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Capillary electrophoretic behaviour of recombinant human erythropoietin and darbepoietin.

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Introduction

Direct differentiation of recombinant human erythropoietin (rHuEPO) from the natural human urinary EPO is, at present, based on their electrophoretic differences, apparently due to their different sugar composition. The method is carried out by in-gel iso-electric focusing (IEF) separation, followed by an immuno-blotting procedure and a chemiluminescent detection[1,2]. As it is based on the similar separation mechanism to the gel IEF, capillary electrophoresis (CE) can be a powerful, easy-to-use technique for identifying the different glycoform compositions between the natural EPO and recombinant EPO. Thus, the aim of present work is to explore robust capillary electrophoresis conditions for analyzing glycoform compositions of human erythropoietin and its related derivatives, such as darbepoietin (NESP) (figure 1).

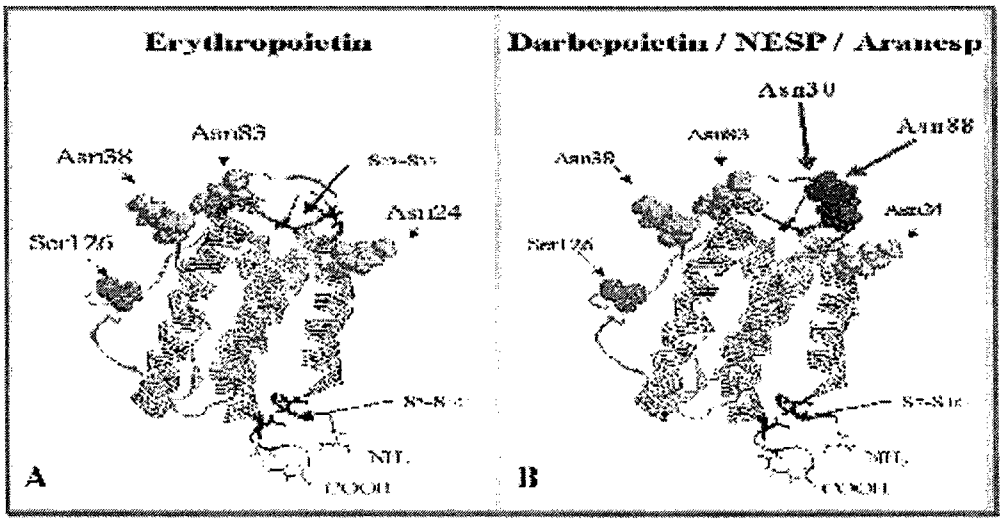

Figure 1. 3D NMR solution structure[3] of (A): human EPO. Highlited are the three N and one O-glycosylation sites as well as the S-S bridges. (B): Darbepoetin. The new additional glycosylation sites are also indicated.

Experimental

Sample preparation:

250 mg of rHuEPO (European Pharmacopoeia)[4] or 50 mg of NESP (Amgen) are dissolved in 250 ml of water (Milli-Q, Millipore).

The solution is desalted by filtration through a Microcon-10 cartridge (Amicon, Millipore).

The recovered retentate was adjusted to 250 ml.(or 100 ml for NESP, so that the concentration of the final aqueous solution obtained are 1 mg/ml for rHuEPO (and 0.5 mg/ml for NESP).

Instrumental conditions

Instrument: HP ^{3D}CE capillary system (Agilent Techonologies)

Capillary: Uncoated 50 μm (I.D.) fused–silica capillary. Effective length: 40 cm

Detection: UV, 214 nm

Buffers: (a) 0.01 M tricine, 0.01 M NaCl, 0.017 M NaOAc, 7 M urea, 0.025 mM putrescine. Final pH 5.5[4];

(b) the same as above, but 25 mM putrescine, pH 5.5;

(c) the same as above and 25 mM putrescine but pH 4.0.

Injection: Hydrodynamic, 50 mbar during 4 seconds at the anodic end.

Results

In bare silica, a high concentration of putrescine (25mM), as compared with other authors[5], resulted essential for a good and reproducible electrophoretic behaviour of both molecules (Table I). pH 5.5 (just above the pI of all isoforms) was optimum, allowing the separation of up to 8 different "bands" from rHuEPO (only 4 from NESP). Figure 2 illustrates the results obtained.

Table I. Reproducibility of the electrophoretic mobility of rHuEPO under the conditions developed in this work.

