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Case study: Erythropoietin

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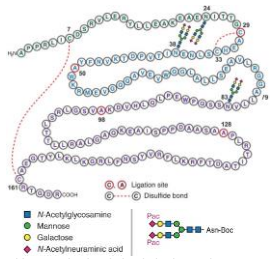
Part 1 rhEPO and plasmid construct

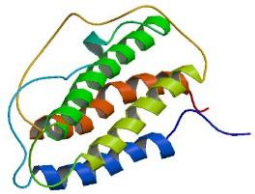
1. What is the *in vivo* physiological effect of EPO? What are the indications for using EPO therapy
2. What are the structural characteristics of EPO?
3. Does EPO undergo post-translational modifications? Which ones?
4. If so, what type of host cell is required for this post-translational modification?
5. In fig 3, what are the specificities of the plasmid used?
6. How can this specific construct be created from the commercial plasmid?
7. What methods can be used to control the recombinant plasmid?

1) Primary structure of EPO



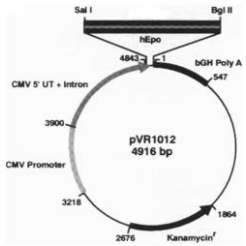
Adapted from Murakami et al Chemical Science Advances 15 Jan 2016 Vol. 2, no. 1, e1500678 DOI: 10.1126/sciadv.1500678

2) Human erythropoietin, NMR minimized average structure



Retrieve from http://www.rcsb.org/pdb

3) Schematic illustration of the pVRhEPO plasmid

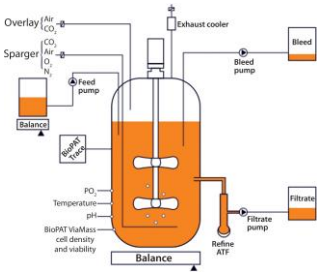


Tripathy SK et al Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. Proc Natl Acad Sci U S A. 1996 Oct 1;;93(20):10876-80.

Part 2 : Production and Purification

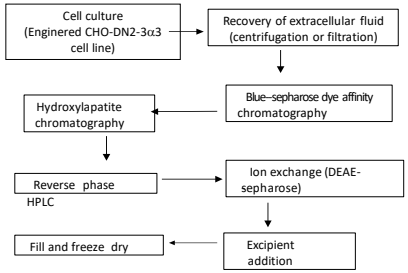
8. What are the different stages of fermentation and purification?
9. What are the different parameters to be controlled in bioreactors? What does fed-batch means?
10. Why is there no cell lysis step in the purification process?
11. At which step the downstream process is starting?
12. What are the possible contaminants/impurities present after the bioproduction step?
13. What is the principle of each of the steps described (fig 2) in the purification scheme?
14. What are the different roles of excipients in protein formulation? Cite examples

1) Set-up of a concentrated perfusion or fed--batch set-up using the Biostat STR single-use bioreactor



Sherman et al , Continuous Cell Culture Operation at 2,000-L Scale. BioProcess International, November 17, 2016

2) Schematic overview of the production of the EPO-based product « Neorecormon ».

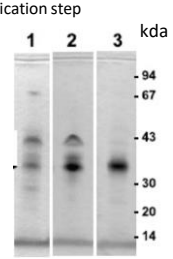


Adapted from G Walsh. Biopharmaceuticals 2d edition. 2003. Wiley

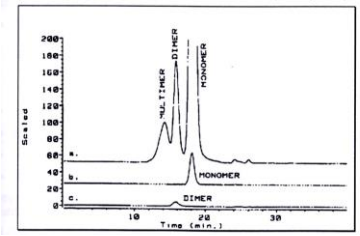
Part 3 Identity, purity and security

15. Why is glycosylation control important for EPO specialities?
16. Describe the SDS-PAGE technique and explain how it can be used to monitor the purity of EPO during its production?
17. Explain what specific observations the Size Exclusion Chromatography technique allows.
18. Cite two (other) identity and purity controls that can be performed on rhEPO
19. How can we control its biological activity?
20. Describe two examples of techniques to verify that a batch of EPO is not contaminated with a given microorganism.
21. How is the absence of endotoxins in the final product ensured?

