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Design of a Stable Cell Line Producing Recombinant Darbepoetin Alpha based on *CHO* Cells

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Abstract—A stable cell line that is based on *CHO* cells and produces 100 mg per liter of culture medium of recombinant darbepoetin alpha with a target glycosylated isoforms effective yield of about 30% has been selected. The expression product of the cell line was characterized and compared to the originator (Aranesp, Amgen). It was shown that the obtained preparation contained all isoforms characteristic of the originator. The created cell line can be used for the development of industrial cultivation and a purification scheme for recombinant darbepoetin alpha production.

Keywords: cell line, *CHO* cells, darbepoetin, erythropoetin, expression, transfection

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INTRODUCTION

A critical task in clinical medicine is the search for and implementation of therapeutic agents that can serve as an alternative to donor blood and its components. The use of drugs based on recombinant proteins for the treatment of anemia of various origins (nephrogenic, oncological, etc.) is more safe and efficient compared with transfusion therapy. Since the late 1980s, the implementation of recombinant human erythropoietin (rhEPO) in clinical practice revolutionized the approach to the correction of anemic conditions [1].

Darbepoetin alfa (DARB) is an analog of the human EPO-alpha, which has an increased biological half-life due to the increased degree of glycosylation when compared with the native and recombinant forms of erythropoietin [2]. The mechanism of action of both proteins is identical: they bind to the same receptor on the surface of the membrane and trigger the same chain of molecular intracellular interactions. However, DARB has higher stability in vivo , and the time of its biological half-life is three times higher than that of EPO-α [3]. Because of these properties, the use of DARB in medical practice allows less frequent administration to patients [4–6].

Protein glycosylation is the linkage of oligosaccharide chains to asparagine residues (N-glycosylation) or to serine/threonine residues (O-glycosylation). These terminal sugars are sialic acid residues [7, 8], which represent only one of the studied group of charged sugars, and their role is to protect glycoproteins from the action of proteases and to prevent aggregation [9].

DARB contains two additional glycosylation sites compared with EPO-alpha, and these sites significantly affected the properties of the protein, in particular the isoelectric point [2]. About 25–30 glycoforms of DARB form as a result of their production in mammalian cells, and 6–8 forms with the highest number of acidic groups (i.e. with the highest stability and activity) remain in the final product. Since a correct glycosylation profile is one of the main quality criteria for DARB, this parameter must be monitored at all stages of the production of the protein.

DARB isoforms also differ by the degree of sialylation and, as a result, by the charge; it is a prerequisite for their separation by isoelectric focusing gel and capillary electrophoresis.

Because DARB biological activity in vivo (including the effectiveness and biological half-life) depends on the extent and nature of glycosylation [2, 3, 10, 11], it is produced exclusively using eukaryotic cell cultures, mainly *CHO* [12, 13]. The benefits of *CHO* cells are correct glycosylation and their high rate of proliferation, easy cultivation, and sufficient characterization of the cells, which simplifies the registration of recombinant drugs in the relevant regulatory bodies [14].

Abbreviations: HPLC–High Performance Liquid Chromatography; DARB–darbepoetin alfa; ELISA–enzyme-linked immunosorbent assay; IEF–isoelectric focusing; CCF–cell culture fluid; CZE–capillary zone electrophoresis; PAGE-polyacrylamide gel; rhEPO–recombinant human erythropoietin; EPO–erythropoietin; *CHO*-Chinese hamster ovary cells; CMV–cytomegalovirus; SDS–sodium dodecyl sulfate.

A *CHO* cell culture suspension is the best choice for production [12, 15, 16]. The suspension culture allows the achievement of high cell densities (10^7–10^8 cells/ml medium) without the use of blood cells, which provides biological safety of the product and facilitates the subsequent stages of chromatographic purification [15, 17].

The goal of this study was the production of a stable cell line that produces recombinant darbepoetin alpha and characterization of the purified target protein in comparison with the original DARB-containing drug Aranesp (Amgen).

MATERIALS AND METHODS

pCI-dEPOs-2x expressing plasmid was produced based on pCI-neo plasmid (Promega, USA). In the first stage, a pCI-EF1α-dEPOs1 plasmid containing a synthetic dEPO gene was obtained as described previously [18]. Then the pCI-EF1α-dEPOs1 plasmid (recipient) was restricted with *SdaI* and afterwards by T4-polymerase for production of the blunt-ends of the linearized vector. After the inactivation of T4-polymerase by heating for 5 min at 80°C, the vector was restricted with *BglI*I. The resulting pCI-EF1α-dEPOs1 recipient vector contained on one side a sticky end, *BglII*, and the other end remained blunt. The pCI-EF1α-dEPOs1 donor vector was treated with restriction enzymes, *NruI* and *BglII*, and restriction products were separated by electrophoresis in 1% agarose gel (Helicon, Russia). Sites containing the promoter of *dEPOs1* gene, terminator, and S/MAR-element were cut; they were 2500 bp in size. The 5'-site of this fragment contained the sticky end after treatment with *BglII*, and the 3'-site contained the blunt end as a result of treatment with *NruI*. Vector pCI-EF1α-dEPOs1, containing the blunt *BglII*- end and a pCI-EF1α-dEPOs1 (*NruI* and *BglII*) fragment, was ligated to produce pCI-dEPOs-2x (pCI-EF 1α-dEPOs 1-dEPOs 1) expression plasmid. All restriction endonucleases and enzymes of nucleic acid metabolism were produced by Fermentas (Lithuania).

