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Doping control analysis of filgrastim in equine plasma and its application to a co-administration study of filgrastim and recombinant human erythropoietin in the horse

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ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor regulating granulopoiesis. The recombinant human granulocyte colony-stimulating factor (rhG-CSF) is widely used for the treatment of granulopenia in humans. Filgrastim is a rhG-CSF analogue and is marketed under various brand names, including Neupogen® (Amgen), Imumax® (Abbott Laboratories), Neukine® (Intas Biopharmaceuticals) and others. It is banned in both human and equine sports owing to its potential for misuse. In order to control the abuse of filgrastim in equine sports, a method to identify unequivocally its prior use in horses is required. This study describes an effective screening method for filgrastim in equine plasma by enzyme-linked immunosorbant assays (ELISA), and a follow-up confirmatory method for the unequivocal identification of filgrastim by analysing its highly specific tryptic peptide ^{1}MTPLGPASSLPQSFLLK^{17}. Filgrastim was isolated from equine plasma by immunoaffinity purification. After trypsin digestion, the mixture was analysed by nano-liquid chromatography–tandem mass spectrometry (LC/MS/MS). Filgrastim could be detected and confirmed at 0.2 ng/mL in equine plasma. The applicability of the ELISA screening method and the LC/MS/MS confirmation method was demonstrated by analysing post-administration plasma samples collected from horses having been co-administered with epoetin alfa as recombinant human erythropoietin (rhEPO) and filgrastim as rhG-CSF. rhEPO and filgrastim could be detected in plasma samples collected from horses for at least 57 and 101 h respectively. To our knowledge, this is the first identification of filgrastim in post-administration samples from horses.

1. Introduction

Granulocyte colony-stimulating factor (G-CSF), also known as colony stimulating factor 3 (granulocyte), is a glycoprotein produced by monocytes, fibroblasts, and endothelial cells. It regulates the production of neutrophils within the bone marrow and affects neutrophil progenitor proliferation [1,2], neutrophil progenitor differentiation [1,3] and selected end-cell functional activation (including enhanced phagocytic ability [4], priming of the cellular metabolism associated with respiratory burst [5], antibody dependent killing [6], and the increased expression of some functions associated with cell surface antigens [7]). The mature excreted form of natural human G-CSF (hG-CSF) is an 18.8 kDa protein consisted of 174 amino acids. It has two intra-molecular disulphide bonds and one free cysteine [8]. A single O-glycosylation at Thr133 was present protecting the protein from aggregation. The recombinant hG-CSF (rhG-CSF), namely filgrastim, is manufactured by recombinant DNA technology [9]. It is a 175 amino acid protein produced by *Escherichia coli* bacteria into which the hG-CSF gene has been inserted. This protein has an amino acid sequence identical to natural hG-CSF, except for the addition of a N-terminal methionine necessary for expression in *E. coli* as well as being non-glycosylated. Apart from filgrastim, there are also other rhG-CSF products, such as Nartograstim, Lenograstim and PEGylated filgrastim in the market.

rhG-CSF is an inevitable therapy for granulopenia and represents a major step forwards in oncology. It is used to accelerate recovery from neutropenia after chemotherapy, and to increase the number of hematopoietic stem cells in the blood of the donor

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before transplantation. In the horse, there have been reports on subcutaneous administration of rhG-CSF to stimulate *in vitro* bone marrow cells in Standardbred horses with familial neutropenia, and to accelerate bone marrow production of neutrophils in foals with alloimmune neonatal neutropenia [10]. Besides, rhG-CSF has been used in combination with recombinant human erythropoietin (rhEPO) for the treatment of anaemia, peripheral blood progenitor cell or autologous stem cell transplantation in human [11–14]. In all these reports, the rhEPO plus rhG-CSF treatments gave promising results.

Erythropoietin (EPO) is the major glycoprotein regulator for erythropoiesis, the process that controls the production of red blood cells in mammals. rhEPO has been used as an erythropoiesis-stimulating agent (ESA) with a history of abuse in human and even equine sports, despite there has been report showing the use of such human proteins in horses could cause severe, and sometimes fatal, anaemia [15]. Apart from anaemia, neutropenia was apparently another problem encountered during intermittent ESA administrations, resulting in low-grade infections.

More recently, there was a report on the influence of rhG-CSF on blood parameters relevant for the Athlete Biological Passport [16]. It is reasonable to believe the ESA plus rhG-CSF treatment regimen might also be used in doping. Indeed, a US harness racing trainer was charged with the possession of Epogen (rhEPO) and Neupogen (rhG-CSF) in 2004 [17], which was basically direct proof of their abuse in racing. In 2009, there was also a relevant incident in Hong Kong. Some empty syringes labelled as containing Neupogen®, the active ingredient of which is recombinant human granulocyte colony-stimulating factor (rhG-CSF), were mysteriously found in a trainer's stable. Neupogen® is neither an authorised medication in the Hong Kong Jockey Club nor a registered equine therapeutic in Hong Kong. The source of supply of the syringes could not be established, nevertheless this incident has arisen our concern of using rhG-CSF in doping. The use of rhG-CSF combined with ESA might give a 'more balanced' blood cell production by the bone marrow during the blood boosting treatment regimen and avoid possible problems caused by relatively low neutrophil levels, including chronic mild infections. Both ESAs and rhG-CSF are prohibited in horse racing as implicit in Article 6 of the International Agreement of Breeding, Racing and Wagering (published by the International Federation of Horseracing Authorities) [18].

