Erythropoietin (EPO) and protein based drug

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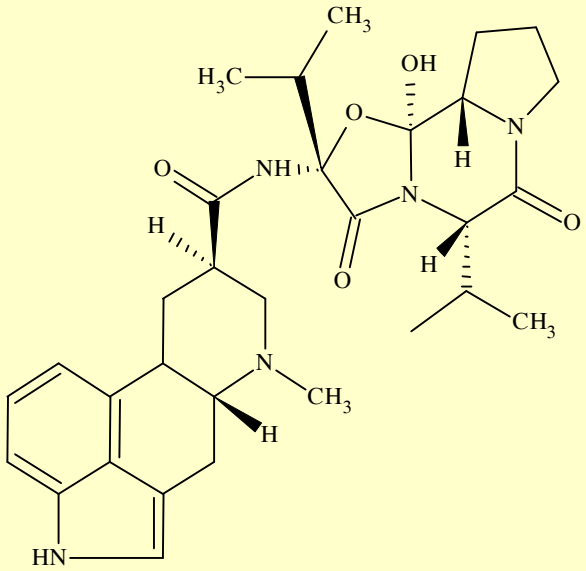
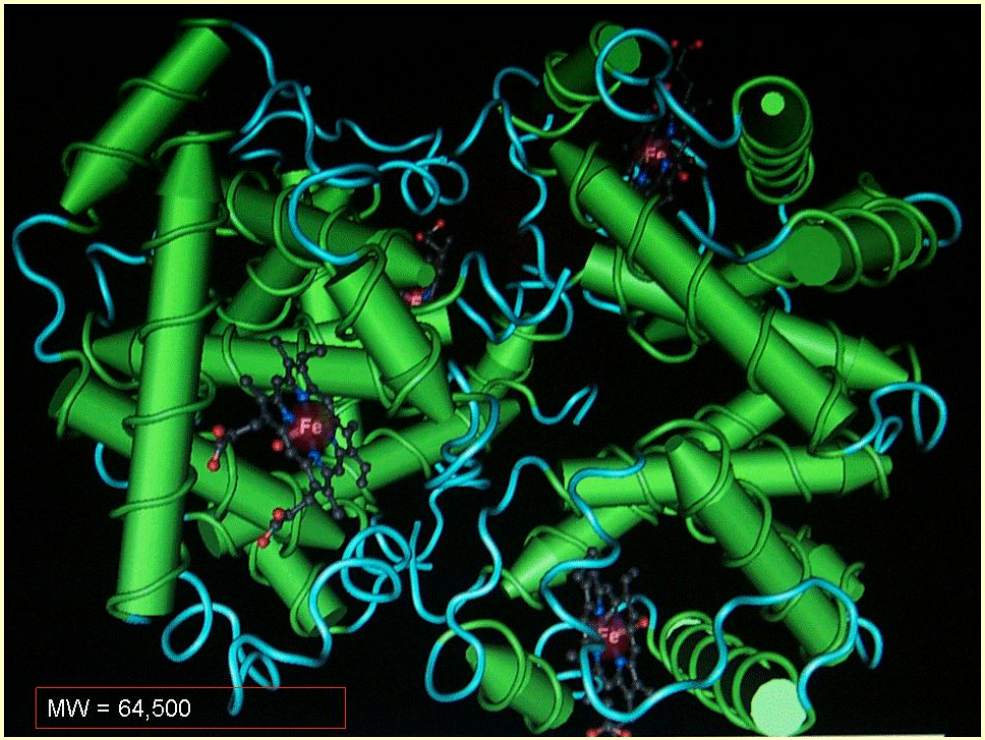
Size: Small vs. large molecules

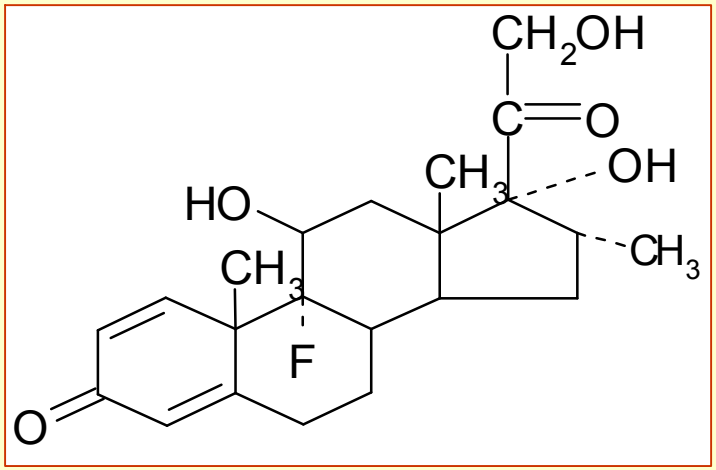


Dihydroergocornine MW 564



Oxyglobin™ (64,000 Daltons)

Size: Small vs. large molecules



Dexamethasone (392.5)

