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High-performance anion-exchange chromatography combined with intrinsic fluorescence detection to determine erythropoietin in pharmaceutical products

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Abstract

A high-performance anion-exchange chromatography (HPAEC) method was developed for determination of recombinant human erythropoietin (EPO) in pharmaceutical products. A fluorescence detector was added to the HPLC system as intrinsic fluorescence detection compared favourably to UV detection regarding sensitivity and selectivity. The HPLC method has been successfully applied to analyse erythropoietin products even in the presence of albumin as excipient. The intrinsic fluorescence chromatograms of both proteins revealed various peaks attributed to either micro-heterogeneous erythropoietin or albumin variants. The intrinsic fluorescence signal was linear over the range 10–200 μg/ml erythropoietin corresponding to pharmaceutically relevant concentrations. The HPLC method appeared to be a suitable method for differentation between recombinant human erythropoietin epoetin-alpha and -beta as they revealed different intrinsic fluorescence elution profiles. In conclusion, this study contributes to the development of a straightforward physicochemical method for specific quantification of recombinant human erythropoietin in pharmaceutical preparations.

Keywords: HPLC; HPAEC; Intrinsic fluorescence; Erythropoietin; Epoetin-alpha; Epoetin-beta; Albumin

1. Introduction

Erythropoietin (EPO) is a glycoprotein produced primarily by the kidney and it is the main factor regulating red blood cell production [1]. The protein has a molecular mass of 30–34 kDa and 40% of the molecular weight represents carbohydrates of which 17% are sialic acids [1–3]. The sugars are attached to the protein via one O-linked (serine) and three N-linked (asparagine) glycosylation sites [4]. Since the carbohydrates vary in amount, size and structure, EPO has a heterogeneous structure. Since the mid 1980s EPO has been expressed applying recombinant DNA technology (rhEPO) and is now one of the most successful biopharmaceutical products. Besides, rhEPO is known for the extensive misuse as performance-enhancing agent in endurance sports. The biological activity of EPO in vivo is affected by the glycosylation pattern (sialic acid content). Since production system and process conditions for rhEPO affect the glycosylation pattern the production process should be carefully validated and monitored to assure consistency of the biological activity throughout different production batches [5].

At present the content of rhEPO preparations is typically tested by complex in vivo potency assays which measure the relevant biological activity. For instance, the European Pharmacopoeia describes an assay for rhEPO bulk solutions in which the effect of rhEPO on mice kept under low oxygen conditions is monitored by measuring incorporation of radio-labelled ferric chloride [6]. For assaying the content of rhEPO preparations in a routine setting these types of bioassays require a significant number of animals. A rapid and less resource demanding physico-chemical assay may

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not specifically mimic bioactivity but it would provide a wider forum for controlling the quality of these common pharmaceutical products. Moreover, from an analytical point of view content assays based on physicochemical technology will be more precise than bioassays. Developing a suitable physicochemical assay for rhEPO preparations is hampered by the low dose of the micro-heterogeneous glycoprotein in presence of relatively large amounts of excipients. Particular difficulties are encountered when human serum albumin (HSA) is present. The protein HSA is obtained from large pools of human plasma and cannot be considered chemically homogeneous. Although HSA is not present in currently licensed rhEPO preparations in the European Union, HSA containing preparations are still widely available on the market such as in the USA. Candidate physicochemical assays should have a high degree of selectivity for rhEPO and assay reproducibility. So far capillary electrophoresis (CE) methods have been developed to characterize the rhEPO glycoform pattern and a capillary zone electrophoresis method has now been prescribed by the European Pharmacopoeia as an identification test for rhEPO in concentrated bulk solutions [6]. In addition to this method, another CE method has been developed that is capable of analysing rhEPO pharmaceutical preparations containing salts and HSA, and in the concentration range of 0.03–1.92 mg rhEPO/ml [7].

HPLC in combination with UV-detection is a separation method that provides a powerful means for characterising the homogeneity of common biopharmaceuticals such as somatropin, insulin and interferons. Because of its high resolution, reversed-phase HPLC is often applied for quantification of the active pharmaceutical ingredient and for the analysis of closely related protein variants or degradation products (e.g., oxidised, deamidated) [8–10]. High performance size exclusion chromatography may also be used for quantification but it is normally applied to determine the native size of the protein and to reveal possible dimers, oligomers and aggregates [11–13]. Ion exchange chromatography provides another principle for separating closely related monomeric species in a protein preparation. High-performance anion-exchange chromatography (HPAEC) separates proteins according to their negative electric charge. In combination with pulsed amperometric detection HPAEC has often been applied to analyse carbohydrates cleaved from glycoproteins [14]. Methods to analyse an intact glycoprotein by HPAEC are not common although the various negative electric charges of the glycoforms, contributed by the sialic acid groups, provide conditions for separation of the EPO glycoforms by HPAEC.

