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Mass spectrometric detection of peginesatide in human urine in doping control analysis

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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 anaemic patients. 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 were developed as conventional detection assays for EPO do not allow for the analysis of the EPO-mimetic peptides. However, as urine specimens are the most frequently provided doping control samples and pharmacokinetic studies conducted in rats and monkeys revealed the excretion of the pegylated peptide into urine, a detection method for peginesatide in urine would be desirable. A mass spectrometric assay in human urine was developed consisting of protein precipitation with acetonitrile followed by proteolytic digestion after the removal of the acetonitrile fraction under reduced pressure. Purification and concentration of the resulting proteotypic target peptide was accomplished by means of solid-phase extraction on strong cation-exchange resin prior to liquid chromatographic–tandem mass spectrometric analysis. Method validation was performed for qualitative purposes and demonstrated specificity, precision, linearity as well as sufficient sensitivity (limit of detection: 0.5 ng/ml) while proof-of-concept for the applicability of the assay for the determination of peginesatide in authentic urine samples was obtained by analyzing animal *in vivo* specimens collected after a single i.v. administration of peginesatide over a period of 4 days.

1. Introduction

Peginesatide (formerly referred to as Hematide™) is an erythropoiesis-stimulating agent (ESA) which has been approved recently by the U.S. Food and Drug Administration (FDA) 'for the treatment of anaemia due to chronic kidney disease (CKD) in adult patients on dialysis' [1] and is marketed under the trade name Omontys®.

Unlike the glycoprotein erythropoietin (EPO), it consists of a homodimeric peptide (~5 kDa) which is coupled to a branched polyethylene glycol (PEG) derivative of approximately 40 kDa [2,3] to achieve a considerably longer half-life as well as a reduced immunogenicity in the body [4]. Despite its peptidic structure dissimilar to that of EPO, specific binding to the EPO-receptor was observed eventually resulting in the stimulation of erythropoiesis and an enhanced production of red blood cells [5,6]. Thus, peginesatide was considered as promising drug candidate for the treatment of anaemia [7–9] and demonstrated to be effective in maintaining the haemoglobin level in patients with chronic kidney disease on dialysis previously treated with epoetin while the side effect profile was considered non-inferior to the comparator [10].

In view of the presumed effects analogous to EPO, peginesatide is expected to have an advantageous effect on endurance performance [11] and has thus been explicitly banned by the World Anti-Doping Agency (WADA) under paragraph S2.1 *Erythropoiesis-Stimulating Agents* since 2009 [12].

Until now, two different approaches have been developed for detecting peginesatide in blood: one mass spectrometric based assay after proteolytic digestion applicable to plasma/serum or dried blood spots [13,14] as well as an enzyme-linked immunosorbent assay (ELISA) screening method for serum and plasma samples employed in combination with Western double blotting detection after gel electrophoretic separation for confirmatory purposes [15]. However, as the majority of doping control samples provided are urine specimens, a detection method for peginesatide in that matrix would be desirable. Pharmacokinetic studies in rats and monkeys revealed the kidney as primary route of elimination and described the excretion of a significant amount of the pegylated peptide into

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urine [2,16]; to the best of our knowledge comparable data from human administration have not been published so far. As the data provided for rats and monkeys were quite similar with regard to the recovered dose over a period of two weeks [2,16], there is no indication to assume an entirely different behaviour in the human body.

However, all data were based on radioactivity measurements after an i.v. application of ^{14}C-peginesatide, bearing the radiolabel at the lysine residue located between the two PEG monomers (Fig. 1) [2]. This does not provide conclusive information on whether the peptidic moiety of peginesatide being of particular importance for both detection approaches (mass spectrometry as well as ELISA) is excreted intact into urine. Therefore, in the present study, a specific and sensitive mass spectrometric detection method for peginesatide in human urine was developed and an administration study with laboratory rodents conducted in order to obtain proof-of-concept of the applicability for the determination of the target peptide in authentic urine samples.

2. Experimental

2.1. Chemicals and reagents

Sodium hydroxide (NaOH), hydrochloric acid 32% (HCl), sodium chloride (NaCl), sodium phosphate monobasic dihydrate ($NaH_2PO_4{\cdot}2H_2O$), sodium phosphate dibasic (Na_2HPO_4), ammonia solution 25% (NH_3) and glacial acetic acid were purchased from Merck (Darmstadt, Germany) and formic acid, trifluoroacetic acid (TFA), urea, thiourea, CHAPS, Trizma® base as well as the protease from *Bacillus licheniformis* (type VIII, ~12 units/mg, subtilisin) were obtained from Sigma–Aldrich (Steinheim, Germany). Methanol and acetonitrile (both high-performance liquid chromatography (HPLC) grade) were purchased from VWR (Leuven, Belgium) and deionised water for aqueous buffers and solutions was of Milli-Q grade. The strong cation-exchange cartridges Strata SCX (100 mg, 1 ml) were obtained from Phenomenex (Aschaffenburg, Germany), the internal standard (AcGGLYA**C**HM[$^{13}C_2$, ^{15}N]GPIT(1-nal)V**C**QPLR(MeG)K-NH2, oxidised form) was ordered from BMFZ (Düsseldorf, Germany) and the pentapeptide *GPIT* (*1-nal*) from PANATecs (Tübingen, Germany) whereas the pegylated EPO-mimetic peptide peginesatide was synthesised in-house as described elsewhere [13].

