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Comparison of the Isoelectric Focusing Patterns of Darbepoetin Alfa, Recombinant Human Erythropoietin, and Endogenous Erythropoietin from Human Urine,

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Novel erythropoiesis-stimulating protein (Aranesp™; darbepoetin alfa) is a glycoprotein hormone with a longer serum half-life than recombinant human erythropoietin (rHuEPO) *(1)*. The polypeptide backbone of the human EPO molecule has an invariant amino acid sequence; however, the carbohydrate side chains exhibit microheterogeneity in sugar content and structure *(2–4)*. A negatively charged sialic acid molecule typically caps the end of each arm of a carbohydrate chain. As a consequence, the variable nature of the sialic acid content gives rise to EPO isoforms with differences in charge *(3)*. After purifying isoforms of rHuEPO, Egrie and coworkers *(5, 6)* discovered a direct correlation between the number of sialic acid groups on the carbohydrate part of rHuEPO and both its serum half-life and biological activity, as well as an inverse relationship with receptor binding. These data showed that pharmacokinetic factors have a greater influence on biological activity than receptor binding affinity. These principles explain the increased half-life and increased in vivo activity of darbepoetin alfa, which contains 5 N-linked carbohydrate chains and up to 22 sialic acids *(5, 7)*. In contrast, rHuEPO has 3 N-linked carbohydrate chains and a maximum of 14 sialic acids *(5, 7)*.

Similar clinical responses can be achieved by administering darbepoetin alfa once a week or rHuEPO three times a week *(8, 9)*. The efficacy of darbepoetin alfa in the treatment of anemia associated with chronic renal failure has been shown *(10)*, and in 2001 it was approved by the US Food and Drug Administration for that indication. Darbepoetin alfa is under investigation for the treatment of anemia in cancer patients *(11)* and other applications. Although darbepoetin alfa was approved only recently, we detected darbepoetin alfa in the urine of three athletes competing in the 2002 Winter Olympic Games in Salt Lake City. To date, it has not been reported in human urine.

The isoelectric focusing (IEF) patterns of standard rHuEPO, endogenous human EPO in urine extracts, and administered rHuEPO in urine extracts have been reported *(12)*. This report describes the IEF pattern observed after applying the same method to standard darbepoetin alfa and post-administration urine extracts.

The pooled urine of two healthy, drug-free males was used as the endogenous HuEPO control urine (QC1). The rHuEPO positive control urine (QCP) was pooled urine from healthy individuals (eight males and seven females) who received rHuEPO on nine visits over 19 days (50 IU/kg at each visit). Some, but not all, urines were included in the pool. A urine collected from a female cancer patient 1 week after a single dose (0.675 μg/kg) of darbepoetin alfa (Aranesp; Amgen Inc., Thousand Oaks, CA) was used as the darbepoetin alfa control urine. The participants gave written informed consent under applications approved by the UCLA Office of Human Subject Protection.

Aranesp (60 mg/L) containing human serum albumin was obtained from a pharmacy. EPO Biological Reference Preparation (BRP) was obtained from the European Directorate for the Quality of Medicines (Strasbourg, France). Tris base, phosphate-buffered saline tablets, glycine, 100 mL/L Tween 80R (low peroxide), dithiothreitol, sucrose, and bovine serum albumin (RIA grade) were purchased from Sigma. Protease inhibitor (Complete) was purchased from Roche Diagnostics. Urea, Ready-Mix IEF acrylamide/bisacrylamide (29:1 by weight), ammonium persulfate, and *N,N,N,N*-tetramethylethylenediamine were purchased from Amersham Biosciences, and the ampholytes Servalyt 2-4, 4-6, and 6-8 were purchased from Serva. Nonfat dry milk was purchased in a supermarket. The primary antibody (AE7A5; monoclonal mouse anti-hEPO) was obtained from R&D Diagnostics, and the secondary antibody conjugate [biotin–goat anti-mouse IgG (H+L)] and horseradish peroxidase–streptavidin conjugate (both Zymax grade) were obtained from Zymed Laboratories. The chemiluminescence substrate (ChemiGlow) was obtained from Alpha Innotech Corp. Phosphoric acid was obtained from Aldrich Chemicals, glacial acetic acid (HPLC grade) was from Mallinckrodt Chemical, and black ink (Tusche A) was from Pelikan.

Unless specified, we used electrophoresis or higher grade chemicals.