1) SDS PAGE 10-15% polyacrylamide gel performed at different purification steps:
1, crude culture supernatant
2, intermediate purification step
3, final step



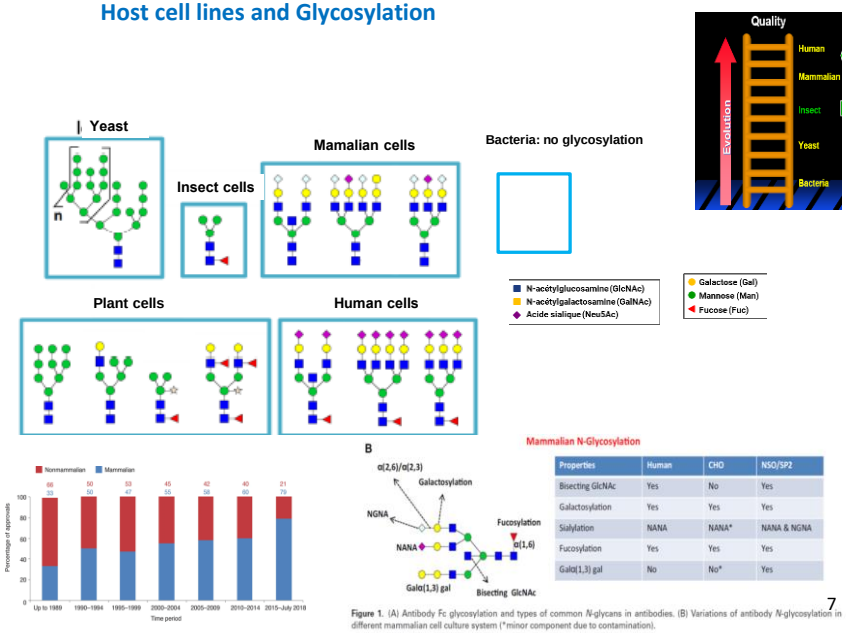
2) Data obtained from Size Exclusion Chromatography of EPO (b) monomer (c) dimer and a (a) mixture



Part 4 EPO biobetter

22. What is the blood half-life of human EPO, or classical rhEPO
23. What are the administration routes for rhEPO, formulation and excipients?
24. What strategies have been used to improve rh EPO?
25. What are the different kinds of commercialized biobetter

Host cell lines and Glycosylation



Properties	Human	CHO	NSO/SP2
Bisecting GlcNAc	Yes	No	Yes
Galactosylation	Yes	Yes	Yes
Sialylation	NANA	NANA*	NANA & NGNA
Fucosylation	Yes	Yes	Yes
Galα(1,3) gal	No	No*	Yes

Figure 1. (A) Antibody Fc glycosylation and types of common *N*-glycans in antibodies. (B) Variations of antibody *N*-glycosylation in different mammalian cell culture system (*minor component due to contamination).

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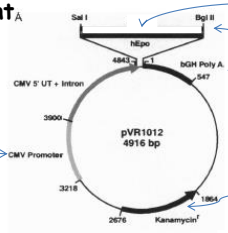
Genetic development



coding Sequence of human EPO = ADNc

Restriction site for the insertion of the sequence of interest

Polyadenylation sequence of mRNA transcripts
The bovine growth hormone polyadenylylation signal (bGH Poly A) is located immediately 3' of the Epo cDNAs.

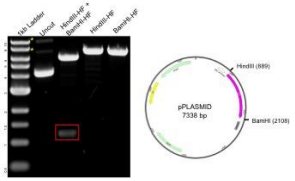
Promoter sequence from cytomegalovirus: allows a high level of transcription of adjacent cDNA

Selection gene: inclusion of the plasmid in the host cell

Production of recombinant EPO

- ✓ Transfection of the expression vector into the host cell: mammalian cell (CHO type) because the glycosylation of EPO must be as close as possible to the human form; the plasmid must be recombinantly inserted into the cell genome in order to be transmitted to the daughter cells during divisions
- ✓ Creation of cell banks: MCB and WCB
- ✓ Production phase: cell culture in bioreactor (control of temperature, agitation, pH, supply of nutrients, etc.) secretion of the protein into the culture medium

***In Vitro* Analysis of pVRmEpo.** To construct a plasmid expression vector that could program high-level production and secretion of recombinant proteins from skeletal myofibers *in vivo*, we cloned the hEpo and mEpo cDNAs into pVR1012 (24), a plasmid vector that contains a eukaryotic expression cassette controlled by the cytomegalovirus immediate early (CMV-IE) promoter and the CMV-IE 5′ untranslated and intron A sequences followed by the bovine growth hormone polyadenylylation signal (Fig. 1*A*) (24). pVR1012 was used in these experiments because this plasmid backbone has been



