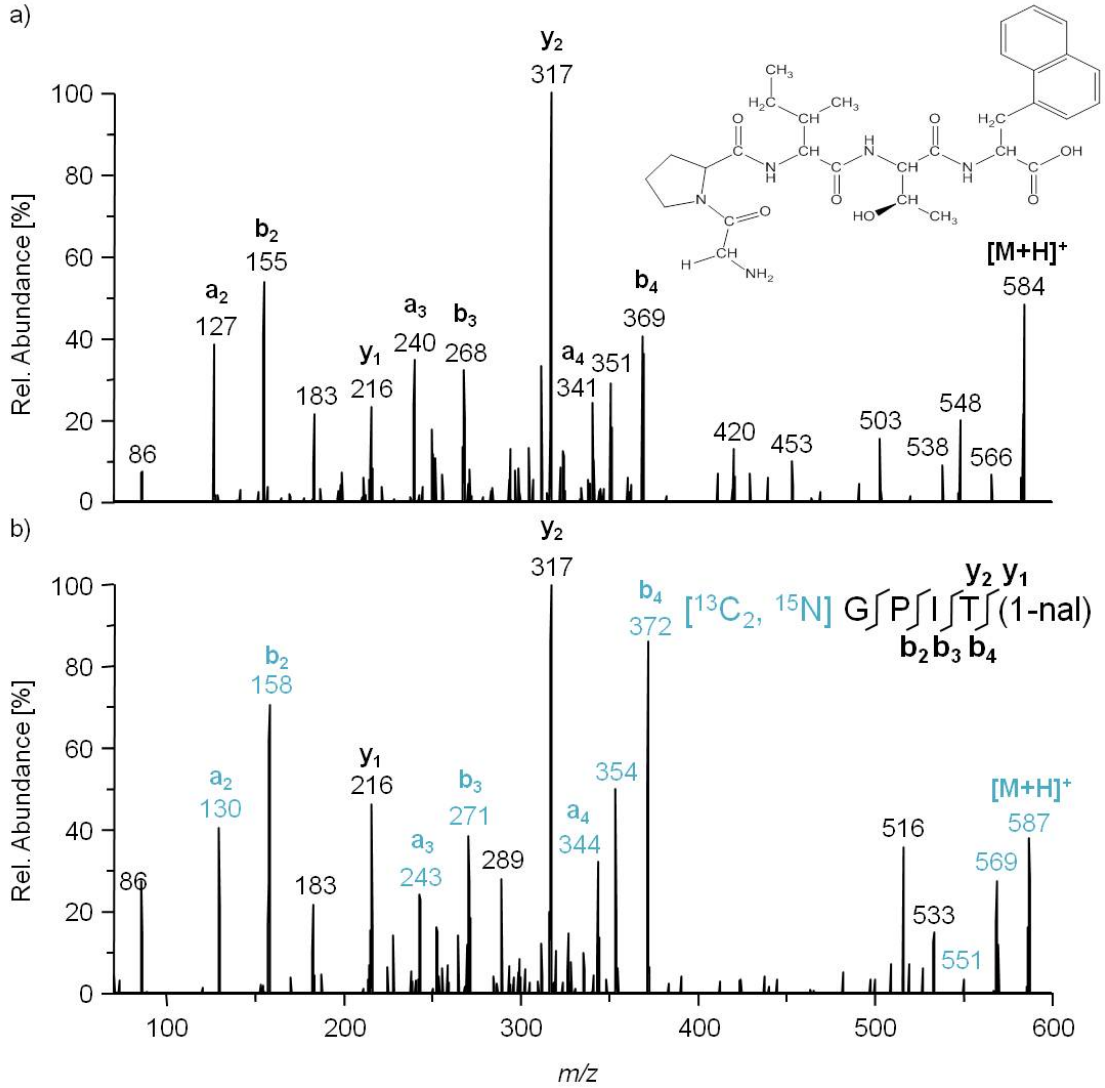

Figure 2: ESI product ion spectra of the protonated proteotypic pentapeptide fragment of the analyte (a) and the internal standard (b) after subtilisin digestion, recorded on a QTrap mass spectrometer at a collision energy of 35 eV. The stable isotope labeling of the internal standard in b) is clearly observable by a +3-Da shift as well as the coloring in light-blue.

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Comprising of a protein precipitation, a proteolytic digestion with subtilisin, and a purification by means of solid-phase extraction on strong cation-exchange resin prior to LC-MS/MS analysis, the assay is simple, rapid, and cost-effective, especially as the final cation-exchange purification step can be omitted in routine analysis, while, by performing the digestion in half of the volume, an equal sensitivity can be achieved.
Using the MRM mode, the proteotypic peptide fragment was unambiguously detected on the API4000 while no interferences were observed in a blank specimen as depicted in Figure 3, showing a blank serum specimen as well as a serum sample fortified with 50 ng/mL of peginesatide.

