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Rapid detection of erythropoiesis-stimulating agents in urine and serum

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ABSTRACT

A rapid and easy-to-use test kit, EPO WGA MAIIA, which can be used for distinguishing various endogenous human erythropoietins (hEPOs) and several recombinant hEPO and EPO analogues, has been evaluated. The test is based on chromatographic separation of the glycosylated isoforms of EPO using wheat germ agglutinin (WGA) and a sensitive immunoassay using anti-EPO carbon black nanostrings and image scanning for quantification. All of the reactions take place along the porous layer of a lateral flow microcolumn containing WGA and anti-EPO zones. The presence of molecules resembling hEPOs, such as Mircera, was detected by the aberrant affinity interaction with the antibody zone on the strip. It was possible to distinguish nine recombinant hEPOs expressed in hamster and human cell lines, as well as Aranesp and Mircera, from endogenous urine hEPO. The required amount of EPO in the samples, a few picograms, is very low compared with other methods for EPO isoform identification. This EPO isoform determination method opens the possibility to monitor recombinant EPO therapy for clinical research and seems to be a valuable candidate to the arsenal of EPO doping control tests.

Erythropoietin (EPO)[1] is a glycoprotein hormone responsible for the homeostatic regulation of red cell production, being up-regulated by hypoxia through a recently described oxygen-sensing mechanism [1,2]. Assays for measurement of endogenous erythropoietin were developed both as tools for studies of pathophysiology of anemia and polycythemia and for diagnostic purposes in the same conditions. Biological assays, such as the polycythemic mouse assay, were specific in the sense that they measured biologically active EPO but had low sensitivity and were expensive and time-consuming [3]. Radioimmunoassays with better sensitivity and lower cost were developed and honed into measuring only bioactive EPO [4,5]. In addition to the medically motivated need for detection and measurement of endogenous EPO, a growing need for corresponding methods for exogenous erythropoiesis-stimulating agents (ESAs) has emerged as result of the use of these agents as performance-enhancing agents in endurance sports [6,7] and the health risks incurred by such use [8–10]. In this regard, the detection of ESA doping is dependent on the possibility to distinguish between endogenous EPO and exogenous ESAs in test samples from athletes.

Furthermore, there is a need to distinguish between EPO produced in liver and kidneys for research in EPO physiology. In fetal life EPO is produced solely in the liver, and shortly after birth it is shifted to be produced in the kidney [11,12]. For adults, more then 90% of EPO comes from the kidney, whereas the liver produces up to 10% during normal conditions. During extreme hypoxia, the production from the liver may account for up to 50% [13]. The different endogenously produced forms of human EPO (hEPO) show microheterogeneities in their carbohydrate structures. EPO circulating in the blood during the evening contains less negatively charged isoforms than during the morning [14]. Liver-produced EPO in blood from newborn infants contains less negatively charged isoforms than EPO produced in the kidney [14]. Kidney tumors also seem to produce hEPO with the same charge as liver EPO and recombinant hEPO (rhEPO).

Doping in sports using hormones and the development of EPO doping control procedures have been reviewed recently [15–17]. The isoforms of the glycoprotein hormone hEPO from various

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[1] *Abbreviations used:* EPO, erythropoietin; ESA, erythropoiesis-stimulating agent; hEPO, human EPO; rhEPO, recombinant hEPO; p*I*, isoelectric point; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; s.c., subcutaneous; WADA, World Anti-Doping Agency; GlcNAc, *N*-acetylglucosamine; CBNS, carbon black nano-strings; dbpp, delta blackness per pixel; PMI, percentage of migrated isoforms; RAM, relative analyte migration; AbQ, antibody line quotient; IEF, isoelectric focusing; CHO, Chinese hamster ovary; BHK, baby hamster kidney; SD, standard deviation; CV, coefficient of variation; SAR, sarcosyl; PEG, polyethylene glycol.

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production sites (e.g., human cells, Chinese hamster cells) show differences in charge [14,18] or isoelectric point (p*I*) [19] related to the presence of various numbers of sialic acids and sulfonate structures in the carbohydrate side chains. Minor differences in apparent molecular mass of rhEPO compared with endogenous EPO have been revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20,21]. Affinity chromatography separation, using the lectin wheat germ agglutinin (WGA), has been used to distinguish hEPO forms [22] by their varying content of polylactosamine structures [23]. The use of mass spectrometry for measuring size differences of intact or fragmented EPO, or glycan composition, seems not (so far) to have sufficient sensitivity to measure the presence of low concentrations of hEPO in biological samples [24–26]. All of these different methods require skilled technicians, several hands-on steps, and expensive equipment and also take several days to perform.

There is a large number of ESA variants on the market [8,27,28] that differ in biological activity and structure (e.g., glycosylation) due to cell line expression and details in the manufacturing process such as culture conditions and purification methods. New ESAs are continuously entering the market, and this will make doping control even more difficult than it is today [29]. The recently described MAIIA technology [30] seems to be a suitable technology for improved isoform identification of glycoproteins, such as hEPO, because it can distinguish minor differences between subpopulations. The technology is flexible, and several types of ligands can be applied to distinguish subpopulations through affinity chromatography interaction [31–33]. The detection sensitivity and specificity have been shown to be high [34,35]. It seems possible to adapt the technology to high-throughput testing.