2.2. Liquid chromatography–mass spectrometry

Liquid chromatography–mass spectrometry (LC–MS) measurements were performed either on a triple quadrupole instrument (API4000 QTrap, Applied Biosystems, Darmstadt, Germany) or a Q Exactive (Thermo, Bremen, Germany), a triple quadrupole Orbitrap hybrid instrument.

The API4000 QTrap mass spectrometer was coupled to an Agilent 1100 series liquid chromatograph (Waldbronn, Germany) which was operated with 0.2% formic acid (A) and acetonitrile (B) as solvents and a Nucleodur C18 Pyramid analytical column (2 mm × 50 mm, 3 μm, Macherey-Nagel, Düren, Germany). Gradient elution was done at a flow rate of 250 μl/min starting at 90% A and declining to 40% A over 9 min, from which it further decreased, after a 1 min hold, to 0% A within 0.25 min. After another 0.75 min at 0% A, the column was re-equilibrated at starting conditions for 5 min. Using a spray voltage of 5.5 kV and a capillary temperature of 450 °C, the mass spectrometer was operated in multiple reaction monitoring (MRM) mode with positive electrospray ionisation (ESI+) and nitrogen gas was provided by a nitrogen generator (CMC Instruments, Eschborn, Germany). Further details regarding mass spectrometric parameters and ion transitions are listed in Table 1.

Table 1
Ion transitions and parameters applied at the API4000 for MRM experiments; the dwell time was set to 50 ms.

Compound	Declustering potential (V)	Ion transition (*m/z*)	Collision energy (eV)
Peginesatide	60	584.3–127.1	60
		584.3–155.1	40
		584.3–216.1	45
		584.3–317.1	30
		584.3–341.2	30
		584.3–351.2	30
		584.3–369.2	25
Internal standard	60	587.3–130.1	60
		587.3–158.1	40
		587.3–317.2	30
		587.3–372.2	25

The Q Exactive was equipped with a Thermo Open Accela liquid chromatograph and a heated ESI source (HESI-II), which was operated at 350 °C. With a capillary temperature of 320 °C, a ionisation voltage of 4 kV and nitrogen as collision and damping gas obtained from a nitrogen generator (CMC instruments), three MS settings were performed in positive ionisation mode with a resolution of 35,000 (full width at half maximum): a full scan experiment (*m/z* 400–600) as well as two targeted MS^2 experiments at *m/z* 584.31 and 587.31 applying a collision energy of 30 eV and a precursor isolation window of 1.2 Da. Mass accuracy was tested prior to the analyses using the manufacturer's calibration solution containing Ultramark, caffeine and MRFA. The liquid chromatographic separation was realised on a Thermo Hypersil Gold C18 column (2.1 mm × 50 mm, 1.9 μm) and the respective pre-column (2.1 mm × 10 mm, 2 μm) with a flow rate of 200 μl/min. The gradient decreased from a composition of 95%, 0.2% formic acid (A) and 5% acetonitrile (B) to 60% A within 9 min, maintained there for 1 min before it further declined to 0% A over 0.5 min followed by a 0.5 min hold and re-equilibration (5 min).

2.3. Sample preparation

To a volume of 500 μl of urine, placed in a 1.5 ml Eppendorf tube, 5 μl of the internal standard solution (c = 0.25 μg/ml) as well as 500 μl of acetonitrile were added. After 5 min shaking on a rotating wheel and centrifugation at 17,000 × *g* for 7 min, the supernatant was transferred to a fresh tube and the acetonitrile removed using a vacuum centrifuge (~20 min). To the remaining liquid remnant, 100 μl of a subtilisin solution (c = 2 mg/ml in PBS buffer) and further 400 μl of PBS buffer (containing 137 mM NaCl, 8 mM Na_2HPO_4 and 1.3 mM $NaH_2PO_4{\cdot}2\ H_2O$, pH 7.4) were added before the sample was incubated in a thermomixer at 37 °C for 3 h with gentle shaking (450 rpm). Subsequently, the sample, acidified with 10 μl of TFA (pH ~ 1.5) and centrifuged (1 min at 17,000 × *g*), was purified and concentrated by means of a strong cation-exchange cartridge preconditioned with 1 ml of 0.2 N NaOH, 0.1 N HCl and acetonitrile/water (1:3, v/v), respectively. Then, washing with 1 ml of acetonitrile/water (1:3, v/v) as well as 1 ml of methanol was accomplished before the cartridge was eluted twice with 0.5 ml of 5% NH_3 in methanol (v/v, freshly prepared). The combined eluate was evaporated to dryness in a vacuum centrifuge and resuspended in 40 μl of 2% acetic acid before analysis, where 10 μl of the solution were injected.

For frozen urine samples, sample preparation was performed as described above, but in contrast to this procedure, multiple chaotropic sample solution (MCSS, 7.7 M urea, 2.2 M thiourea, 4.4% CHAPS (w/v), and 44 mM Trizma® base) was added after the first centrifugation step to the pellet, instead of it being discarded, until it was completely resuspended (here 50 μl). To ensure acceptable conditions for subtilisin digestion, the mixture was diluted 1:7 (v/v)