In order to control the abuse of ESAs and rhG-CSF in equine sports, methods for their effective detection and identification in horses are required. A number of methods have already been reported for the screening and confirmation of ESAs in biological samples [19–28]; however there is limited report on the detection of rhG-CSF. The only study available was the analysis of rhG-CSF in rat serum using immunochemical detection, with the lower calibration limit at 12.5 ng/mL [29]. The objective of this study was to evaluate an immunoassay for the screening of rhG-CSF in equine plasma, and to develop a sensitive mass-spectrometry based method for the confirmation of filgrastim (one of rhG-CSF products) in horse blood samples. Immunoassay is a simple, efficient and practical technique for screening protein-based drugs. It can detect suspicious samples containing the particular drugs of interest from a large number of samples within a short period of time. Several enzyme-linked immunosorbent assays (ELISA) from different suppliers for the screening of rhG-CSF were evaluated. When a sample suspicious for rhG-CSF was detected, it would be subjected to LC/MS confirmatory analysis, which could provide unequivocal proof for the presence or otherwise of filgrastim in the sample. Filgrastim was isolated from equine plasma by immunoaffinity purification followed by trypsin digestion. The trypsin digest was then analysed by nano-liquid chromatography–tandem mass spectrometry (LC/MS/MS). Confirmation of filgrastim was achieved by monitoring a highly specific tryptic peptide ^{1}MTPLGPASSLPQSFLLK^{17}. In order to demonstrate the applicability of this method in doping control, post-administration blood samples collected from two horses co-administered with filgrastim and rhEPO were analysed by this method.

2. Experimental

2.1. Materials

Filgrastim (Neupogen®, rhG-CSF, 300 μg/0.5 mL syringe) was obtained from Roche (Basel, Switzerland). Epoetin alfa (EPREX®, rhEPO, 2000 IU/mL and 10,000 IU/mL) was purchased from Jassen-Cilag Ltd (Saunderton, High Wycombe, Bucks, UK). Darbepoetin alfa (DPO) (Aranesp®, 100 μg/0.5 mL) was obtained from Amgen Inc. (Thousand Oaks, CA, USA). Methoxy polyethylene glycol-epoetin beta (PEG-EPO) (Mircera®, 150 μg/0.3 mL) was purchased from Roche Products Pty Limited (Dee Why, NSW, Australia). All the stock solutions were stored in Eppendorf vials at 4 °C before use. Peptides VNFYAWK (for human EPO) and MTPLGPASSLPQSFLLK (for filgrastim) were synthesised by Synbiosci (Livermore, CA, USA). Polyclonal anti-human EPO antibody (P/N AB-286-NA, produced in rabbits immunised with purified, Chinese hamster ovary (CHO) cell-derived, recombinant human erythropoietin), monoclonal anti-human G-CSF antibody (P/N MAB 214, Monoclonal Mouse IgG1 Clone # 3316) and ELISA kits for hEPO (P/N DEP00) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Sequencing grade modified trypsin was purchased from Roche (P/N 11418025001) (Indianapolis, IN, USA). Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Pierce (Rockford, IL, USA). PEG4000 was obtained from BDH (Radnor, PA, USA). ELISA kits for hG-CSF were purchased from eBiosicence (P/N BMS2001INST, Vienna, Austria), Genway Biotech (P/N 40-006-224001, San Diego, CA, USA) and Invitrogen (P/N KHC2032, Camarillo, CA, USA). Working standard solutions of individual protein standards were prepared by consecutive dilution of the respective stock solutions. Magnetic beads, Dynabeads M-280 tosylactivated, and magnetic particle concentrator (MPC) were obtained from Invitrogen (Carlsbad, CA, USA). Igepal CA-630 (biochemical grade) was obtained from Fluka (Buchs, Switzerland). Sodium hydroxide (pellets, analytical grade) and boric acid (analytical grade) were purchased from Riedel-de Haën (Seelze, Germany). Acetic acid (100%, Suprapur) hydrochloric acid (30%, Suprapur) and formic acid (>98%, Suprapur) were obtained from Merck (Darmstadt, Germany). Low-binding sample vial (2-mL) was purchased from Eppendorf (Hamburg, Germany). Polypropylene (PP) plastic tubes (100 mm × 13 mm) were obtained from Sarstedt (Numbrecht, Germany). Autosampler vial (PP Snap ring micro-vial, 0.3-mL) were obtained from La-Pha-Pack (Langerwehe, Germany). HPLC grade deionised water was obtained from a Millipore water purification system (Milli-Q, Molsheim, France). Acetonitrile (ACN) (Chromasolv; gradient grade), phosphate-buffered saline (PBS), EDTA, sodium azide and Trizma base were purchased from Sigma (St. Louis, MO, USA). Buffers used for linking the polyclonal anti-human EPO antibody or the monoclonal anti-human G-CSF antibody to the magnetic beads and subsequent immunoaffinity purification of the plasma samples were prepared according to the Invitrogen's manual and another reference [28]. These included PBS, pH 7.4; borate buffer, 0.1 M, pH 9.5 (*buffer* B in the Invitrogen's manual); PBS (pH 7.4), plus 0.1% (w/v) bovine serum albumin (BSA), 2 mM EDTA, and 0.02% (w/v) sodium azide (*buffer* C); Tris buffer (0.2 M, pH 8.5) plus 0.1% (w/v) BSA (*buffer* D); washing buffer, 1% (w/v) Igepal CA-630 in PBS (pH 7.4); and elution buffer, PBS (adjusted to pH 2.0 with concentrated HCl). All the buffers were stored at 4 °C.