In the present study we investigate the possibilities of HPAEC for determination of rhEPO at pharmaceutically relevant concentrations. Our preference for HPAEC chromatography was based on its expected selective properties for charged compounds such as rhEPO isoforms and the possibility HPAEC offers to analyse the protein under native conditions which would not be the case in reversed-phase chromatography [15,16]. For this purpose several rhEPO preparations containing epoetin-alpha and/or epoetin-beta in absence or presence of HSA were analysed. Previously, we reported about the enhanced sensitivity and selectivity properties of intrinsic fluorescence detection compared to UV detection in the analysis of biopharmaceuticals [16]. Therefore, in this study a fluorescence detector was introduced to the HPAEC system and its performances were compared to a UV detector. Next to monitoring the amount of protein, intrinsic fluorescence spectroscopy also provides information on changes in the local environment of the aromatic side chains (tertiary protein structure). Eventually, this investigation contributes to the development of a straightforward and fast physicochemical method for determining the amount as well as the type of EPO in pharmaceutical products.

2. Materials and methods

2.1. Reagents

'EPO BRP' (batch 2) was purchased from the European Directorate for the Quality of Medicines (EDQM, Strasbourg, France). The vials contained 250 μg of rhEPO being a mixture of equal amounts of epoetin-alpha and epoetin-beta. 'Eprex 10000 IE/ml' (Janssen-Cilag, charge number 02BS09T) was a licensed solution for injection provided in a 1.0 ml syringe having a label claim of 10,000 IU/ml corresponding to 84.0 μg/ml epoetin-alpha (10,000 IU/ml). 'NeoRecormon 10000 IE' (Roche, charge number MH64761 05) was a licensed solution for injection provided in a 0.6 ml syringe having a label claim of 10,000 IU/0.6 ml (16,667 IU/ml) corresponding to 83 μg/0.6 ml epoetin-beta (138 μg/ml). Both Eprex and NeoRecormon did not contain HSA as excipient. The HSA preparation 'Cealb' (Sanquin, The Netherlands) contained 20% protein predominantly (>95%) albumin. Tween-80 (polysorbate-80) was purchased from Merck. Phosphate buffered saline, pH 7.2 (PBS), consisted of 8 mM $Na_2HPO_4{\cdot}2H_2O$, 2 mM $NaH_2PO_4{\cdot}H_2O$ and 154 mM NaCl. Solvents for chromatography were HPLC grade and salts were analytical grade chemicals.

2.2. Sample preparation

EPO BRP (250 μg) was dissolved in 250 μl water. Subsequently, this 1 mg/ml protein solution was diluted with PBS to obtain 10, 30, 60, 100, 150, 200, 300 and 400 μg/ml solutions before 100 μl was applied to the HPAEC column. One prepared protein solution contained 100 μg/ml EPO BRP and 3 mg/ml HSA.

All samples and solutions were filtered before use over a 0.45 μm filter.

2.3. HPLC

2.3.1. Instrumentation

HPLC experiments were performed on an Agilent 1100 system including a G1379A micro vacuum degasser, G1312A

binary pump, G1329A auto-sampler, G1330B auto-sampler thermostat, G1316A thermostatted column compartment, G1315B diode array detector and G1321A fluorescence scanning detector. This Agilent HPLC system was operated by ChemStation software. UV detection was performed at 214 and 280 nm, intrinsic fluorescence emission detection at 343 nm with an excitation wavelength of 295 nm (excitation of tryptophan). The fluorescence spectra were measured at 1 nm intervals over the range 320–380 nm with a scanning speed of 80 nm/s (PMT-gain 12).