7. What methods can be used to control the recombinant protein?

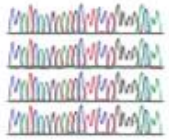
DNA ANALYSIS

RE Digest

OR

Colony PCR

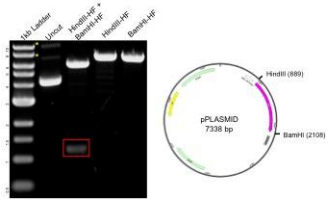
OR

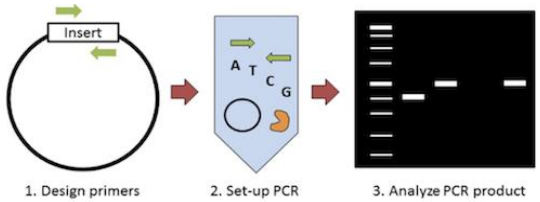


Sequencing

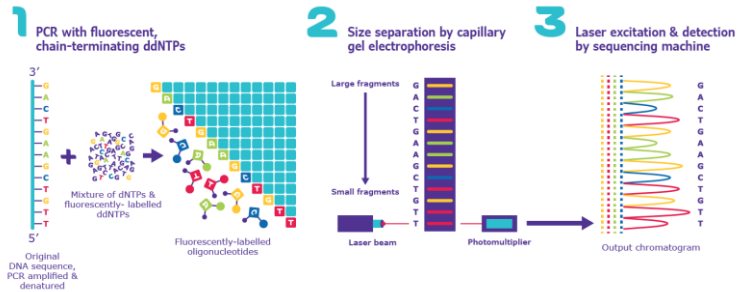
1) incubating your DNA with restriction enzymes which cleave the DNA molecules at specific sites and

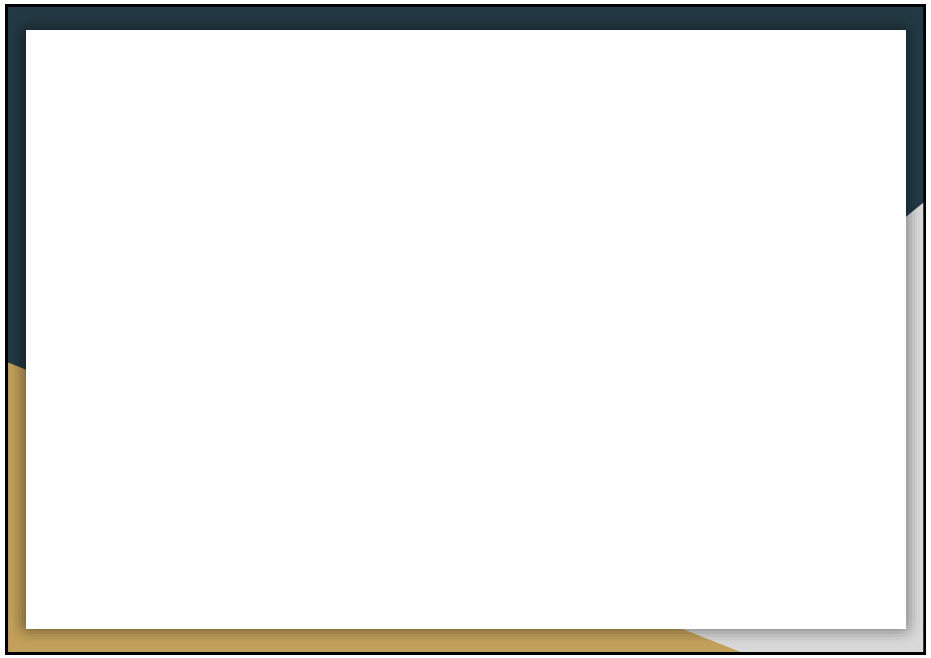
2) running the reaction on an agarose gel to determine the relative sizes of the resulting DNA fragments.



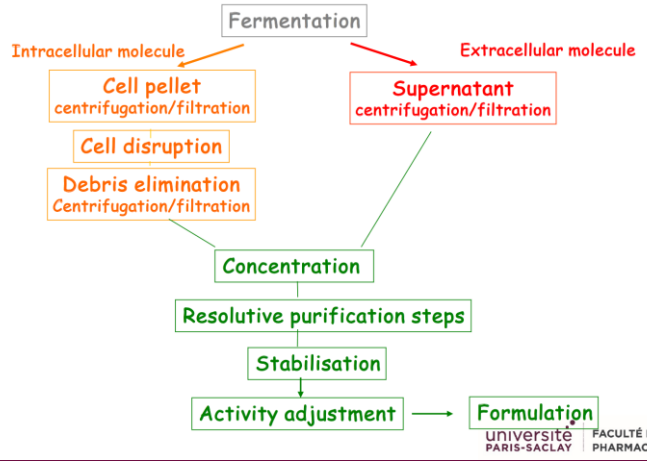


Sanger sequencing determines the precise order of nucleotides within a DNA molecule, in this case a plasmid. Sequencing is one of the surest ways to know that your insert is what you expect it to be





Downstream processing (DSP)