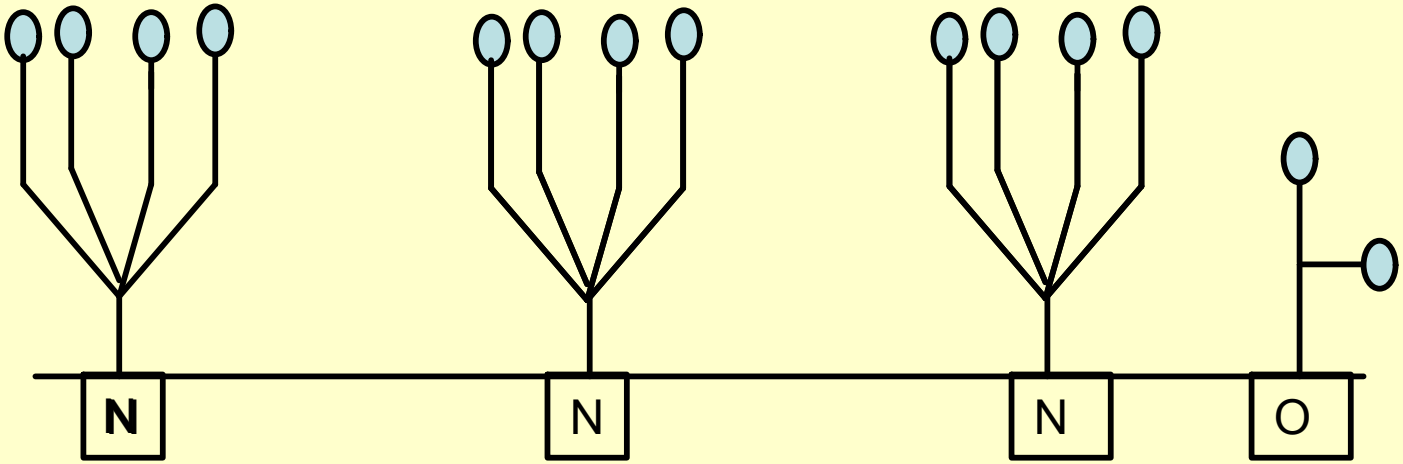
Erythropoietin (34,000 Daltons)

Proteins & protein based drugs

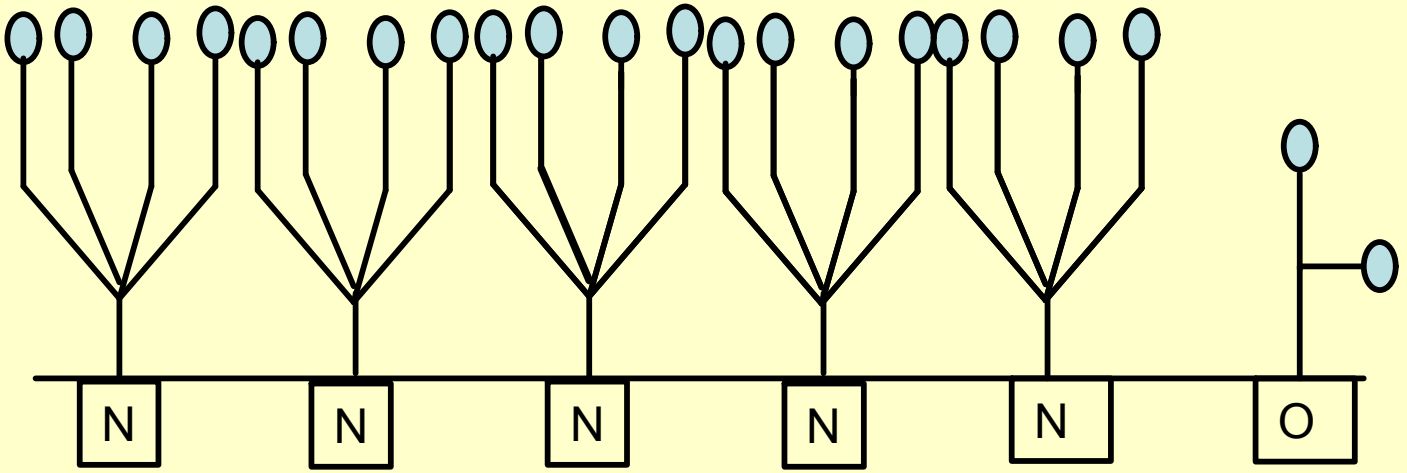
- Proteins are the bodies functional machinery are made according to the DNA blueprints that carry out most cell functions
- All naturally occurring proteins are made from ~20 amino acids
- Proteins are regulators of body functions
- Protein can be a bio-marker for a disease or metabolic disorder
- Deficiency can result in a metabolic disorder
- Protein based drugs are subsituites 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.

