THE SEPARATION OF EPO FROM OTHER PROTEINS IN MEDICAL PRODUCTS FORMULATED WITH DIFFERENT STABILIZERS

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Abstract: The RP-HPLC method, useful for the separation of a protein of the erythropoietin formulations with different stabilizers, was elaborated. Using this method, rhEPO, HSA and other proteins, can be determined, depending on the sample composition. The method is sensitive and rapid. Our results indicate that this method is useful for quality control of rhEPO in final drug preparations with albumin as stabilizer. The method was also capable of distinguishing products from different manufacturers.

Keywords: recombinant erythropoietin (rhEPO); human serum albumin (HSA); HPLC

Erythropoietin is a glycoprotein hormone produced in kidneys in approximately 90%. Relative deficit of the hormone is the main cause of anemia in chronic renal disease, which is treated with erythropoietin products, manufactured using biotechnological methods (1, 2).

The EPO molecule is a protein complex containing a primary biological disulfide bridge and four sugar chains, attached in the process of glycosylation. The configuration of carbohydrate structures in the erythropoietin molecule (carbohydrate chain length, complexity and branching, as well as the content of sialic acid) is characteristic of a given biosynthesis process and determines biological activity (3-5).

The microheterogeneity of the rhEPO molecule is the main explanation of various types of epoetins, referred to as alfa, beta and omega, used as the active substances in medicinal products.

Since a recombinant protein molecule readily undergoes structural changes as a result of oxidation, deamination and aggregation (dimer and polymer formation), appropriate formulation of a medicinal product (6-8), ensuring its stability in the declared shelf life, is an important issue. The drugs used should not be only effective, but first of all safe. In addition to identity and activity confirmation, purity determination is a very important issue. Schellekens and Patten (9, 10) have shown that the products of protein degradation or polymerization may be the reason for immunogenicity of biotechnology products. Therefore, the admissible level of impurities should be specified in quality requirements for a given product.

To ensure appropriate stability of rhEPO-containing medicinal products, some manufacturers use human serum albumin as a stabilizer, in a quantity of 1-2 mg/mL of the product i.e., 100 × more by weight than the active substance content. These quantity proportions of the stabilizing protein to the active protein enable the use of analytical methods for determining a single protein as part of the quality control of these medicinal products. Albumin is a strong interfering agent in these tests.

The objective of the study was to develop a method, using HPLC technique, enabling determination of proteins in rhEPO-containing medicinal products.

EXPERIMENTAL

Instrumentation

HPLC analysis was performed using a Shimadzu liquid chromatograph equipped with two LC-10AT vp pumps, an SCL-10A vp controller connected to a computer (Class-VP 5.03), an SIL-10AD vp autosampler, a UV-Vis SPD-10A vp detector and a CTO-10AC vp column oven.

Material and reagents

The recombinant human erythropoietins used expressed in CHO and BHK cells, were present in medical products with different stabilizer. CHO-EPO was named as epoetin alfa and epoetin beta and BHK-EPO was named as epoetin omega. Ampoules of formulated rhEPO were supplied by the manufacturers.
The Erythropoietin BRP European Pharmacopoeia Standard and human serum albumin with 4% globulin fraction (Sigma) and human serum albumin without globular protein fraction (Fluka) were used. All other chemicals used were of the highest purity available.

**Method**

A reverse phase high performance liquid chromatography (RP-HPLC) method was used to determine rhEPO and other proteins in various pharmaceutical preparations. In order to optimize the conditions of epoetin, contained in the sample along with other substances, a number of experiments were performed, in which the influence of the solid phase (column type) and the mobile phase (composition and profile gradient) were measured.

The specific liquid chromatographic parameters for the assay were chosen as follows. The column was a J. T. Baker, Wide Pore Octadecyl, 250 × 4.6 mm with 5 µm particle size packing. The mobile phase flow rate was kept constant at 1 mL/min. Mobile phase A was a 0.1% (v/v) solution of trifluoroacetic acid in water and phase B was a 0.1% (v/v) solution of trifluoroacetic acid in acetonitrile/water (70:30 v/v). With the aim of establishing suitable resolution between albumin and rhEPO, and related proteins, gradient elution was determined, as follows:

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>0</th>
<th>12</th>
<th>19</th>
<th>43</th>
<th>44</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>%B</td>
<td>30</td>
<td>60</td>
<td>60</td>
<td>75</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

All determinations were performed at ambient temperature, detection was at wavelength 214 nm. Standards and samples were diluted to a concentration of 1000 IU EPO/mL and were injected in the volume 100 µL directly on to the column.