Intra-assay precision (t_m / t_{EOF}, n =10)	**0.87 % to 1.12 %**
Inter-assay precision (t_m / t_{EOF}, n = 5)	**2.03 % to 3.38 %**

Regarding stability, no changes were observed when the rHuEPO solution was kept for 2 weeks at –20°C or 4°C. At room temperature, very minor changes if any, could be seen in 2 weeks. After 2 months the changes in electrophoretic profiles were evident. Figure 3 shows those profiles.

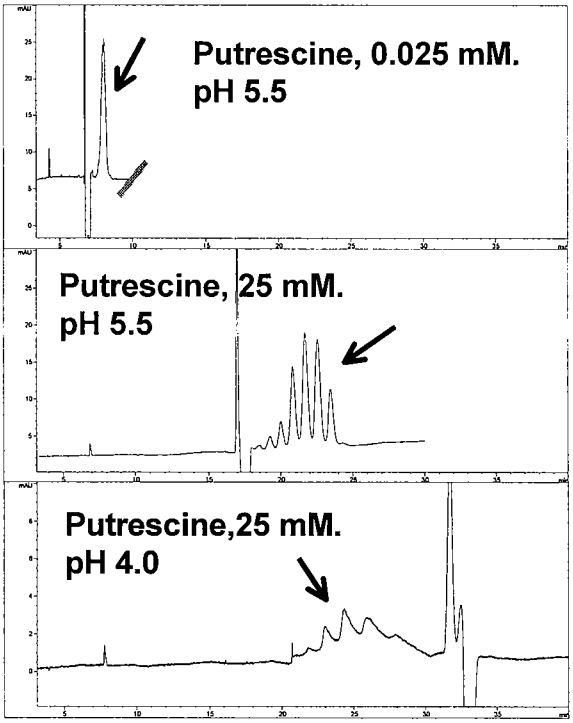

Figure 2. Influence of pH and concentration of putrescine on electrophoretic behaviour or rHuEPO.

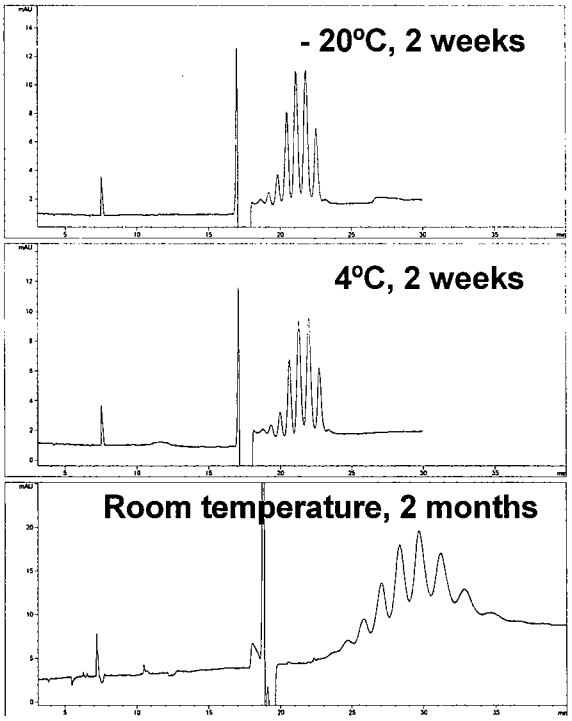

Figure 3. Stability of rHuEPO under different storage conditions.

Differences in electrophoretic behaviour are very clear when rHuEPO and NESP are analysed (Figure 4).

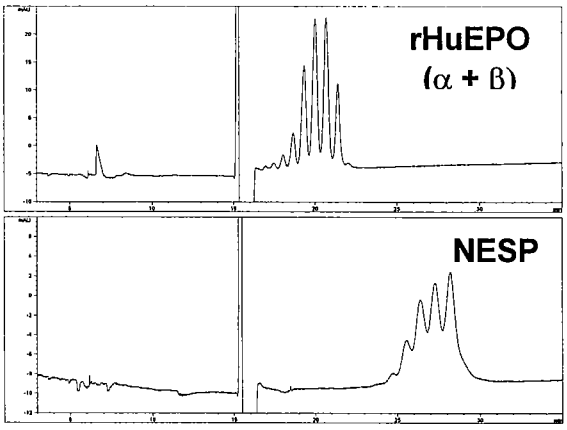

Figure 4. Analysis of rHuEPO and NESP under the conditions of the method developed.

Conclusion

Although the use of CZE method for detecting HuEPO biological samples is limited by the sensitivity of UV detection at present, it can be used as a FAST, EASY AND POWERFUL means for identifying those differences in glycoform compositions of rHuEPO or its analogues from different manufacturers. Also, it can be a very feasible approach for studying the stability of rHuEPO, uHuEPO and analogues in urinary samples under different storage conditions.

References

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