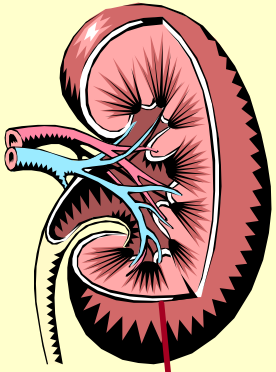


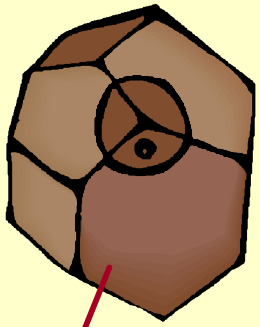
Additional carbohydrates

- 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



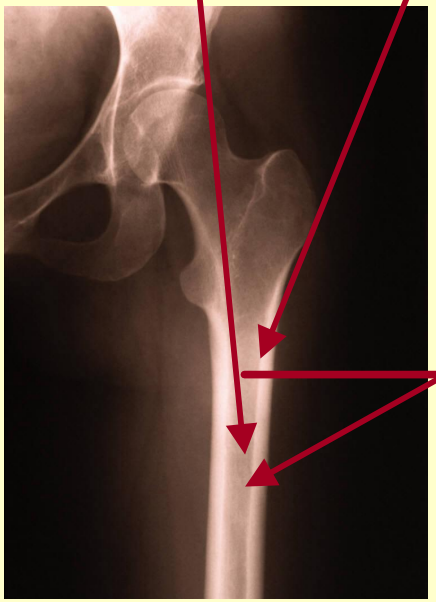


Liver Cell

EPO

EPO

OR injection of rHu-EPO

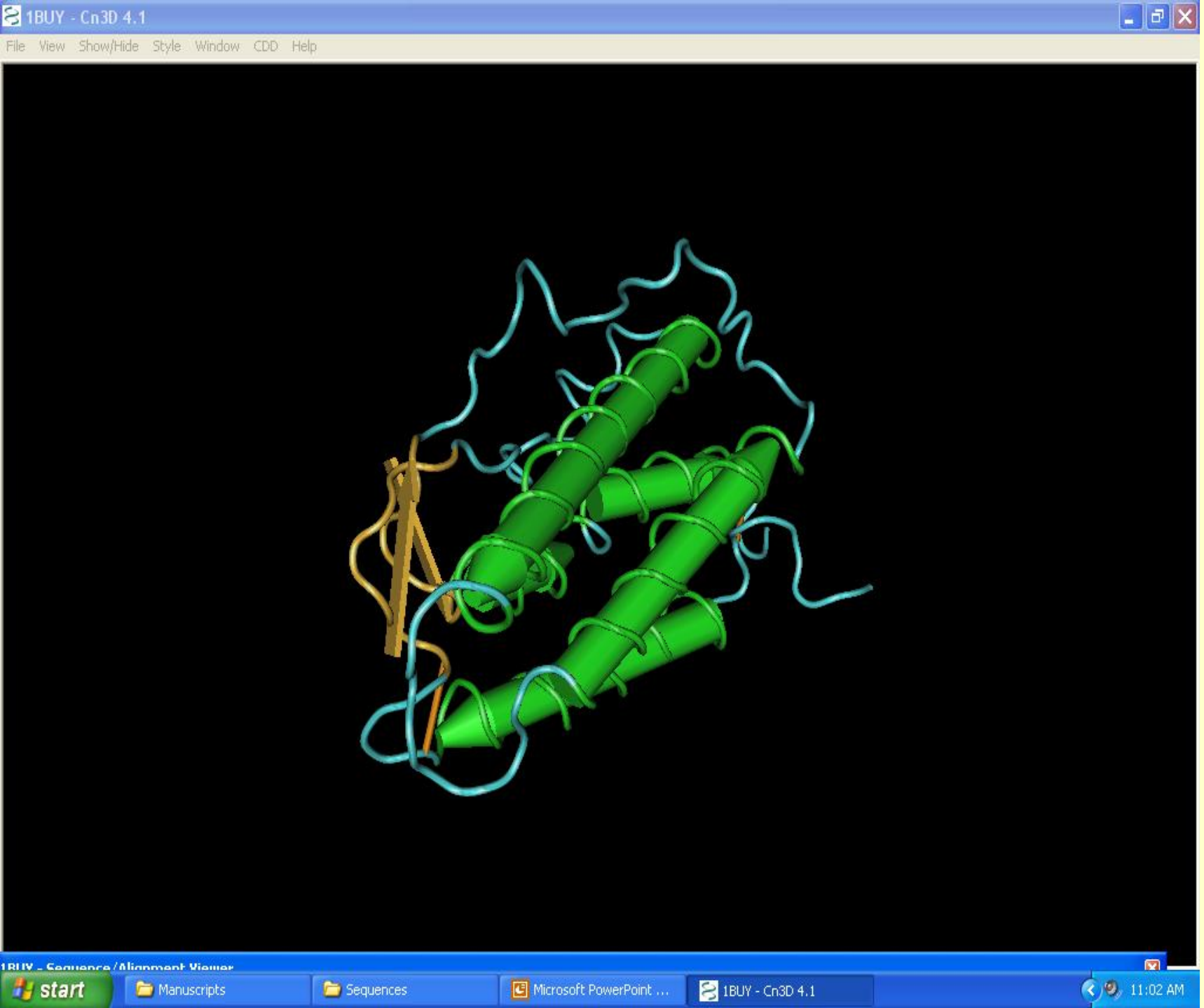


Man vs. horse