The method was originally described by Lasne *(13)*. All modifications are detailed below. A minimum of 20 mL of urine was adjusted to near neutral pH with 3.75 mol/L Tris (pH 7.4) to inhibit any acidic protease activity. The activities of other proteases were inhibited by adding Complete. Any particulate matter was removed from the urine by centrifugation and microfiltration (0.22 μm) of the supernatant. The filtrate was reduced to the smallest possible retentate volume with a two-step ultrafiltration [Millipore Centricon Plus-20 + Centricon YM-30 (molecular weight cutoff, 30 000)]. The volume reduction included one washing step with 50 mmol/L Tris (pH 7.4) and Complete. The final retentate (20 μL) was applied to an IEF gel after adjustment of the apparent EPO concentration to a maximum of 500 IU/L.

A polyacrylamide gel (250 × 120 × 1 mm; 5% T, 3% C; 50 g/L sucrose, 50 mL/L Servalyt 2-4, 50 mL/L Servalyt 4-6, 7 mol/L urea) was prefocused for 30 min at 250 V and 8 °C, with 50 mL/L Servalyt 6-8 as the catholyte and 0.5 mol/L H_3PO_4 as the anolyte. We then applied 20 μL of either a 0.1 nmol/L standard (EPO BRP or Aranesp) or the urine extracts (heat inactivated for 3 min at 80 °C) containing 10 mL/L Tween 80R approximately 5 mm from the cathode. The gel was focused for 4000 Vh with maximum settings of 2000 V, 50 mA, and 30 W.

The focused proteins were detected by "double-blotting" *(13)*. In this procedure, in which the primary antibody (monoclonal mouse anti-hEPO) is electroblotted (1 mA/cm^2 for 10 min) to a second membrane, nonspecific binding of the secondary antibody [biotin-goat anti-mouse IgG (H+L)] is markedly decreased. After incubation with streptavidin–horseradish peroxidase and ChemiGlow substrate, the emitted light was captured with a chemiluminescence imaging system (FluorChem 8000; Alpha Innotech Corp.).

An isoform of EPO is a subset of the EPO molecules that has a defined charge. The isoforms appear in the electropherogram as bands. An isoform pattern consists of bands, specifically their number, positions, and densities relative to each other. The number of isoforms and their positions result directly from the structural characteristics of the molecules.

The number of charged molecules, such as the sialic acid content of the carbohydrate, influences the isoelectric point (pI), which in turn determines the final position of the isoform on the gel. Within one lane, the denser the isoform, the more of that particular isoform is present in that lane.

Fig. 1 is an electropherogram showing the patterns of isoforms from rHuEPO and darbepoetin alfa standards, endogenous urinary EPO, and administered rHuEPO and darbepoetin alfa. The isoform pattern of a urine extract from QC1 (Fig. 1, lane 2) contained at least 10 isoforms. The isoforms closest to the anode and cathode are less dense than the isoforms in the middle.

As predicted from the chemical differences between rHuEPO and darbepoetin alfa standards, the migration patterns and pIs of rHuEPO and darbepoetin alfa differed greatly. Darbepoetin alfa appeared in the anodic region, and there was no overlap with rHuEPO, which appeared in the cathodic region.

The isoform pattern of pharmaceutical darbepoetin alfa is shown in lanes 5 and 7 (Fig. 1). It contains four dominant isoforms clustered in the acidic area of the electropherogram. Isoform density increases from the least to the most acidic band. The isoform pattern of an extract of a urine from a cancer patient who received darbepoetin alfa (Fig. 1, lane 6) matched that of pharmaceutical darbepoetin alfa in terms of the number of isoforms, their positions, and their relative intensities. The match establishes the identity of the compound in the urine extract (Fig. 1, lane 6) as darbepoetin alfa.

Although there are faint isoforms of endogenous EPO in the anodic region (Fig. 1, lane 2), the density in this region is minimal, and the overall isoform pattern is distinctly different from that of the darbepoetin alfa lanes. In contrast to the isoforms of the darbepoetin alfa standard (Fig. 1, lanes 5 and 7), the isoforms of the EPO BRP standard (lanes 1 and 4) are in the less acidic area of the electropherogram. The pattern of isoforms in urine obtained after rHuEPO was administered to individuals is shown in lane 3. This pattern is characterized by very dense isoforms in the least acidic area and lighter isoforms moving toward the anode.

In our experience with electrophoresis performed on urines, obtained from >300 healthy control individuals, lane 2 is a typical normal pattern, which was first published by Lasne and de Ceaurriz *(12)*. This work demonstrates that both rHuEPO and darbepoetin alfa appear in the urine. Differences in the isoform patterns of these pharmaceuticals compared with endogenous (urinary) EPO are readily apparent. The fact that a strong darbepoetin alfa signal is observed in a urine sample from a patient 7 days after administration of the drug is consistent with its mean terminal half-life of 25.3 h *(1)*.