2.3.2. High-performance anion-exchange chromatography

EPO BRP dilutions (10, 30, 60, 100, 150, 200, 300 and 400 μg/ml), 100 μg/ml EPO BRP in presence of 3 mg/ml HSA and Eprex were applied in injections of 100 μl to a DNAPac PA-100 analytical column (4 mm × 250 mm) (Dionex), equilibrated with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer containing 25 mM NaCl, pH 7.0. The DNAPac PA-100 anion exchange column is specifically designed to provide high-resolution separations and is composed of 100 nm quaternary amine functionalised MicroBeads™ bound to a 13 μm solvent-compatible, non porous substrate. NeoRecormon was applied to the column in a volume corresponding to a protein loading of 84 μg. The elution was performed at 25 °C and at a flow rate of 1 ml/min, and with an increasing NaCl gradient as described in Table 1 (program 1). In case of EPO BRP (100 μg/ml), Eprex and NeoRecormon also a second gradient program was applied (Table 1, program 2).

Table 1
Gradient programs applied to the HPAEC analysis

Time (min)	A (%)	B (%)
Program 1		
0	97.5	2.5
3.0	97.5	2.5
3.1	88.0	12.0
21.0	88.0	12.0
27.0	82.5	17.5
33.0	82.5	17.5
39.0	50.0	50.0
45.0	50.0	50.0
45.5	0	100
51.5	0	100
52.0	97.5	2.5
55.0	97.5	2.5
Program 2		
0	97.5	2.5
3.0	97.5	2.5
3.1	89.5	10.5
5.1	89.5	10.5
5.2	89.0	11.0
7.2	89.0	11.0
7.3	88.5	11.5
9.3	88.5	11.5
9.4	88.0	12.0
15.0	88.0	12.0
16.0	0	100
22.0	0	100
23.0	97.5	2.5
27.0	97.5	2.5

Buffer A: 20 mM HEPES, pH 7.0. Buffer B: 20 mM HEPES containing 1 M NaCl, pH 7.0.

3. Results

3.1. Performances of detection systems

EPO BRP (100 μg/ml) was applied to a DNAPac PA-100 column and the eluate was monitored with an UV and intrinsic fluorescence scanning detector to test the suitability of both detection systems. UV detection was performed at 214 and 280 nm and intrinsic fluorescence emission detection at 343 nm (Fig. 1). The HPAEC intrinsic fluorescence chromatogram showed a main peak between 5 and 10 min and minor peaks at the void volume and at high salt concentrations (Fig. 1A, Table 1). The main peak was attributed to EPO as the minor peaks were also observed at the same intensity in a blank (PBS) run. The HPAEC UV_{214} profile of EPO BRP showed a signal at the void volume and a small peak around 6 min (Fig. 1B). These UV_{214} signals appeared to be artefacts since a blank (PBS) run revealed an identical chromatogram (data not shown). It was also shown that the signal to noise ratio was not satisfactory. With respect to the HPAEC UV_{280} profile (Fig. 1B), a significant peak appeared at the same retention as was observed for EPO in the fluorescence emission chromatogram. Although this peak showed a low absorbance, it was significantly

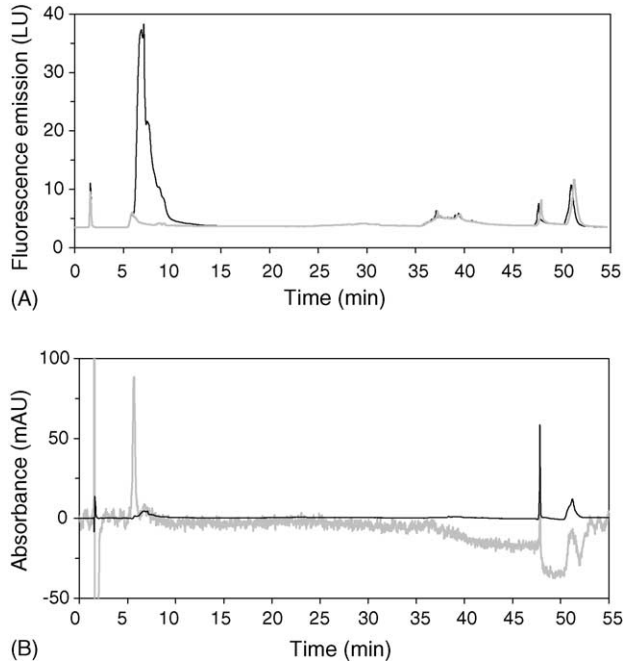

Fig. 1. EPO BRP, 100 μg/ml (black line) and PBS (grey line) applied to HPAEC and monitored by intrinsic fluorescence (A), EPO BRP, 100 μg/ml applied to HPAEC and monitored by UV absorbance at 214 nm (grey line) and 280 nm (black line) (B).

present compared to the corresponding blank elution profile.