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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File View Show/Hide Style Window CDD Help
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Manuscripts
Sequences
Microsoft PowerPoint ...
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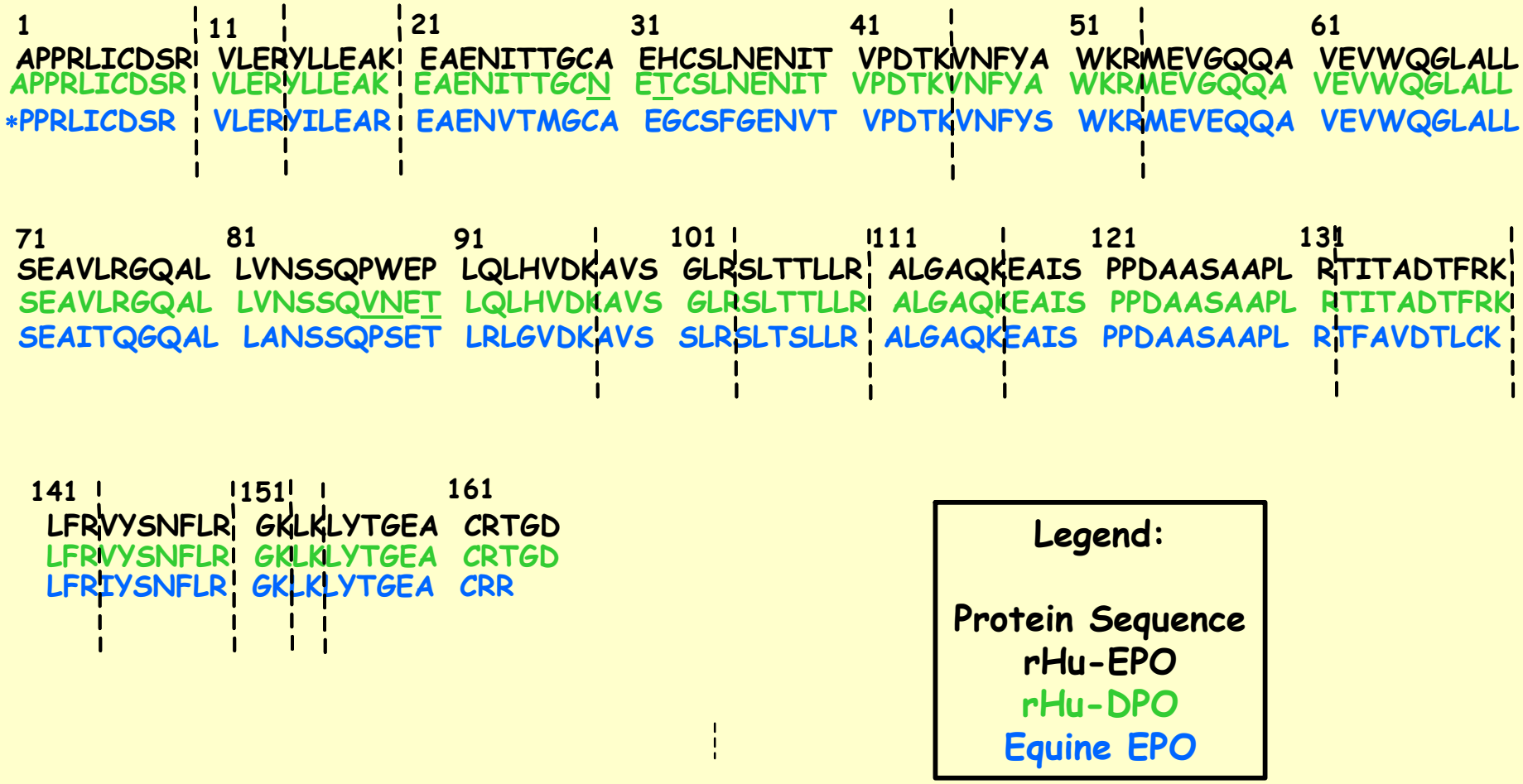
Amino Acid Sequences of Human and Equine EPO