**RESULTS AND DISCUSSION**

Adverse changes, consisting in formation of high molecular weight compounds i.e., dimer and polymers, occur during storage of medicinal products containing active proteins. As these compounds shown immunogenic activity, their number should be strictly limited. They are determined using polyacrylamide gel electrophoresis (SDS-PAGE) or size exclusion chromatography (SEC). In the European Pharmacopoeia monograph, the SEC method to determine aggregates in erythropoietin concentrated solutions is used. The same technique is also suitable for the determination of albumin aggregates. However, in products containing both proteins, this determination becomes a problem, because molecular weights of recombinant human erythropoietin (rhEPO) and human serum albumin (HSA) are similar. No HPLC method enabling quality evaluation of products containing rhEPO and albumin has been found in the bibliography, so it was undertaken to develop such method.

Based on the different hydrophobic properties of both proteins in a non-polar stationary phase, an RP-HPLC in gradient method was used.

![Typical chromatograms obtained using the RP-HPLC method under chosen conditions for the tested samples containing: (a) epoetin omega stabilized with albumin (b) epoetin alfa stabilized with albumin, (c) epoetin alfa stabilized with polysorbate 80, (d) epoetin beta stabilized with polysorbate 20, (e) Erythropoietin BRP standard. It presents peaks for: 1 – globular protein fraction, 2 – human serum albumin, 3 – albumin aggregate, 4 – fully glycosylated rhEPO, 5 – glycoform of rhEPO (not to be glycosylated at Ser 125), 6 – erythropoietin aggregate.](image-url)
The selectivity of the developed method for separating both proteins was tested on a model sample represented by water solution of standard albumin and erythropoietin, achieving satisfactory separation of the tested compounds, as evidenced by retention times, approximately 17 and 33 min for HSA and rhEPO, respectively. In order to show method usability, the dependence between peak area and concentration of the discussed compounds was calculated for quantitative determinations. A linear dependence was obtained, described by $y = 727474.5x + 2575.9$, $R^2 = 0.999$ and $y = 0.4532x - 69.5$, $R^2 = 0.999$ equation for albumin in the range 0.15-1.25 mg/mL and rhEPO in the range 100-6500 IU/mL, respectively. High regression coefficients have confirmed the method usability for the assay of these proteins.

In the next step, the usefulness was checked of the method for separation of active substance from other proteins in four marketed products. Two of these products contained epoetin alfa, one contained epoetin beta and one contained epoetin omega. The tested products also differed in the composition of excipients. Various stabilizers of the active protein were used in their formulations, in order to minimize the EPO polymer formation process. Two pro-

![Figure 2. Chromatograms of: (a) rhEPO solution and (b) rhEPO solution after thermal stress, obtained with (A) SEC-HPLC and (B) RP-HPLC. Commentary: comparison of peaks for the solution before and after heating enabled determination of the retention time for aggregates, which in RP-HPLC conditions elute directly after the peak of erythropoietin.](image-url)
ducts contained human serum albumin, and two other products contained polysorbates 20 or 80. All products tested had the same dosage form, and solution for injections. The samples for tests were prepared by appropriate dilution with water, to obtain approximate concentrations of the active substance (the active substance activity in each product was adopted based on the manufacturer’s declaration). A pharmacopoeial standard, Erythropoietin BRP, was used in the study.

For all samples, the chromatograms (Figure 1) showed peaks at retention times corresponding to the erythropoietin standard i.e., approximately 33 min, and the peak area was proportional to the concentration. Albumin present in two tested products did not interfere, because it was eluted before the rhEPO peak, at approximately 17 min, similar to the reference substance. The adequate specificity of the developed method was shown for both proteins, and the usefulness of the method for qualitative and quantitative determinations of rhEPO in medicinal products stabilized with albumin was confirmed, which had been impossible with the SEC method.