3.2. Separation of erythropoietin-albumin

The HPAEC intrinsic fluorescence profiles of 100 μg/ml EPO BRP and 3 mg/ml HSA are shown in Fig. 2A and B, respectively. Whereas, EPO BRP eluted between 6 and 10 min (around 0.1 M NaCl), HSA eluted between 11 and 43 min. In both cases it was demonstrated that the proteins are not homogeneous although EPO showed a higher level of homogeneity than HSA. The elution profile of the EPO preparation containing an excess of HSA (=1:30 mixture) (Fig. 2C) resembled the profiles of the individual proteins when combined. The total peak area of the various HSA peaks appeared to be approximately 14 times higher than the peak area of the EPO BRP peak. It was shown that the applied HPAEC method is a suitable method to separate rhEPO from amounts of HSA commonly present in various pharmaceutical preparations.

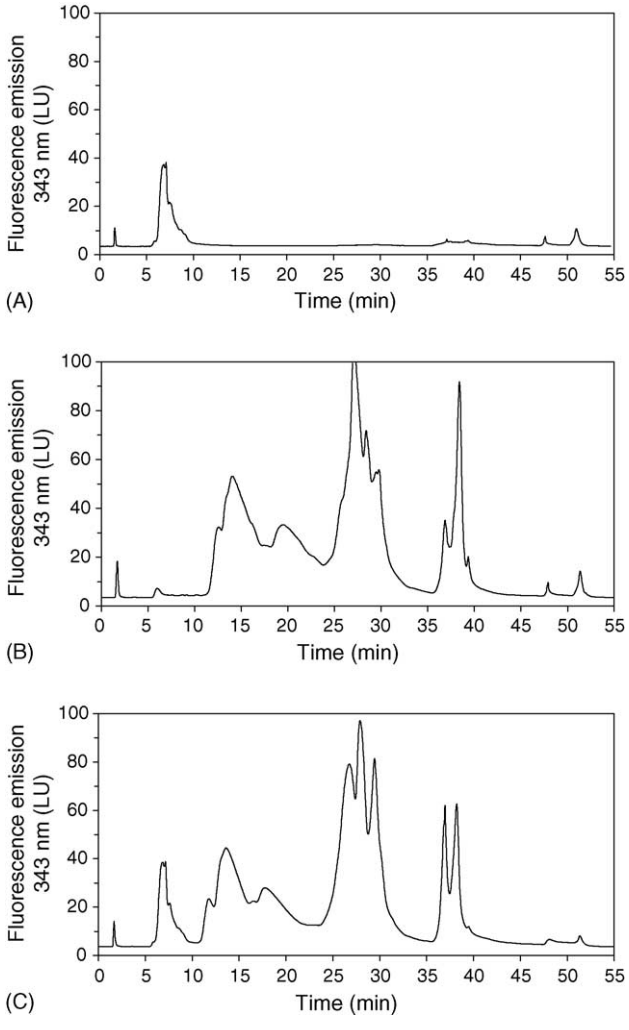

Fig. 2. HPAEC intrinsic fluorescence chromatogram of 100 μg/ml EPO BRP (A), 3 mg/ml HSA (B) and 100 μg/ml EPO BRP containing 3 mg/ml HSA (C).

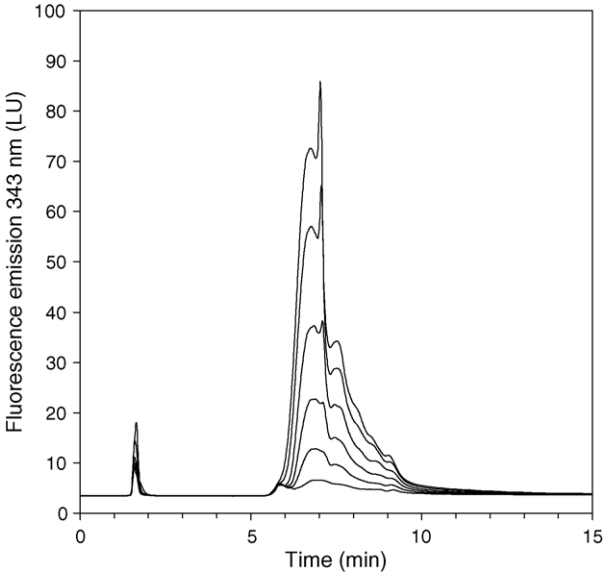

Fig. 3. HPAEC intrinsic fluorescence chromatogram of 10, 30, 60, 100, 150 and 200 μg/ml EPO BRP.