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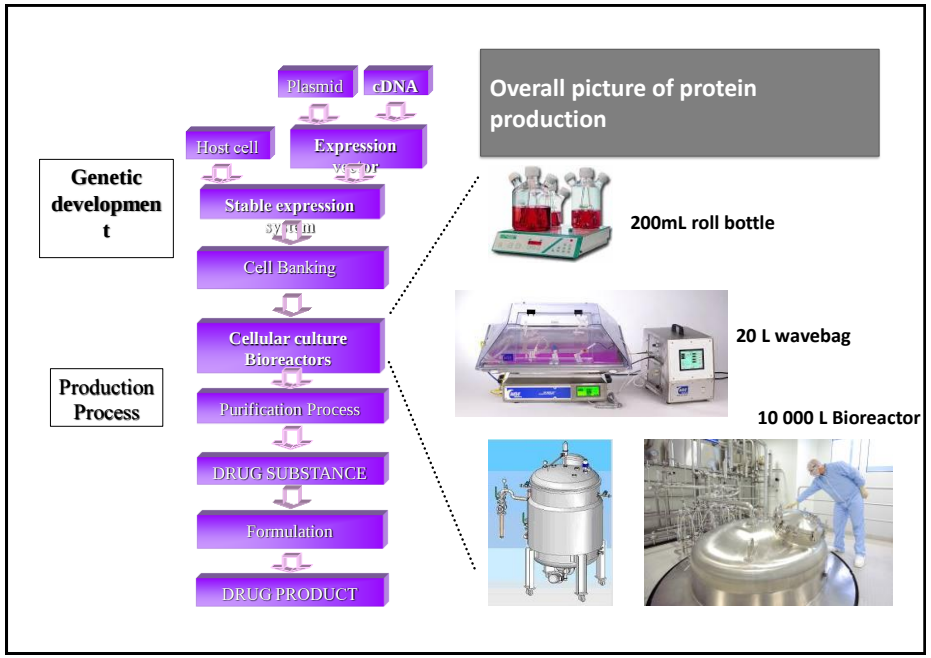
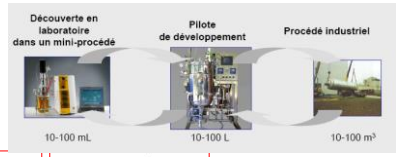
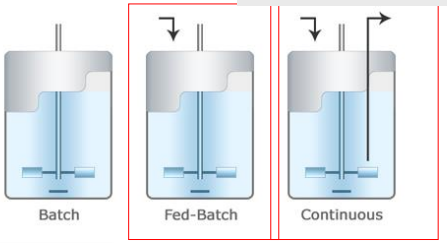
From Isabelle Turbica lecture



3 systems to feed cultivated cells

Bioreactor Volumes: 15L; 250L…20 000L.





- Simple
- Low risk of contamination
- Low investment cost
- Small production only
- Duration : a few days

- Compatible with large bioreactors
- 2-3 weeks

- Automatisable
- Risk of contamination ++
- More expensive
- Bigger production volumes

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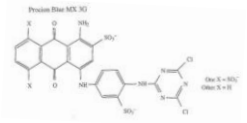
Différent kind of affinity columns for protein purification

Ligands	Affinity	Examples
Antibodies	Very specific, need drastic /harsh elution conditions	Anti facteur VIII
Prot A (Prot G)	Protéin from staphylocoque Auréus having strong affinity for Fc fragments of IgG	**Monoclonal antibodies**
lectins	Proeéins having affinities for specific sugar motives	Concanavaline A : capture **glycoproteins** (terminal mannose ou glucose)
Chelating metals	Coordination bonds through metals	➢Metalloprotéins ➢Prot with basic AA
Dyes (analogs of triazin)	Électrostastiqc interactions (sulfonates) ,sometimes hydrophobic and H bondings too Spécificity not predictible	Procion blue



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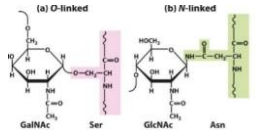
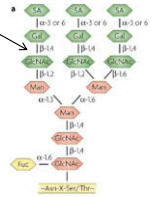
Structure/activity: Glycosylation a major post-translational modification (PMT)



N and O-glycosylation
N-glycosylation: Asn (consensus sequence: Asn-X-Ser/thr)
O-glycosylation: Ser; Thr

N-glycans: 3 types

antenna

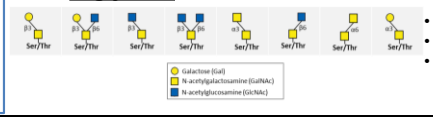


core

Complex hybrid oligomannose

balzarini ;Nature Reviews Microbiology 5, 583-597 (2007)

O-glycans



- Many cores
- Shorter
- GalNac

Why glycosylation is important for biopharmaceuticals?