Proper assembly of the expression vector was verified by expanded restriction analysis with the participation of Evrogen (Russia). The nucleotide sequence of both the genes and the adjacent sites were verified by sequencing. In both cases, the results coincided with what was expected.

Culturing of the CHO-S cell line (Invitrogen, USA) was done in 125 and 250 mL Erlenmeyer flasks in a Multitron Cell shaker-CO_2-incubator (Infors HT, Switzerland) with a shaking speed of 125 rpm in an atmosphere of 5% carbon dioxide at a temperature of 37°C and humidity of 95%. Refreshment of the medium was performed every 2–3 days; the suspension was diluted in density to 0.3×10^6 cells/mL. We used a serum-free 2 Power CHO-CD medium (Lonza, Switzerland) supplemented with 4 mM alanyl-glutamine, 16 mM thymidine, 0.1 mM hypoxanthine (Invitrogen). Counting of the number of cells and analysis of their viability analysis was performed after staining with trypan blue (Panreac, Spain) in a Goryaev chamber using a CKX41 microscope (Olympus, Japan).

Transfection of the CHO-S cell line by the pCI-dEPOs-2x vector was done using a lipophilic agent FreeStyle MAX (Invitrogen). For this, *CHO-S* cells were replated one day prior to transfection to obtain a suspension with a cell density of 0.5×10^6 cells/mL. On the day of transfection, the cell density was determined and the cells were pelleted by centrifugation at 200 g for 10 min at room temperature using a 25 Allegra-R centrifuge (Beckman, Germany). The supernatant was removed by decantation, and the cells were suspended in FreeStyle™ CHO Expression Medium, containing 8 mM alanyl-glutamine (both reagents Invitrogen), to a final density of 1.0×10^6 cells/mL. Further transfection was performed in 6-well plates, according to the manufacturer's instructions (FreeStyle CHO-S Cells, Invitrogen, catalog number R800-07).

The selection of a stable clone producing DARB was performed by the limiting dilution method. Cell suspension was diluted 24 h after transfection by a 2 Power CHO-CD medium (Lonza) to densities of 10000, 3000, and 1000 cells/mL, and each dilution was added to a sterile solution of antibiotic G418 (Lonza) up to a final concentration of 500 mg/L. The suspension (0.1 mL) was placed in wells of 96-well plates (Corning, USA), using 30–40 plates for each dilution. Plates were placed in the CO_2 incubator for culturing in 5% carbon dioxide at 37°C and with 95% humidity for 20–30 days. Starting from 12 days of cell growth, they were controlled using microscope and marking wells, in which the growth from a single cell was observed. Since the number of cells in the well corresponds to the Poisson distribution, the probability of the absence of clonality (2 or more cells in one well) can be calculated based on the proportion of positive wells according to the formula:

$$P(n > 1) = 1 - \Sigma(e^{-\lambda}\lambda^n/n!),$$

where $\lambda = -\ln(P(0))$, the number of wells in which positive cell growth was observed; $n = 0.1$, the number of cells per well.

Individual clones were transferred to new 96-well plates when 80–100% confluence was reached. After 5 days CCF samples were analyzed for the presence of DARB. Clones with the highest productivity during cultivation were transferred into 24-well plates with 500 μL selective media (containing 500 mg/L G418 antibiotic) and cultured for 5 days. After the determination of cell viability, the clones were transferred to 125 mL flasks for suspension of the culture. Clone productivity was assessed according to the results of product cycle in flasks. Based on the criteria

of growth, viability, and productivity, the best clones were selected and a cell bank was prepared for them.

Determination of the DARB concentration was performed using a solid phase enzyme-linked immunosorbent assay (ELISA) and analytical affinity chromatography. The kit Erythropoietin-EIA-BEST (Vector-Best, Russia) was used for ELISA and calibration was performed using Aranesp (Amgen).