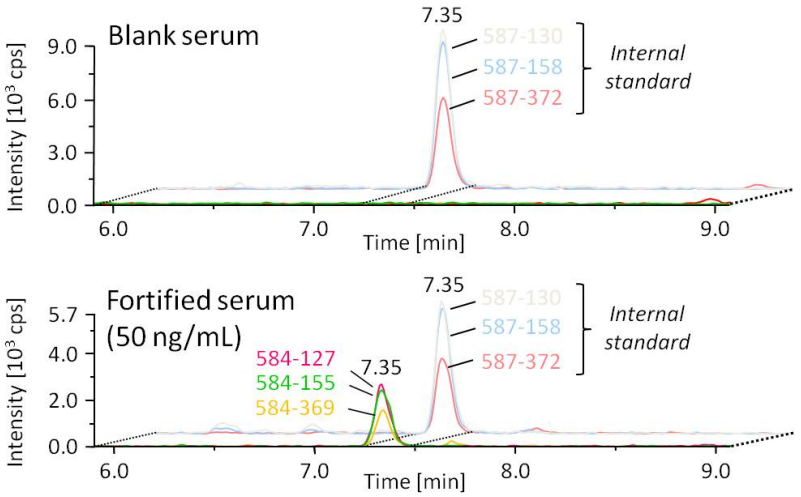

Figure 3: MRM chromatograms recorded on an API4000 QTrap mass spectrometer depicting three diagnostic ion transitions for the proteotypic pentapeptide after subtilisin digestion of the analyte and the internal standard, respectively, of a human blank serum sample (a) and a serum specimen fortified with 50 ng/mL of peginesatide (b).

Method Validation. To evaluate its fitness for purpose in the sports drug testing arena, the assays' most important method characteristics for qualitative purposes were determined in accordance with international guidelines [9]. The main results are summarised in Table 3.

LOD (ng/mL)	Precision at LOD (n = 6)	Recovery (%) at 100 ng/mL (n = 5 + 5)	Linearity			Intraday Precision (n = 30)		Interday Precision (n = 30 + 30 + 30)		Stability	
			Slope	*Intercept*	*Corr. Coeff.*	*Conc. (ng/mL)*	CV (%)	*Conc. (ng/mL)*	CV (%)		
10	9.2	39	0.0076	0.0181	0.997	*10*	12.0	*10*	11.9	*4 °C*	≥ 28 d
API4000			*(n = 7, 1-50 ng/mL)*			*25*	6.8	*25*	7.2		
						50	4.1	*50*	7.4		
1	20.6		0.0086	-0.0015	0.999	*200*	3.7	*200*	4.7	*- 18 °C*	≥ 28 d
Q Exactive			*(n = 7, 10-1,000 ng/mL)*			*500*	4.8	*500*	5.1		

Table 3: Summary of validation results for the detection method of peginesatide in human serum.

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Specificity and LOD. The method proved to be specific as no interfering signals were observed at the respective retention time of the analyte neither at the API4000 nor at the Q Exactive by analysing ten blank serum specimens. The LOD at the API4000 was estimated to be 10 ng/mL as the signal-to-noise ratio, determined by evaluating the signal heights of ten blank specimens and six serum samples fortified with peginesatide at this concentration level, exceeded a value of three. As this criterion was not applicable to the Q Exactive due to non-existent background noise on some ion traces, the interpretability and heights of the analyte signals were used instead to estimate the LOD at 1 ng/mL.

Recovery. By fortifying five serum specimens with peginesatide before and with the corresponding amount of the target pentapeptide, obtained by the proteolytic digestion, after sample preparation, the overall recovery of the method was calculated with approximately 39%.

Linearity. The assay was found to be linear according to Mandel both, over a wider concentration range between 10 and 1,000 ng/mL and for the lower concentration range from 1 to 50 ng/mL with slopes of 0.0086 and 0.0076, intercepts of -0.0015 and 0.0181 as well as correlation coefficients of r = 0.999 and 0.997, respectively.

Precision. To determine inter- as well as intraday precisions, relative standard deviations were calculated by preparing and analysing six aliquots each of a set of five fortified serum specimens on three consecutive days. While the relative standard deviations varied between 4.7 and 11.9% for interday precision (Table 3), they ranged from 6.2 to 12.0% (10 ng/mL), 4.8 to 6.8% (25 ng/mL), 2.3 to 4.1% (50 ng/mL), 2.8 to 3.7% (200 ng/mL), and 3.9 to 4.8% (500 ng/mL), respectively, within one day.

Robustness. The duration of the incubation time for digestion was found to have no significant influence on the detected signals for analyte and internal standard when varied between 3 and 20 h.

Stability. Neither by storage at +4 nor at -18 °C was any degradation of the analyte in serum detected over a period of four weeks, which is in good accordance with studies of the manufacturer revealing that peginesatide was stable in sodium heparin plasma at -20 °C for at least one year [6].

Ion suppression/enhancement effects. At the respective retention time of the analyte, no ion suppression nor enhancement effects were observed by monitoring the signal intensities of the different ion traces of the analyte while co-infusing a solution of the pentapeptide *GPIT(1-nal)*, being the target analyte after subtilisin digestion.

Conclusions

Based on a protein precipitation, a proteolytic digestion, and a strong cation-exchange purification, a liquid chromatographic-mass spectrometric detection method was successfully developed for peginesatide, the first representative of the class of EPO-mimetic peptides, and fully validated for qualitative purposes. With an LOD of 1 ng/mL, it achieves a comparable sensitivity as the developed ELISA (decision limit of 0.5 ng/mL) [6], but bypassing typical issues associated with immunoassay techniques, e.g., susceptibility to cross-reactivity, and is expected to allow for the detection of therapeutic doses for approximately one week. Thus, within the scope of preventive doping research, a specific, sensitive, rapid, and cost-effective detection method is applicable to routine sports drug testing and furthermore, readily transferable to other doping control laboratories.

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