3.3. Quantification of erythropoietin

The intensity of the intrinsic fluorescence signals (peak areas) increased when the amount of EPO BRP was increased (Fig. 3). The presence of distinct forms of rhEPO was more clearly observed when higher concentrations of rhEPO were applied. To reveal a possible linear relation between the peak area and the concentration of EPO, the obtained HPAEC intrinsic fluorescence profile of the blank was subtracted from the profiles obtained with 10–400 μg/ml EPO BRP. Eventually, the fluorescence emission signals were linear over the range 10–200 μg/ml EPO ($Y = 30.74X - 47.04$, $R^2 = 0.9996$) and the regression line crossed the origin ($p > 0.05$). In case EPO concentrations above 200 μg/ml were applied to the HPAEC column, the signals started to deviate from linearity i.e. the corresponding peak areas were lower than expected. The measured peak areas of two commercial rhEPO preparations were calculated against the peak area of the EPO BRP reference preparation. This revealed a concentration of 94 μg/ml for the Eprex sample and 155 μg/ml for the NeoRecormon sample.

By using a fluorescence scanning detector it was not only possible to monitor one fluorescence emission wavelength but also to measure fluorescence spectra corresponding to the various peaks on-line. In this way the average fluorescence emission maximum (F_{max}) for 10–200 μg/ml EPO was determined to be 343 nm.

3.4. Selectivity regarding EPO-alpha and -beta

The HPAEC intrinsic fluorescence profiles of EPO BRP, Eprex and NeoRecormon by applying gradient program 2 (Table 1) are shown in Fig. 4. Although the peak profiles of the various rhEPO preparations differed by applying gradient

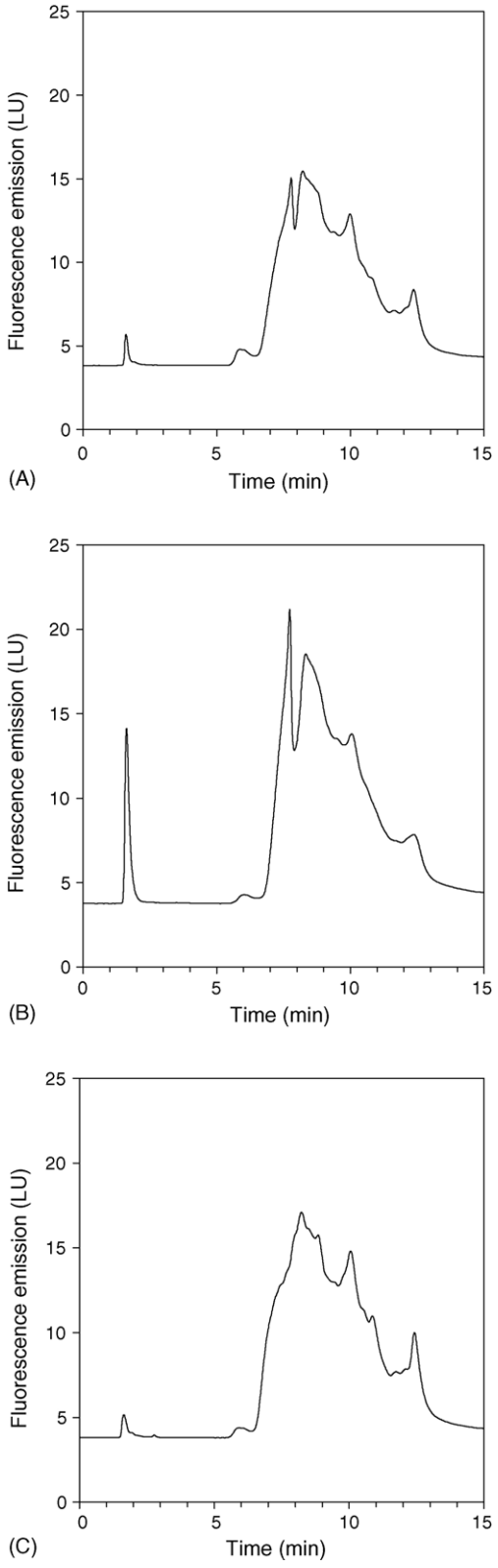

Fig. 4. HPAEC intrinsic fluorescence chromatogram of EPO BRP (A), Eprex (B) and NeoRecormon (C) by applying gradient program 2 (Table 1).

program 1 (data not shown), the differences in peak profiles were more pronounced when gradient program 2 was applied. In the latter case Eprex showed a clear peak at 7.5 min and NeoRecormon at 10.0 and 12.5 min. Next to this, the peak pattern of NeoRecormon revealed more minor peaks in comparison to that of Eprex. The intrinsic fluorescence profile of EPO BRP resembled the combined profiles of Eprex and NeoRecormon. When the various elution profiles were compared it was also noticed that in case of Eprex the void volume peak around 2 min was significantly higher. The corresponding intrinsic fluorescence spectrum revealed a F_{max} of 349 nm. The latter peak was also observed when 100 μl of a blank solution containing 0.03% Tween-80 was applied to the column. The corresponding F_{max} was 347 nm.