- Solubility and stability
- Folding
- Cell recognition (ligand/receptor)
- Bioactivity
- Pharmacokinetics
- in-vivo clearance
- Antigenicity
- Patent protection

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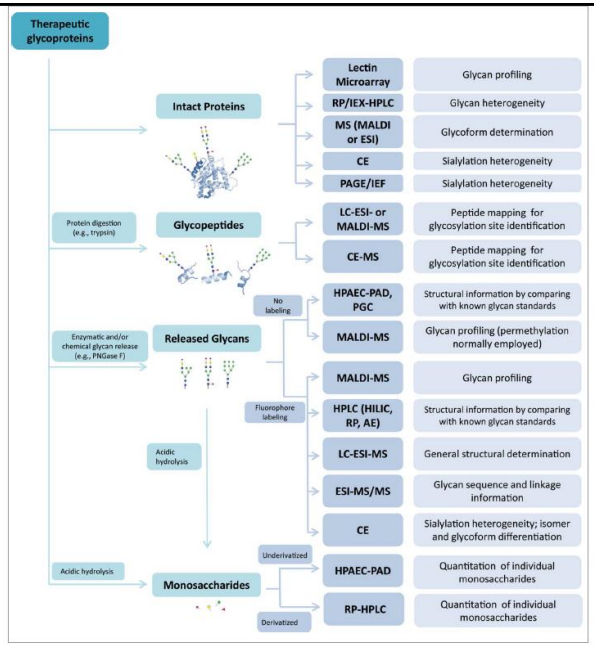
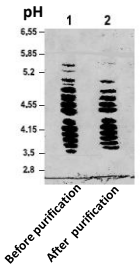
Which strategy for glycan analysis?



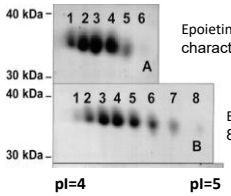
❖Control of **glycosylation: isoelectric focusing**

Isoélectrofocusing (IEF)
1) migration of proteins accrding to their PI
2) Immunodétection of glycoformes using an antibody against d'EPO, coupled to an enzyme for the detection (colorimetric, chimioluminescent)



Au cours de la purification de l'EPO certaines glycoformes sont perdues

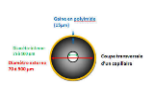
Profils IEF profiles comparing rec EPO of teh first génération:
alpha et beta glycoform profiles differs although they are both produced from CHO celles but from different producers
But in vivo activity is identical



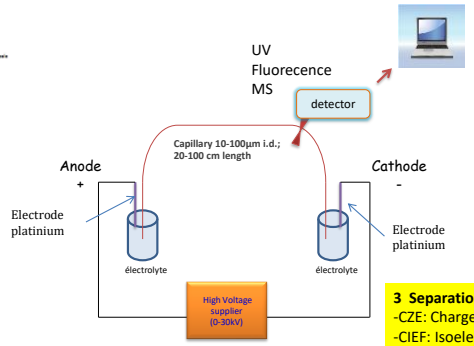
Epoietin alpha (Eprex®): 6 characteristic glycoformes

Epoietin beta (NeoRecormon®): 8 characteristic glycoforms

Capillary electrophoresis



Silica capillary



3 Separation modes for Intact glycoproteins
-CZE: Charge/mass ratio
-CIEF: Isoelectric point
-SDS-CGE: Molecular Mass

❖Identity Control of EPO:

Peptide mapping of EPO:

1) Digestion of purified EPO vy trypsine
2) RP-HPLC of resulting peptides
3) Chromatogramlme compared to the one of the reference (EPO)

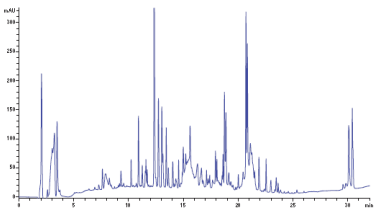


Figure 1. Reversed-phase rhEPO peptide map using a 2.1 × 250 mm, 2.7 µm Agilent AdvanceBio Peptide Mapping column. Protease digestion was accomplished by adding trypsin
protease to a solution including approximately 4.2 mg EPO. The ratio of substrate and enzyme
was 50:1 (w:w). The mixed solution was incubated at 37 °C for 12 hours. The digestion was stopped by storing the sample at –70 °C.

Glycoform mapping by capillary electrophoresis

1)l'échantillon à analysé est soumis à une éléctrophorèse capillaire de zone: les glycoformes sont séparées en fonction de leur Pi
2) Les glycoformes sont repérées en sortie de capillaire en fonction de leur temps de migration
3) Le profil et le nombre de glycoformes est compér à une référence