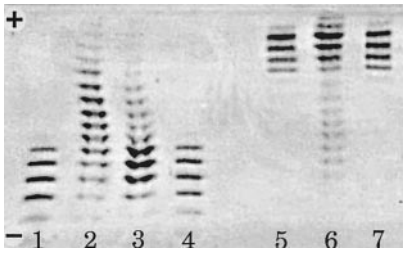

Fig. 1. Electropherogram of rHuEPO and darbepoetin alfa standards and extracts of urine obtained from healthy controls and individuals treated with rHuEPO and darbepoetin alfa.

The anode and cathode sides of the electropherogram are indicated as + and −, respectively. *Lanes 1* and *4*, rHuEPO standard; *lanes 5* and *7*, darbepoetin standard; *lane 2*, extract from a healthy individual showing the normal pattern of endogenous hEPO; *lanes 3* and *6*, urine extracts obtained after the administration of rHuEPO and darbepoetin alfa, respectively.

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Neopterin Concentrations in Cord Blood: A Single-Cohort Study of Paired Samples from 541 Pregnant Women and Their Newborns, *Harald Schennach,[1] Christian Murr,[4] Clara Larcher,[5,6] Werner Streif,[2] Erika Pastner,[3] Daniela Zaknun,[7] Diether Schönitzer,[1] and Dietmar Fuchs[4,6*]* ([1] Central Institute for Blood Transfusion, and Departments of [2] Pediatrics and [3] Gynecology, University Hospital Innsbruck, A-6020 Innsbruck, Austria; Institutes of [4] Medical Chemistry and Biochemistry and [5] Hygiene and Social Medicine, Leopold-Franzens University, and [6] Ludwig Boltzmann Institute for AIDS Research, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria; [7] Department of Pediatrics, University of Vienna, A-1090 Vienna, Austria; * address correspondence to this author at: Institute of Medical Chemistry and Biochemistry, Leopold-Franzens University, and Ludwig Boltzmann Institute for AIDS Research, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria)

Neopterin, a product of interferon-γ-activated monocyte-derived macrophages, is a sensitive indicator of cell-mediated immune activation *(1)*. In humans, increased concentrations of neopterin in serum and urine have been found in various malignant disorders and autoimmune diseases as well as during allograft rejection episodes and viral infections, including HIV type 1 *(2–8)*. Serum neopterin concentrations have also been investigated during pregnancy and in the neonatal period *(9–11)*.

In this study, serum neopterin was measured in women with uncomplicated pregnancies, and concentrations were compared with cord-blood concentrations after delivery. A total of 541 women with a median age of 29.0 years (range, 15.5–44.3 years) who delivered at the University Hospital Innsbruck between October 1997 and July 1999 and who had all examinations during pregnancy performed at the same institution were included in the study. All of them took part in the Austrian healthcare program called "Mutter-Kind-Pass", which is recommended to every pregnant woman and is supported by the public health system. This program includes at least five gynecologic examinations and one internal medical investigation during pregnancy. In addition, all pregnant women are tested for antibodies against rubella virus, *Treponema pallidum*, and *Toxoplasma gondii* and are screened for hepatitis B surface antigen. None of them had medical or obstetric complications. All pregnancies were uncomplicated singleton gestations that produced (with one exception) healthy term infants (290 males and 251 females), whose growth was appropriate for gestational age. In keeping with customary healthcare practice in Austria, the development of all the children was checked by medical investigations at least five times beginning with the neonatal period up to the age of 14 months. In addition to this routine program, EDTA-blood samples collected from all newborns by heel lancing in the first week after birth were tested for cytomegalovirus (CMV) by the qualitative Amplicor CMV test (Roche Molecular Systems). This PCR assay amplifies a 365-bp fragment of the CMV polymerase gene and has a limit of detection of ~1000 copies/mL *(12)*.

Blood samples were drawn by venipuncture of the mother in the 28th week of gestation. Immediately after delivery, blood samples were drawn by puncture of the umbilical artery of the cord before the placenta was discarded. The blood was allowed to clot at room temperature, and serum was obtained by centrifugation at 3220*g* for 15 min. Neopterin analyses were performed within 1 day after blood collection. Serum neopterin was measured by a commercially available ELISA (ELItest® Neopterin; BRAHMS Diagnostica) with a detection limit of 1 nmol/L neopterin and an interassay CV ranging from 3.9% to 8.2% *(13)*. Upper reference limits (95th percentiles) for neopterin concentrations are age-dependent and range from 8.7 nmol/L (19–75 years) to 13.5 nmol/L (<19 years) and 19.0 nmol/L (>75 years) as described previously *(13)*. The study was approved by the local ethics committee, and consent was obtained from all participating women before all procedures were performed.

Correlation between variables was assessed by the nonparametric Spearman rank correlation method because the distributions of observed values were generally