This article evaluates a rapid and easy-to-use EPO isoform determination method based on the novel MAIIA technology combining WGA affinity separation and ultra-sensitive EPO immunoassay in a thin lateral flow strip. The antibody interaction profile, formed between EPO in the analyzed sample and anti-EPO antibody present on the detection zone of the strip, can also be used to reveal the presence of EPO analogues.

Materials and methods

Biological samples

Urine specimens from healthy volunteer donors (men and women between 20 and 60 years of age) and from patients receiving EPO therapy were collected after approval by the local ethics committee. The 14 tested patients from the hematology department each received a subcutaneous (s.c.) dose of 10,000 to 30,000 IU of epoetin or 150 μg of Aranesp at each injection, for some patients every day but for most of them once a week, as shown together with the results. Urine samples from patients with kidney failure receiving Aranesp (30 μg/every third week to 50 μg/week, $n = 18$) and NeoRecormon ($n = 8$) were also obtained, but without information about injection day and sample collection day. From participants in a major sports competition (athletics), urine samples ($n = 101$) were collected and an EPO doping test was performed by a World Anti-Doping Agency (WADA)-accredited anti-doping laboratory in accordance with WADA technical document TD2004EPO. Blood samples ($n = 150$) were also collected. For the imprecision study, 405 affinity-purified urine and plasma samples from horses and humans injected with epoetin β was used, with approximately 50% of the samples containing rhEPO in excess. These studies had approval from the local ethics committee. The serum and plasma samples used were leftovers from a health control. The specimens were stored in aliquots at −20 °C.

Recombinant EPO

Eprex, recombinant epoetin α, was purchased from Janssen–Cilag (Sollentuna, Sweden). NeoRecormon, recombinant epoetin β, and Mircera, methoxy polyethylene glycol-epoetin β, were obtained from Roche (Mannheim, Germany). Aranesp, the recombinant EPO analogue darbepoetin α, was purchased from Amgen (Thousands Oak, CA, USA). For Eprex and NeoRecormon, 1 IU of epoetin corresponds to 8.4 and 8.3 ng of recombinant EPO, respectively. Retacrit, recombinant epoetin ζ, was purchased from Hospira Enterprises (Hoofddorp, Netherlands), and Dynepo, recombinant epoetin δ, was obtained from Shire Pharmaceuticals (Basingstoke, UK). Epomax, recombinant epoetin ω (Lek Pharmaceutical and Chemical Company, Ljubljana, Slovenia), was obtained from Slovenia, and EPIAO (3SBio, Shenyang Sunshine Pharmaceutical, Shenyang, China) was obtained from Jia Lin Hao(Shandong E-Hua, Shandong, China), Ji Mai Xin (NCPC GencTech Biotechnology, Shijiazhuang, China), and Ning Hong Xin (Huaxin Pharm, Leshan, China).

Affinity purification of EPO from biological fluids

An EPO Purification Kit (cat. no. 0250) was obtained from MAIIA Diagnostics (Uppsala, Sweden, www.maiiadiagnostics.com), and EPO from urine and serum samples was purified according to the instructions from the producer using the recommended addition of bovine serum albumin (BSA) and detergent to the reagents. The urine samples (5–33 ml) collected from athletes during a major sports competition were purified with a prototype to this kit using the same procedure but with a slightly wider anti-EPO monolith, ∅13 mm. The recovery for urine specimens from the competition study, exceeding an EPO concentration of 1.5 ng/L in urine, was 49%, calculated after measuring the EPO concentration in urine and in eluate.

Basic laboratory procedure for EPO WGA MAIIA

An EPO WGA MAIIA prototype kit was obtained from MAIIA Diagnostics and used in accordance with the instructions from the supplier. Fig. 1 describes the test principle. 25 μl sample of diluted affinity purified EPO was dispensed into four microtiter wells for the duplicate determination. Other wells were prepared by dispensing 25 μl each of elution buffer containing low (optimized) and high (100 mM) concentrations of *N*-acetylglucosamine (GlcNAc), anti-EPO-CBNS (carbon black nano-strings), and washing buffer. After mounting and drying the strips, the intensity in the anti-EPO zone of the strip was detected by an Epson Expression 1680 scanner (Epson, Sollentuna, Sweden) used in reflection mode with an optical resolution of 600 ppi and 16-bit sample depth in accordance with the scanner detection instructions (MAIIA Diagnostics). MAIIAcalc software calculated the intensity signal in the anti-EPO zone as delta blackness per pixel (dbpp). The principle for detection of grayscale intensity for the carbon black line has been described previously [34].

Standardization and concentration determination

A standard curve of epoetin β (NeoRecormon) was obtained by dilution from the provided stock solution of 10 μg/L to 3 to 1000 ng/L using the sample dilution buffer. The standard series was measured by the EPO WGA MAIIA method using elution buffer *high,* which releases all EPO for migration to the anti-EPO zone. The EPO concentration of unknown samples was obtained from their elution buffer *high* values. The concentration values for unknown samples using *high* and *low* elution buffer in EPO WGA MAIIA was calculated by using a four-parameter logistic curve fit program (WorkOut 2, PerkinElmer, Turku, Finland) for the signal intensity