Erythropoietin (EPO) and protein based drug

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Size: Small vs. large molecules

Dihydroergocornine MW 564

Oxyglobin™ (64,000 Daltons)
Size: Small vs. large molecules

Dexamethasone (392.5)

Erythropoietin (34,000 Daltons)
Proteins & protein based drugs

- Proteins are the body's functional machinery and are made according to the DNA blueprints that carry out most cell functions.
- All naturally occurring proteins are made from ~20 amino acids.
- Proteins are regulators of body functions.
- Protein can be a bio-marker for a disease or metabolic disorder.
- Deficiency can result in a metabolic disorder.
- Protein based drugs are substitutes for that specific protein.
- Recombinant drugs are replicates of natural occurring protein.
- Example “rHu-EPO”
Forms of Erythropoietin

EPO (Epogen) a protein with a sugars attached (Glycoprotein).

Darbepoetin (Aranesp) differs from EPO contains 5 N-linked carbohydrate.
Additional carbohydrates

- Darbepoetin (Aranesp) - Long-Acting EPO
- The additional carbohydrates result in longer half-life and increased biologic activity.
- Remain in blood stream longer
- Half-life of EPO 6-8 hours; ~ 3X for DAR
- Consequence of longer presence is detection
Erythropoietin is the primary regulator of mammalian RBC production

Low oxygen - stimulation of natural EPO production

OR injection of rHu-EPO

Man vs. horse

Red Blood Cell Increase
Uses

- Treatment of disease that produced anemia - man, dog, cat
- No known medical use in horses
- EPO has been misused as a performance-enhancing drug in endurance athletes.
- EPO has been banned by all sports organizations.
- EPO administration can be dangerous in healthy humans.
- Deaths in the horse have been reported
- Mechanism is different in horse and human
Amino Acid Sequences of Human and Equine EPO

- Human EPO
  - APPR LICDSR VLERYLLEAK EAENITTGCA EHCSLNENIT VPDTKVNFYA WKRM EVGQQA VEVWQGLALL SEAVLRGQAL LVNSSQPWEP LQLHV DKAVS GLRSLTTLLR ALGAQKEAIS PPDAASAAPL RTITADTFRK LFRVYSNFLR GKLKLYTGEA CRTGD

- Amino acids marked in red - carbohydrates attached.

- Equine EPO
  - PPR LICDSRVLERYILEARE AENVTMGCAE GCSFGENVTVPD TKVN FYSW KRMEVEQ QAV EVW QGLALLS EAI LQGQALLANS SQPSetL RLHV DKAVSS LRSL TSLLRA LGAQKEAISPPDAASAAPLR TFAVDTLCKL FRI YSNFLRG KKL KLYTGEAC RRGDR
Enzymatic digest done using trypsin: Cleavage at –R (arginine) and –K (lysine)

Legend:
Protein Sequence
rHu-EPO
rHu-DPO
Equine EPO
LC-MS chromatogram of Darbepoetin alpha digestion
Extraction of rHu-EPO & rHu-DAR from plasma by immunoaffinity separation

- Anti-EPO antibodies linked to magnetic beads.
- The beads are incubated with equine plasma for ~24 h.
- The beads are washed.
- EPO or DAR alpha remaining on the beads are eluted (removed) with a elution buffer.
- The eluate containing EPO or DAR is subject to buffer exchange.
- After buffer exchange, EPO or DAR are ready for digestion.
Trypsin Digestion

- rHu-EPO or rHu-DAR alpha incubated in trypsin at 37 °C for 3 hr.

Liquid chromatography column

- LC column: Zorbax Stable Bond guard column

LC-MS/MS

- Mass spectrometer: LTQ linear ion Trap (Thermo-Finnigan)
Actions of foreign proteins

- In animals rHu-EPO is a foreign protein.
- Body produces antibodies against this protein.
- Reduction in RBC count associated with long term adm.
- Reports of death in horses.
- Antibodies measured in the horse.
Anti-rhEPO antibodies detected in equine plasma during and after IV injection of 8 doses of rHu-EPO (4000 IU/dose).
Anti rHu-EPO in several equine populations

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ELISA R&D Kit

Hours

Plasma Conc (IU/ml)

- t$_{1/2}$: 6-8 hours

- 4000 IU
- 8000 IU
- 16000 IU

Concentrations:
- 0.15 ng/ml
- 0.12 ng/ml
- 0.06 ng/ml
rHu-DAR (25 mg weekly)

Plasma conc. (ng/ml)

24 h last dose

not detected
EPO ELISA Kits

- Neogen
- R & D Systems
- Stem Cell Technologies
Support

- Pennsylvania Horse & Harness Racing Commissions
- Pennsylvania Standardbred Horseman Association at Pocono Downs and the Thoroughbred Horseman Association a Philadelphia Park.
- The authors thank Donna Telies, Anne Hess, and Fengyu Hao for their excellent technical assistance.

Thank you
Existing methods

- rhu-EPO is less negatively charged than natural human EPO.
- Based on this difference a combination of immunoblotting isoelectric focusing method has been developed.
- Time-consuming, expensive, not good for screening multiple samples, very specialized laboratory.
- Not suitable for equine industry?
250 µg/ml oxyglobin spiked to pl

Plasma and horse Hemoglobin

Plasma and Oxyglobin

Plasma