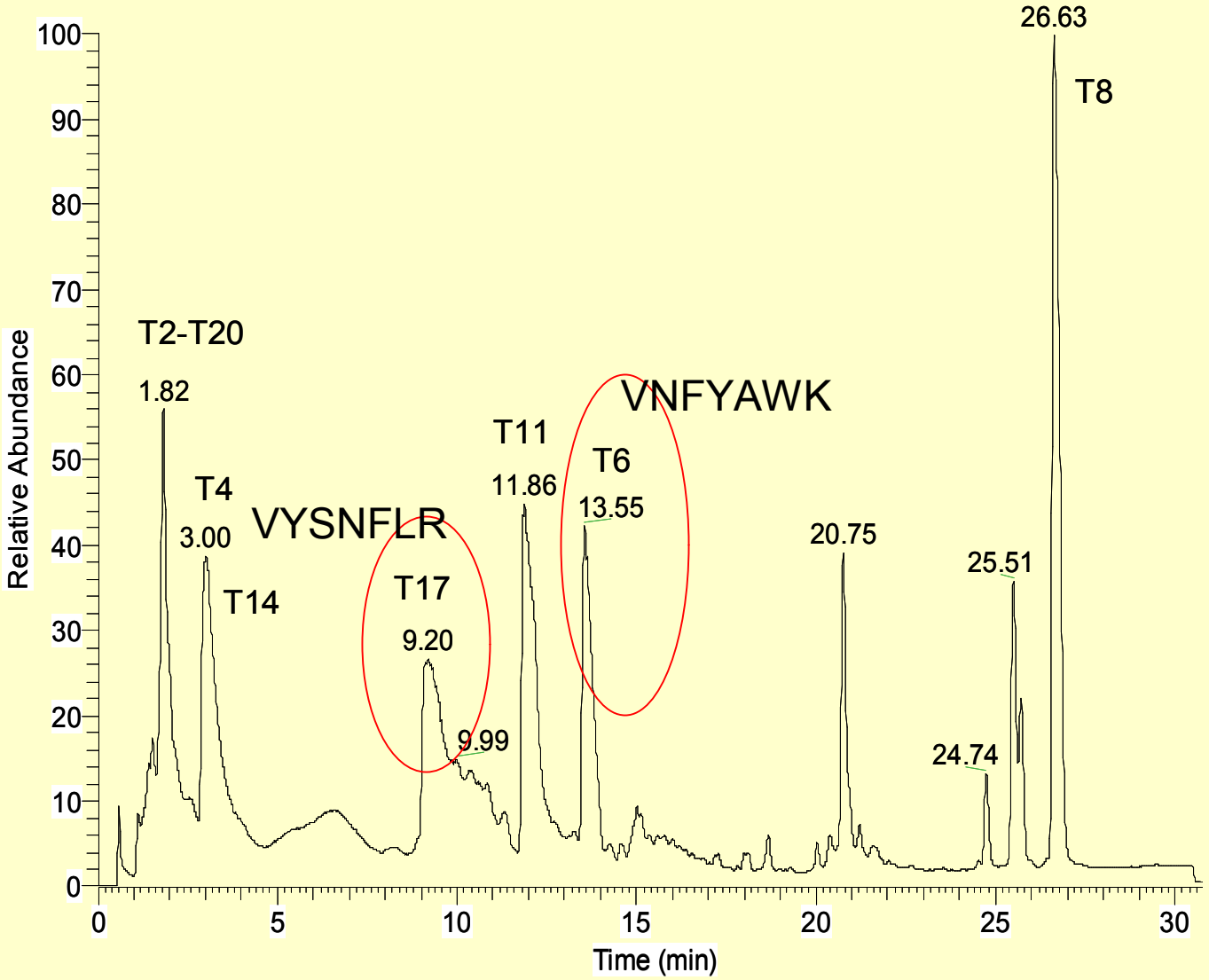
- **Human EPO**
- APPRLICDSR VLERYLLEAK EAENITTGCA EHCSLNENIT VPDTK**VNFYA WK**RMEVGQQA VEVWQGLALL SEAVLRGQAL LVNSSQPWEP LQLHVDKAVS GLRSLTTLLR ALGAQKEAIS PPDAASAAPL RTITADTFRK LFR**VYSNFLR** GKLKLYTGEA CRTGD
- Amino acids marked in red - carbohydrates attached.
- **Equine EPO**
- PPRLICDSRV LERYILEARE AENVTMGCAE GCSFGENVTV PDTK**VNFYSW K**RMEVEQQAV EVW QGLALLS EAI LQGQALL ANS SQPSETL RLHVDKAVSS LRSLTSLLRA LGAQKEAISP PDAASAAPLR TFAVDTLCKL FR**I YSNFLR**G KLKLYTGEAC RRGDR

Enzymatic digest done using trypsin:
Cleavage at –R (arginine) and –K (lysine)



Legend:

Protein Sequence
rHu-EPO
rHu-DPO
Equine EPO



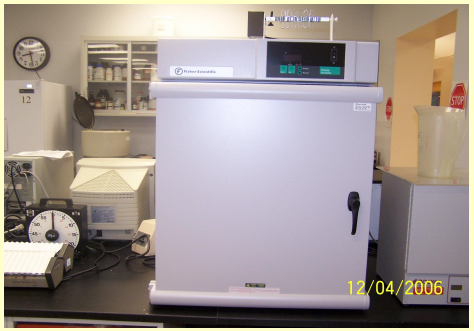
LC-MS chromatogram of Darbepoetin alpha digestion

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 ^{o}C for 3 hr.