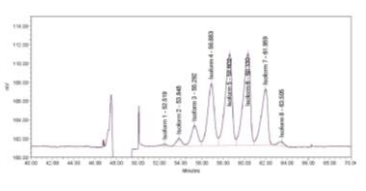
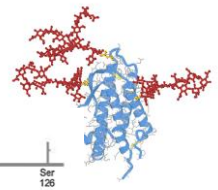
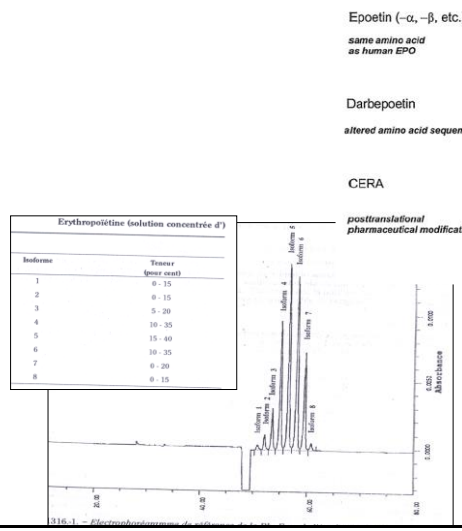


Figure 4 Distribution des isoformes en électrophorèse capillaire de zone (ECZ). *Les différences de glycosylation, en particulier de sialylation, se traduisent par des pH iso-électriques différents. Profil des 6 isoformes caractéristiques de l'Epoïetine alpha.*

European pharmacopeia: CZE of erythropoietin





- Capillary preconditionning : 60min 0.1MNaOH +12h under applied voltage
- BGE: 7M urea, pu 2.5mM Diaminobutane , tricine/acetate/Nacl pH 5.5

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❖Contrôl of **activity**

In vitro Controls: binding to EPO receptor : SPR

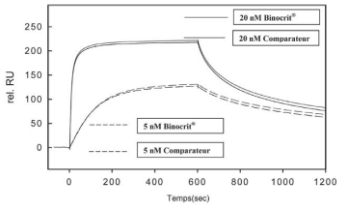


Figure 8 Étude de liaison au récepteur.
Le récepteur à l'EPO a été fixé sur la surface d'un « sensor chips » et la liaison de l'EPO (Binocrit® et Eprex ®) sur son récepteur a été étudiée par résonnance plasmonique : les 2 produits apparaissent hautement comparables, avec des cinétiques très proches.

1) Le récepteur de l'EPO est immobilisé sur une surface
2) L'échantillon à analysé est incubé
3) Mesure de l'association (partie ascendante de la courbe) puis dissociation (partie descendante de la courbe)
4) Calcul des constantes d'association / dissociation

In vivo Contrôl: tests on mice

1) Administrate différents EPO glycoformes
2) The haematocrit of each mouse is measured

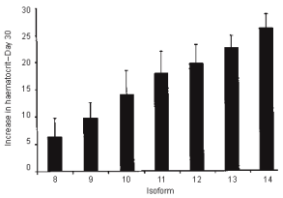
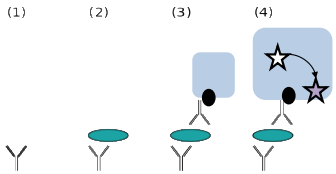


Figure 2 In vivo efficacy of isolated EPO isoforms.
CD-1 mice (n = 20/group) received an equimolar dose (2.5 mcg kg−1 of peptide) of each of the isolated EPO isoforms or vehicle control 3 times per week for 30 days by intraperitoneal injection. The haematocrit of each mouse was determined at baseline and twice-weekly thereafter. The increase in haematocrit over baseline following 30 days of treatment was calculated for each mouse and the group average haematocrit increase (± SD) calculated for each group. There was no significant change in haematocrit in the control group over the course of the study

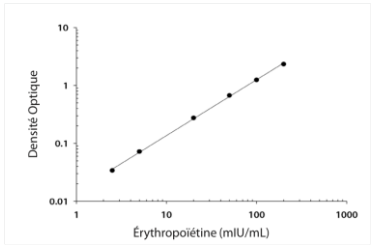
❖ Dosage and identity control:

Dosage of EPO by ELISA:



ELISA **sandwich**
(1) Microplaque sur laquelle à été immobilisé un AcM spécifique de l'EPO
(2) Fixation de l'EPO (échantillon à doser)
(3) Fixation d'un second AcM anti-EPO, couplé à une enzyme
(4) Ajout d'un substrat: l'enzyme transforme le substrat en produit coloré
➔ Lecture d'une absorbance au spectrophotomètre

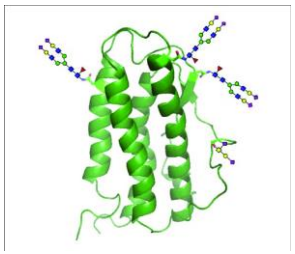
Calibration curve

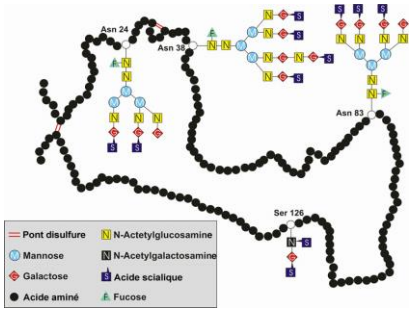


•L'intensité de la coloration est directement proportionnelle à la concentration d'EPO à doser
•On établit une gamme étalon avec des concentrations d'EPO connues.