4. Discussion

Physicochemical methods have been successfully applied to determine the amount, structural identity, purity, integrity and stability of various biopharmaceuticals [7,10,17–21]. However, for rhEPO an in vivo bioassay is the golden standard for measuring the potency. On the other hand, CE methods can be applied for specific identification of rhEPO. CE is known as a highly selective technique that is applicable for the identification, purity and structural evaluation (microheterogeneity) of rhEPO. In general, limitations of CE concern the concentration sensitivity, interference of the excipients and reproducibility. In contrast, HPLC is a more robust and reproducible technique and therefore more suitable for quantitative inter-run comparisons. This explains a general preference of HPLC rather than CE for assaying biopharmaceuticals in QC settings. Therefore, our aim was to develop an HPLC method for specific quantification of rhEPO in pharmaceutical products i.e. at low concentrations without a separate sample clean-up. We expected HPAEC to be able to separate rhEPO under native conditions. Moreover, we considered that an HPAEC method, displaying subtle charge properties, would provide a higher level of specificity to an assay than RP-HPLC or SE-HPLC. After trying several column matrices and buffer systems a DNA-Pac anion exchange column was selected together with HEPES buffer at neutral pH as eluent. In the present study we investigated this HPAEC method in combination with UV and intrinsic fluorescence detection.

To test the suitability of the detection systems for monitoring EPO applied to HPAEC, the UV_{214} and UV_{280} elution profiles were compared to the intrinsic fluorescence elution profile. Intrinsic fluorescence detection was more sensitive and showed a higher selectivity than UV detection (Fig. 1). Normally, HPLC in combination with UV-detection at 214 nm is a powerful method for detecting proteins. However, in our study EPO was not detected this way and a low signal to noise ratio was observed (Fig. 1B). This can be explained by the presence of HEPES in the eluents that interferes with the UV_{214} signal. This inconvenience was not observed with intrinsic fluorescence detection (Fig. 1A).

In general, matrix compounds such as pharmaceutical excipients and buffer components do not interfere in the intrinsic fluorescence chromatogram which specifically displays the fluorescence emitted by the trytophan residues in the protein. Therefore, less complex chromatograms will be obtained and peaks can be readily attributed to be proteinaceous compounds or not. The fact that the sensitivity of intrinsic fluorescence detection of proteins compared favourably to UV detection was demonstrated before [16].

The HPAEC method combined with intrinsic fluorescence detection appeared to be a suitable method to analyse rhEPO in presence of HSA. HSA is added to some biopharmaceuticals as stabiliser during the manufacturing process and/or storage [22,23]. From an analytical perspective particular difficulties are encountered with this excipient since it is another protein, present in large amounts and not homogeneous. Therefore, in size exclusion chromatography rhEPO as well as HSA would elute as single peaks that overlap considering the content ratio and molecular masses of the proteins. The HPAEC method developed by us is capable of analysing rhEPO/HSA mixtures. HSA preparations contain several variants such as mercapto, non-mercapto and glycated forms as well as oxidised, deamidated and polymerised variants of these latter forms [24]. These variants can be separated by charge based techniques such as isoelectric focusing [25] and CE [26]. Since HPAEC separates proteins according to their negative electric charges, HSA eluted at various salt concentrations resulting in various peaks in the HPAEC chromatogram (Fig. 2B). An additional advantage of the HPAEC method is the use of intrinsic fluorescence as detection system. The intrinsic fluorescence intensity is dependent on the amount and location of the Trp residues in the protein molecule. Since rhEPO contains three tryptophan residues and HSA one, rhEPO will have a relatively more intense fluorescence signal compared to HSA. Therefore, less interference of HSA will be observed in the fluorescence chromatrogram (Fig. 2C). Eventually, the advantages of the HPAEC method described above together with an appropriate gradient program provide a suitable method to analyse rhEPO even in the presence of HSA.