Liquid chromatography column

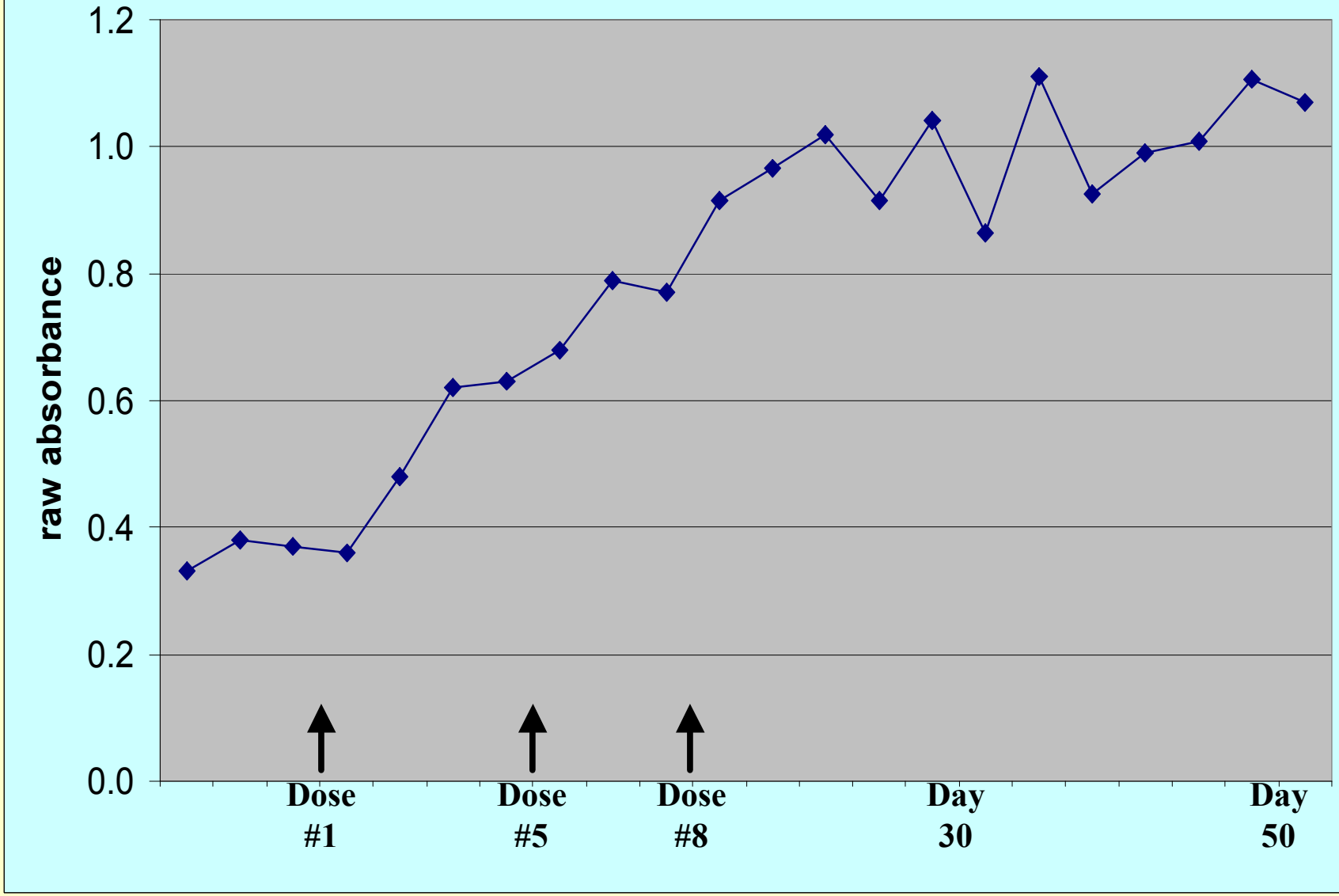
- LC column: Zorbax Stable Bond guard column

LC-MS/MS

- Mass spectrometer: LTQ linear ion Trap (Thermo-Finnigan)

Actions of foreign proteins

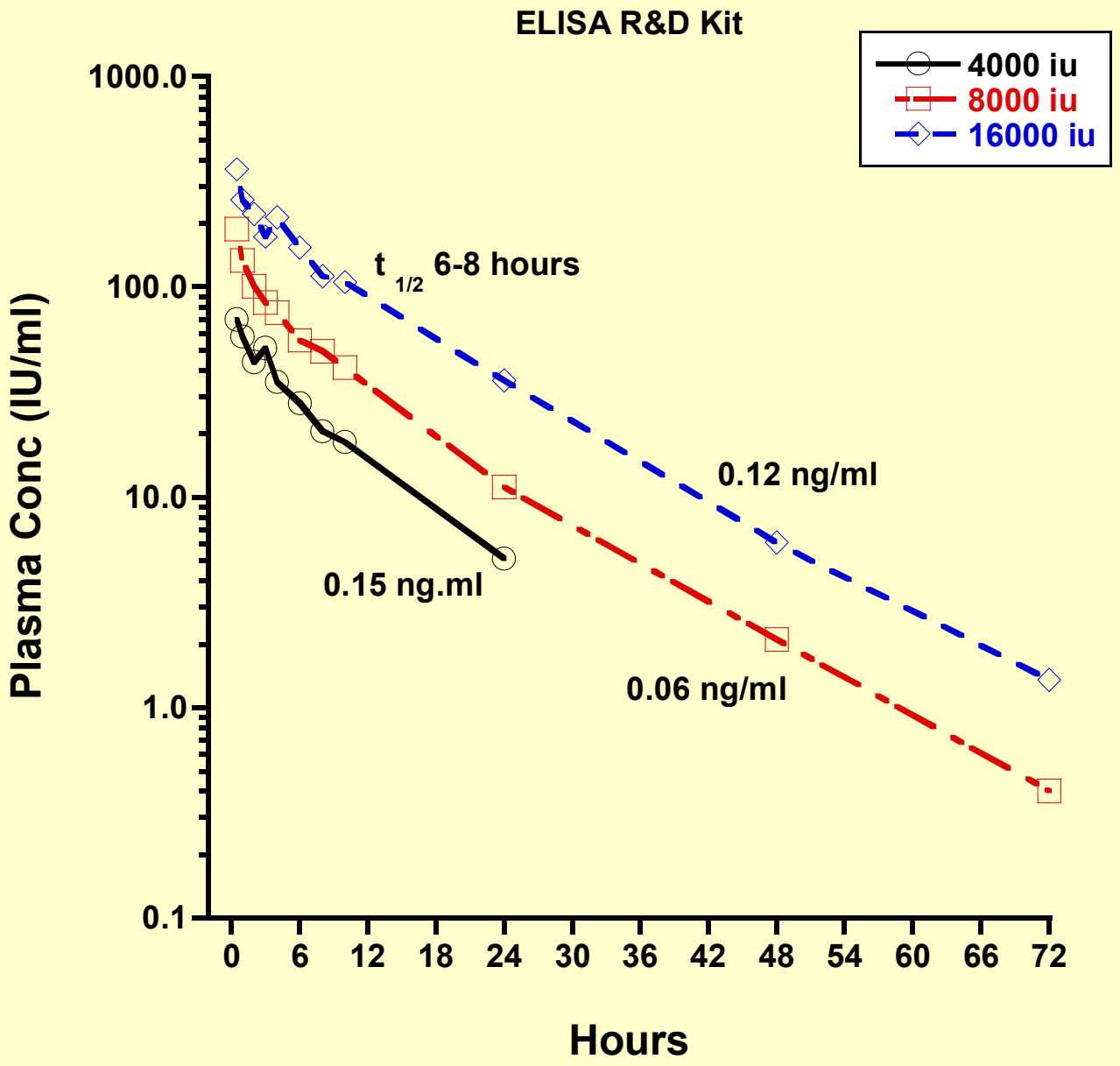
- In animals rHu-EPO is a foreign protein.
- Body produces antibodies against this protein.
- Reduction in RBC count associated with long term adm.
- Reports of death in horses.
- Antibodies measured in the horse.