The story of EPO and analogs

- **1rst generation EPO** (Epoetine, rhu-EPO):





165 aa, 3 sites of N-glycosylation, 1 of O- glycosylation

Kidney elimination
→ $T_{1/2}$ life: 6 to 9 hours
→ Administration: 2 to 3 times a week

The story of EPO and analogs

165 aa, but 5 mutated amino acids: 5 sites of N-glycosylation, 1 of O-glycosylation

Aranesp: 2nd generation or biobetter of EPO
Mutations : create novel consensus sequences Ans-X-Thr/Ser

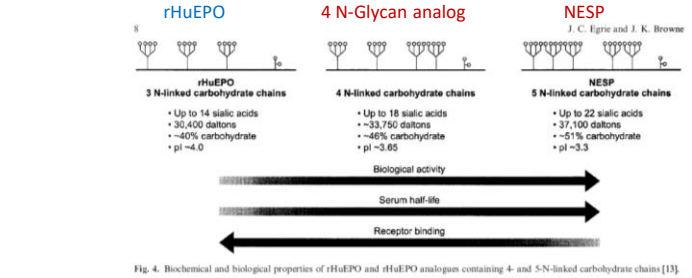


Fig. 4. Biochemical and biological properties of rHuEPO and rHuEPO analogues containing 4- and 5-N-linked carbohydrate chains [13].

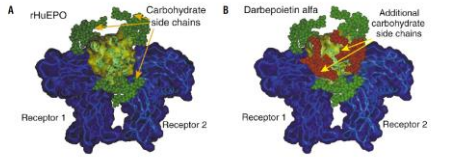


Figure 2. Molecular comparison of rHuEPO (A) and darbepoetin alfa (B). The crystal structure of the extracellular domain of EPO receptor bound to an EPO analog was determined before[1]. A structure of a tetra-antennary carbohydrate containing tetra-sialylated tetra-antennary acid branches was determined by molecular modeling and attached to the original or new N-linked glycosylation sites on the EPO structure. Structures are EPO (yellow), EPO receptors (blue), original three N-linked carbohydrates (green), and new carbohydrates (red).

More sialic acids decrease :
-the affinity of the molecule for its receptor
- the glomerular filtration but still kidney filtration occurs
→ $T_{1/2}$: 24h,
→Administration: once a week

Faster rate of hematocrite rise
Highest plateau

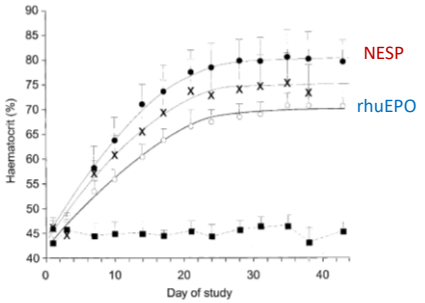


Fig. 5. *In vivo* efficacy of rHuEPO (isoforms 9–14), 4-chain analogue, and NESP. CD-1 mice (8–13/group (2.5 μg/kg of peptide) of rHuEPO (○), 4-chain analogue (×), NESP (●) or vehicle control (■), three ti intraperitoneal injection. Each point represents the group average haematocrit (±SD) for anti-rHuEPO antibo

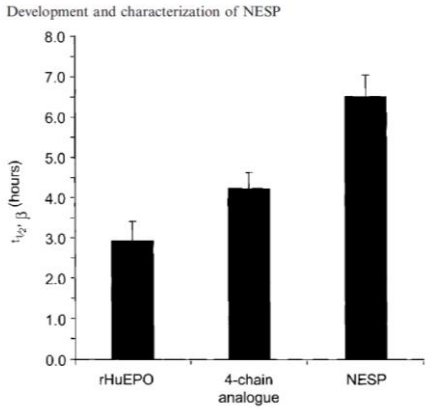
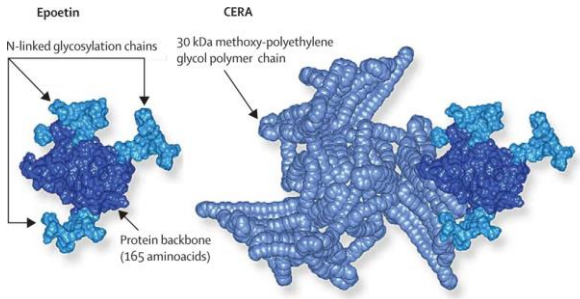


Fig. 6. Serum half-life of rHuEPO (isoforms 9–14), 4-chain analogue, and NESP. The test molecules were iodinated and administered intravenously into cannulated Sprague-Dawley rats ($n=10$ for rHuEPO, $n=6$/group for 4-chain analogue, and NESP). Blood samples were collected periodically and the amount of ethanol-precipitable iodinated test compound remaining in the serum was measured.

