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

Dion M.A.M. Luykx, Pieter J. Dingemanse, Soenita S. Goerdayal, Peter M.J.M. Jongen *

Centre for Biological Medicines and Medical Technology, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands

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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.
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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

* Corresponding author. Tel.: +31 30 2743701; fax: +31 30 2744421.
E-mail address: peter.jongen@rivm.nl (P.M.J.M. Jongen).

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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

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