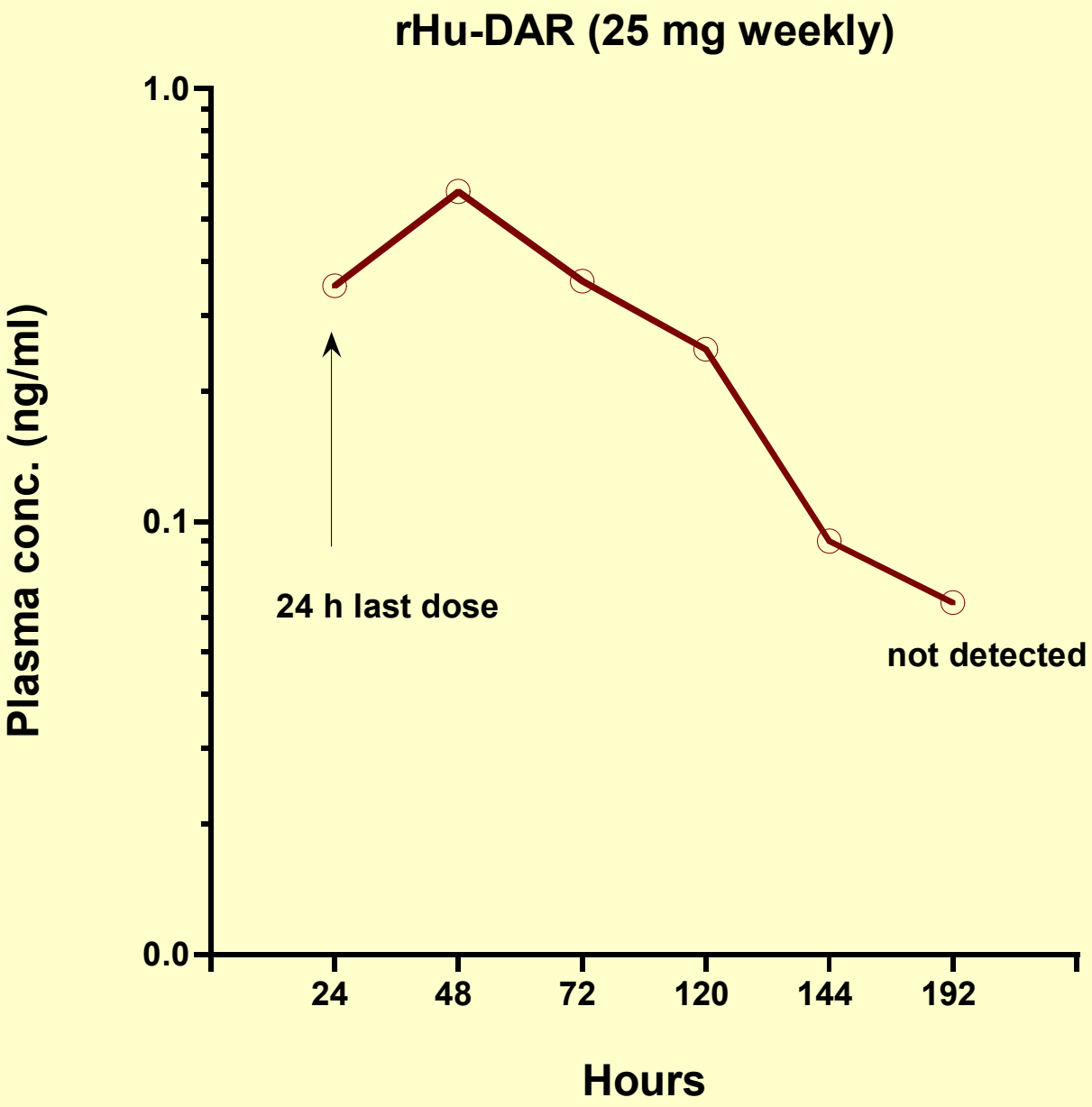


Anti-rhEPO antibodies detected in equine plasma during and after IV injection of 8 doses of rHu-EPO (4000 IU/dose).