Figure 3. Chromatograms of: (a) human serum albumin solution and (b) human serum albumin solution after thermal stress, obtained with (A) SEC-HPLC and (B) RP-HPLC. Commentary: comparison of peaks for the solution before and after heating enabled determination of the retention time for albumin aggregate, which in RP-HPLC conditions elute directly after the peak of albumin.
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By comparing the appearance of rhEPO peaks on chromatograms (Figure 1) obtained for the tested samples and the standard, a clear difference in peak formation for the sample containing epoetin omega was revealed. Near the EPO peak, a clear additional peak is visible, eluting directly after the rhEPO peak. By referring this result to the bibliography data (11), it was adopted that this additional peak is caused by an EPO glycoform in which the carbohydrate chain attached through the oxygen (O-glycosylation at Ser 156 position) is lacking. The above observation allows to conclude that using the presented chromatographic conditions, it is possible to evaluate the degree of EPO protein glycosylation, and thus to assess the homogeneity of the active substance.

As mentioned in the above introduction to this study, an aggregation process occurs during storage of medicinal products containing proteins. In the next phase of the study, the suitability of the presented method for identification of high molecular weight proteins derived from erythropoietin and albumin was checked. The samples containing aggregates were obtained by exposing standard solutions of both proteins to thermal stress conditions (75°C and 24 h). Their presence in the samples was confirmed using the pharmacopoeial method, SE-HPLC. The same samples were analyzed using the developed method, RP-HPLC. The resulting chromatographic profiles are presented in Figures 2 and 3. Comparison of peaks for the solutions of both proteins before and after heating enabled determination of the retention time for aggregates, which in RP-HPLC conditions elute directly after the peak of albumin or erythropoietin, respectively. By analyzing the chromatograms presented in Figure 1, obtained for the tested samples stabilized with albumin, the peaks corresponding to aggregates of both proteins were found. Based on the analysis performed, it was concluded that the developed method at this stage is satisfactory for the confirmation of the presence of high molecular weight proteins, but not for its quantity assay.

A comparison of chromatograms obtained for albumin free from the globular protein fraction and albumin containing globulins (Figure 4) allowed to identify the peak eluting before albumin.

Table 1. Precision of the method (repeatability), determined for a selected tested sample stabilized with albumin

<table>
<thead>
<tr>
<th>n = 6</th>
<th>globulin</th>
<th>HSA</th>
<th>HSA aggregate</th>
<th>EPO</th>
<th>EPO glycoform</th>
<th>EPO aggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak area</td>
<td>736069.3</td>
<td>43284344.8</td>
<td>241247</td>
<td>1285471.8</td>
<td>457494.5</td>
<td>72812.2</td>
</tr>
<tr>
<td>SD</td>
<td>4258.07</td>
<td>8861.91</td>
<td>2208.96</td>
<td>4295.47</td>
<td>2445.58</td>
<td>7237.36</td>
</tr>
<tr>
<td>RSD</td>
<td>0.58%</td>
<td>0.20%</td>
<td>0.92%</td>
<td>0.33%</td>
<td>0.53%</td>
<td>9.94%</td>
</tr>
</tbody>
</table>

By comparing the appearance of rhEPO peaks on chromatograms (Figure 1) obtained for the tested samples and the standard, a clear difference in peak formation for the sample containing epoetin omega was revealed. Near the EPO peak, a clear additional peak is visible, eluting directly after the rhEPO peak. By referring this result to the bibliography data (11), it was adopted that this additional peak is caused by an EPO glycoform in which the carbohydrate chain attached through the oxygen (O-glycosylation at Ser 156 position) is lacking. The above observation allows to conclude that using the presented chromatographic conditions, it is possible to evaluate the degree of EPO protein glycosylation, and thus to assess the homogeneity of the active substance.
Precision of the method was determined by establishing repeatability for a selected tested sample stabilized with albumin. The results are presented in Table 1. The resulting variation coefficients show that the implemented chromatographic separation conditions are appropriate for the determination of proteins present in the sample.

The presented method is specific and may be used for quality evaluation of medicinal products containing various erythropoietins formulated with different stabilizers. It enables assay of the active substance and albumin and determination of other proteins present in the investigate products. It is simple and does not require special preparation of sample for the test, and is characterized by a high precision for main peaks. Obtained results have confirmed the usability of the method.

**CONCLUSIONS**

Difficulties in assessing protein drug products by physicochemical methods are often related to the presence of large amounts of excipients that interfere with the detection and separation of the active ingredient. In the present RP-HPLC in gradient method a complete separation of the two proteins was obtained. The method was found to be useful for quantitative estimation of rhEPO in final drug preparations with albumin stabilization. The method was also capable of distinguishing products from different manufacturers.

**REFERENCES**


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