The HPAEC method showed a linear dose/response relationship in the range of 0.01–0.20 mg/ml rhEPO. This means that the method is sensitive enough to assay the content of licensed pharmaceutical preparations of rhEPO which range between 2000 and 40,000 IU/ml (∼ 0.015–0.4 mg/ml). Prior sample concentration or clean-up is not needed but rhEPO concentrations above 0.20 mg/ml should be diluted with PBS first. When assaying two commercial preparations against the PhEur reference preparation, the measured rhEPO contents only slightly deviated (i.e. 12% higher) from the concentrations declared by their manufacturers. This might be explained by several factors. First, significant interbatch variation exists. Our measurements relate to single production batches while the labelled concentrations are averages of numerous production lots, the actual content of an individual lot may indeed deviate from this average. Secondly, the PhEur reference preparation has not been established for the purpose of physicochemical content assays. The declared content of 250 μg rhEPO used in our calculations is based on the amount filled during its manufacturing process and has not been verified after lyophilisation by independent physicochemical assays (A. Bristow, personal communication). The contents of the pharmaceutical preparations obtained by our method are compared to concentrations (μg/ml) deduced from bioactivity figures (IU/ml) and the specific activity figures (IU/μg) as labelled by the manufacturers. Both preparations have a very similar 'specific bioactivity' i.e. 10.000 IU = 84 μg for epoetin-alpha and 10.000 IU = 83 μg for epoetin-beta. Although both manufacturers apply different types of in-vivo bioassays these assays appear to give equivalent results as was recently shown in a collaborative study [27]. This similarity in specific activity of epoetin-alpha and epoetin-beta provides a basis for corresponding results of physico-chemical and biological activity assays for both compounds. The fact that the results of our physico-chemical method resemble the labelled contents of both EPO preparations supports this.

The HPAEC method also provides selectivity for rhEPO. Two main rhEPO products on the European market are Eprex and NeoRecormon containing epoetin-alpha and -beta, respectively. By adjusting the gradient program in the HPAEC method (Table 1) it was possible to obtain clear differences in the elution profiles for epoetin-alpha and -beta (Fig. 4B and C). These profiles are most probably explained by the various EPO glycoforms present in epoetin-alpha and -beta. These glycoforms contain negative electric charges contributed by the sialic acid groups and therefore they elute at various retention times. Although the glycoforms of epoetin-alpha and -beta were not separated as well as was demonstrated by CE [6,7], the obtained HPAEC intrinsic fluorescence elution profiles of the epoetins provide fingerprints for determining the type of rhEPO. The European Pharmacopoeia reference preparation 'EPO BRP' consists of a mixture of epoetin-alpha and -beta. This was clearly visible in the elution profile of EPO BRP (Fig. 4).

When the HPAEC intrinsic fluorescence elution profiles of the various rhEPO preparations were compared it was noticed that in case of Eprex the peak at the void volume was significantly higher (Fig. 4). The corresponding intrinsic fluorescence spectrum revealed a F_{max} of 349 nm. This value differs from the F_{max} of the main peak (343 nm) and suggests the presence of denatured EPO in the samples. Protein unfolding induces an increase of the F_{max} as the emitting Trp residues will be more exposed to the solvent (more polar environment) [17,19]. Moreover, the measured F_{max} resembles the F_{max}, 351 nm, of denatured EPO species determined by Toyoda et al. [28]. Eprex contains 0.03% of the non-ionic surfactant Tween-80 (polysorbate 80). The higher peak that was observed in the HPAEC intrinsic fluorescence elution profile of Eprex (Fig. 4B) was also observed when a blank solution containing 0.03% Tween-80 was applied to the column. The corresponding F_{max} was 347 nm also suggesting a protein with conformational alterations. This suggests

that the void volume peak in the Eprex elution profile is an analytical artefact due to the presence of 0.03% Tween-80.

In conclusion, we developed an HPAEC method for the determination of EPO in common pharmaceutical preparations. In particular the use of intrinsic fluorescence detection contributes to the selectivity and sensitivity of the method. In addition, it provides conformational information of the studied biopharmaceutical. In its present status the HPAEC method provides a rapid and accurate assay for in-process material and finished products to target and precisely control the amount of EPO-protein. In particular the method is suggested for official medicines control laboratories involved in testing suspect preparations declared to contain EPO. The method is a promising candidate for further establishment and cross-validation against current reference methods which are laborious bioassays involving the use of stressful animal experiments. Moreover, the concept of HPAEC separation combined with intrinsic fluorescence detection may be suitable for analysing other glycoproteins as well.