Anti rHu-EPO in several equine populations

Breed	Status	Numbers	Titer >1:2	%
TB	Racing	389	11	2.8
STB	Racing	274	53	19.3
Various	Non racing	50	0	0
TB	Retired	16	2	12.5
TB	Injected	3	3	100


ELISA R&D Kit
4000 iu
8000 iu
16000 iu
1000.0
100.0
10.0
1.0
0.1
Plasma Conc (IU/ml)
t 1/2 6-8 hours
0.12 ng/ml
0.15 ng.ml
0.06 ng/ml
0
6
12
18
24
30
36
42
48
54
60
66
72
Hours


rHu-DAR (25 mg weekly)
Plasma conc. (ng/ml)
1.0
0.1
0.0
24 h last dose
not detected
24
48
72
120
144
192
Hours

EPO ELISA Kits

- Neogen
- R & D Systems
- Stem Cell Technologies

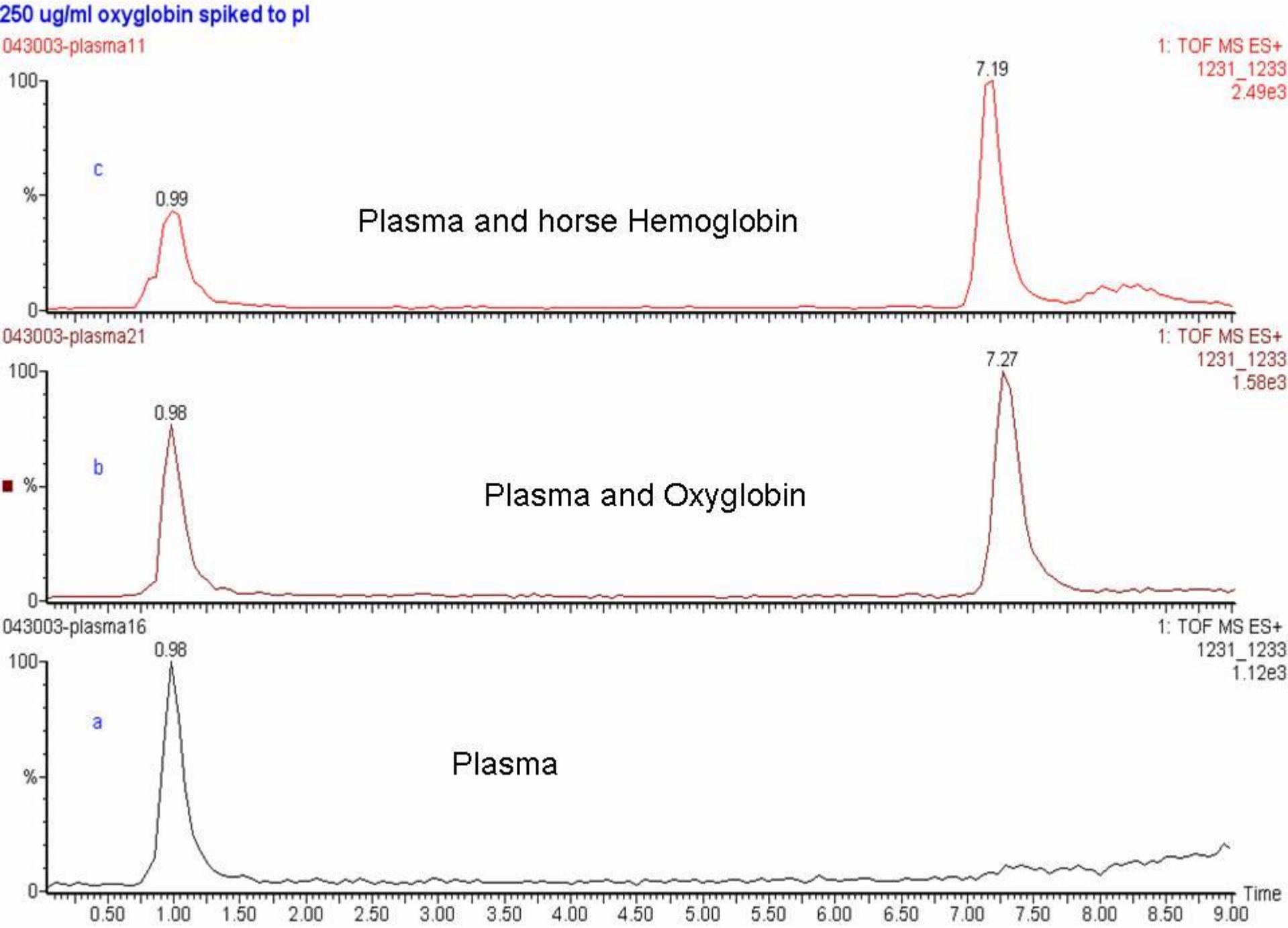
Support

- 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 ug/ml oxyglobin spiked to pl
043003-plasma11
1: TOF MS ES+
1231_1233
2.49e3
7.19
100
%
0
c
0.99
Plasma and horse Hemoglobin
043003-plasma21
1: TOF MS ES+
1231_1233
1.58e3
7.27
100
%
0
b
0.98
Plasma and Oxyglobin
043003-plasma16
1: TOF MS ES+
1231_1233
1.12e3
0.98
100
%
0
a
Plasma
Time
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00
7.50
8.00
8.50
9.00


NO
ACTIVE
CELL PHONES
IN THIS ROOM
GENESIS RSP 200
12/04/2006


NO
ACTIVE
CELL PHONES
IN THIS ROOM
GENESIS RSP 200
12/04/2006


SORVALL LEGEND MACH 1.6R
Kimberly-Clark
SAFESKIN CONTROLLED
12/04/2006


12/04/2006


12/04/2006