Strategies to improve biologics

- **3rd generation:** Methoxy polyethylene glycol-epoietin beta (*mircera*)

Pegylated epoietin: 165 aa, hEPO sequence but chemical binding of PEG residues, PEG allows the increase of the hydrodynamic volume of the molecule and protects from proteolysis.



M_W around 60 kDa
→ No kidney filtration
→ $T_{1/2}$: 134 h
→ Administration: once a month

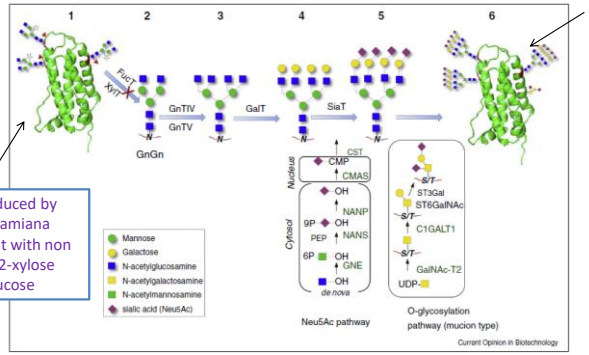
Recombinant EPO commercialised in France

	EPO (natural)	Eprex®/ Binocrit® (biosimilaire) d'Eprex® Neoreocormon® Retacrit®	Aranesp® (Analogue d'EPO)	MirCera® (Analogue d'EPO)
DCI	**erythropoietin**	**Epoétin alfa /beta/zeta**	**Darbépoétine alfa**	**Méthoxy polyéthylène glycol-époiétin bêta Pegylated Epoietin**
Nomber of N-Glycosylation	3	3	5	3
Nb of AA	165	165, séquence identical to the Native human epo $k(on) = 1.1 \times 10^8\ M^{-1}min^{-1}$	165 BUT WITH 5 MUTATED AA FOR AN ADDITIONNAL SITEs OF N-glycosylation $k(on) = 5.0 \times 10^8\ M^{-1}min^{-1}$	165, sequence identical to the NatiVe human EPO BUT +PEG LINKED BY COVALENT CHEMISTRY
Mw	30.4KDa	30.4 KDa	37.1 KDa	60 KDa
Nomber of maxiumum sialic acids	14	14	22	14
T 1/2	6 to 9h	**6 to 9 h**	**24 h**	**134 h**
Elimination	Renal Filtration	Renal Filtration	Affinity for receptor decreased➔ increase of biodisponibility Elimination by renal filtration	Increase of hydrodynamic volume ➔ no glomerular filtration PEG protects against proteolysis
Administration	-	2 à 3 per week	Once a week	Once a month

Glycoingieniery: Genetic modification of the producing host-cell for improving the quality of glycosylation

- block the production of specific enzymes
- allow Expression of new human glycosyltransferases

Exemple :Production of rhEPO in Nicotiana benthamiana



rEPO produced by wt. benthamiana leaves but with non human 1,2-xylose and 1,3-fucose

coexpression de 14 genes present in mammals

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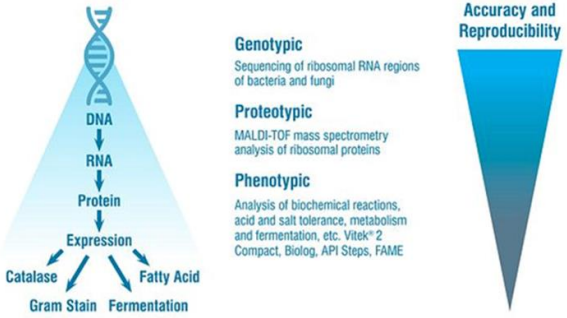
Strasser, (2014) Current opinion in biotechnology,

Microbial Identification Methods



Genotypic
Sequencing of ribosomal RNA regions of bacteria and fungi

Proteotypic
MALDI-TOF mass spectrometry analysis of ribosomal proteins

Phenotypic
Analysis of biochemical reactions, acid and salt tolerance, metabolism and fermentation, etc. Vitek® 2 Compact, Biolog, API Steps, FAME

Identification methodology is a critical factor for accuracy and reproducibility

In recent years Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) microbial identifications have allowed for faster, more cost-effective identifications. And whilst this technology is not as accurate as sequencing, it has the potential to be much more accurate than phenotypic methods, due to analysing ribosomal proteins that are closer to the DNA of the organism rather than analysis of biochemical reactions (such as fermentation, acid and salt tolerance metabolism, etc.).

Endotoxin test