References

[1] S.B. Krantz, Blood 77 (1991) 419.
[2] J.C. Cheetman, D.M. Smith, K.H. Aoki, J.L. Stevenson, T.J. Hoeffel, R.S. Syed, J. Egrie, T.S. Harvey, Nat. Struct. Biol. 5 (1998) 861.
[3] J.M. Davis, T. Arakawa, T.W. Strickland, D.A. Yphantis, Biochemistry 26 (1987) 2633.
[4] W. Jelkmann, Physiol. Rev. 72 (1992) 449.
[5] P.L. Storring, R.E. Gaines Das, J. Endocrinol. 134 (1992) 459.
[6] European Pharmacopoeia, fifth ed., Erythropoietin concentrated solution, Council of Europe, Strasbourg, 2005, pp. 1528–1532.
[7] H.P. Bietlot, M. Girard, J. Chromatogr. A 759 (1997) 177.
[8] J. Brange, L. Langkjaer, in: Y.J. Wang, R. Pearlman (Eds.), Stability and Characterization of Protein and Peptide Drugs, Plenum Press, New York, 1993, p. 315.
[9] K.H. Buchheit, A. Daas, K.H. Jönsson, Pharmeuropa BIO 1 (2002) 7.
[10] R. Pearlman, T.A. Bewley, in: Y.J. Wang, R. Pearlman (Eds.), Stability and Characterization of Protein and Peptide Drugs, Plenum Press, New York, 1993, 1–58.
[11] B.M. Eckhardt, J.Q. Oeswein, T.A. Bewley, Pharm. Res. 8 (1991) 1360.
[12] J.F. Hansen, M. Ezban, Dev. Biol. Stand. 48 (1980) 105.
[13] A. Litzén, J.K. Walter, H. Krischollek, K.G. Wahlund, Anal. Biochem. 212 (1993) 469.
[14] P. Hermentin, R. Witzel, J.F.G. Vliegenthart, J.P. Kamerling, M. Nimtz, H.S. Conradt, Anal. Biochem. 203 (1992) 281.
[15] J. Holthuis, R. Driebergen, in: J.N. Herron, W. Jiskoot, D.J.A. Crommelin (Eds.), Physical Methods to Characterize Pharmaceutical Proteins, Plenum Press, New York and London, 1995, pp. 243–299.
[16] D.M.A.M. Luykx, S.S. Goerdayal, P.J. Dingemanse, W. Jiskoot, P.M.J.M. Jongen, J. Chromatogr. B, in press.
[17] P. Johansen, H.P. Merkle, B. Gander, Biochim. Biophys. Acta 1425 (1998) 425.
[18] C. Jones, D.T. Crane, X. Lemercinier, B. Bolgiano, S.E. Yost, Dev. Biol. Stand. 87 (1996) 143.
[19] D.M.A.M. Luykx, M.G. Casteleijn, W. Jiskoot, J. Westdijk, P.M.J.M. Jongen, Eur. J. Pharm. Sci. 23 (2004) 65.
[20] J.M. Matthews, A. Hammacher, G.J. Howlett, R.J. Simpson, Biochemistry 37 (1998) 10671.
[21] P.L. Storring, R.E. Gaines Das, J.W.M. Mulders, M. Halder, Biologicals 30 (2002) 217.
[22] G.H. Krystal, R.C. Pankratz, N.M. Farber, J.E. Smart, Blood 67 (1986) 71.
[23] T. Moreira, L. Cabrera, A. Gutierrez, A. Cadiz, M.E. Castellano, Acta Pharm. Nord. 4 (1992) 59.
[24] M. Girard, T. Cyr, N. Mousseau, J.-C. Ethier, Chromatogr. Suppl. I 49 (1999) S-21.
[25] B.M. Van Liedekerke, H.J. Nelis, J.A. Kint, F.W. Vanneste, A.P. De Leenheer, J. Pharm. Sci. 80 (1991) 11.
[26] M. Girard, H.P. Bietlot, T.D. Cyr, J. Chromatogr. A 772 (1997) 235.
[27] M.-E. Behr-Gross, A. Daas, A.F. Bristow, Pharmeuropa BIO 1 (2004) 23.
[28] T. Toyoda, T. Itai, T. Arakawa, K.H. Aoki, H. Yamaguchi, J. Biochem. 128 (2000) 731.