Analytical affinity chromatography was performed using HPLC system UltiMate3000 (Dionex, USA) on the Tricorn 5/20 column (GE Healthsare, USA) filled with Sepharose-2B (GE Healthsare) agarose with linked monoclonal antibodies to the recombinant EPO. The protein was identified using a fluorescence detector with the absorption wavelength of 270 nm and an emission wavelength of 340 nm. Step eluent conditions were performed as follows: 0–7 min eluent A (150 mM NaCl, 10 mM Na_3PO_4, pH 7.4), 7–12 min eluent B (3 M NaCl, 10 mM Na_3PO_4, pH 2.0), and 12–17 min eluent C. (2 M glycine, pH 2.0) (all reagents were produced by Sigma, USA). The flow rate was 0.5 mL/min. Aranesp was used as the standard for comparison.

SDS-PAGE electrophoresis under reducing conditions was performed according to Laemmli in a 12% gel, followed by staining with Coomassie-YA250 [19].

Immunoblotting. CCF proteins were separated by SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane with a pore diameter of 0.45 micron (Bio-Rad, USA) using Trans-Blot SD for semi-dry transfer (Bio-Rad). Rabbit polyclonal antibodies to human EPO (E2531, Sigma-Aldrich, USA), goat polyclonal anti-rabbit immunoglobulins conjugated with alkaline phosphatase (A3937, Sigma-Aldrich), and the chromogenic substrate for liquid alkaline phosphatase (Sigma) were sequentially used for immunostaining membranes. The European standard sample EPO-BRP (EDQM, France) and the drug Aranesp were used as standards. The intensity of bands on gels and on membranes was measured using Quantity One (BioRad).

Isoelectric focusing was performed in the gel with a pH gradient from 2.0 to 10.0 using GE Healthcare (Multiphor II, MultiTemp IV, EPS 3501 XL) with the following modes: prefocusing at a voltage of 1000 V, amperage of 24 mA, and power of 20 W for 30 min; entering of proteins into the gel at 500 V, 24 mA, and 20 W for 30 min; and separation of proteins at 1500 V, 24 mA, and 20 W for 60 min. Ampholytes Pharmalyte 3-10 (GE Healthcare) and Servalyt 2-4 (Serva, Germany) were used for gel formation. After the separation of proteins, the gel was placed in a fixation solution containing 0.7 M trichloroacetic acid (Sigma) and 0.16 M sulfosalicylic acid (Merck, Germany) and incubated for 45 min at room temperature. It was then washed in a solution containing 8% acetic acid and 25% ethanol at 90°C and subjected to immunostaining with antibodies to human EPO, as described above.

The isolation of protein from CCF was performed using preparative affinity chromatography in an AKTA Purifier system (GE Healthcare). The proteins were separated on a Pharmacia 10/100 (GE Healthsare) column filled with agarose-Sepharose-2B (GE Healthcare) with linked monoclonal antibody to recombinant EPO alpha.

Capillary electrophoresis with UV detection at λ 214 nm was performed using a Capel 105M (Lumex, Russia). Unmodified quartz capillaries with an external nylon coating (inner diameter 50 mm, total length 102 cm, effective length 93 cm, Agilent, USA) were used. Samples were hydrodynamically injected under pressure 30 mbar for 20 s. Solution containing 10 mM sodium chloride, 10 HM tricine, 10 mM sodium acetate, 7 M urea, and 2.5 M putrescine dichloride, with a pH of 4.5 (all reagents produced by Sigma), was used as background electrolyte. Instrumental monitoring, collection, and recording of electrophoretic data were done using the Multichrom 3.1.1594 system (Ampersand, Russia).

RESULTS AND DISCUSSION

Preparation of a cell line producing recombinant DARB.

We have previously shown that DARB expression during transient transfection of *CHO-S* cells can be increased by the use of a construction that contains twice the dose of a synthetic gene with optimized codon composition and is controlled by the chimeric CMV-EF1α promoter [18]. We decided to use such an approach in order to obtain a stable cell line producing DARB.

At the initial stage, the codon-optimized synthetic DARB gene was introduced into the pCI-neo vector, then the CMV promoter was replaced with the chimeric promoter, CMV-EF1α. Afterwards, the whole transcription unit containing the enhancer, promoter, DARB coding sequence, terminator and polyadenylation signal was duplicated in a pCI-dEPOs-2x vector (Fig. 1). Two S/MAR-containing 5'-regions of human β-interferon gene were added in the expression construct pCI-dEPOs-2x.

The pCI-dEPOs genetic construct obtained during the first stage was transfected into *CHO-S* cells using the lipophilic reagent Freestyle MAX. The selection of cells that had obtained the desired plasmid using the limiting dilutions method was started one day after transfection. After 10 days, cultures in the 96-well plates had their cell growth analyzed by light microscopy. Growth of cell colonies was found in 4% of wells for the dilution with 100 cells/well (1000 cells/mL), in 14% of wells for the dilution with 300 cells/well (3000 cells/mL), and in 59% of wells for the dilution with 1000 cells/well (10000 cell/mL).

Thus, for the dilution of 100 cells/well, we obtained a probability of the absence of clonality of less than

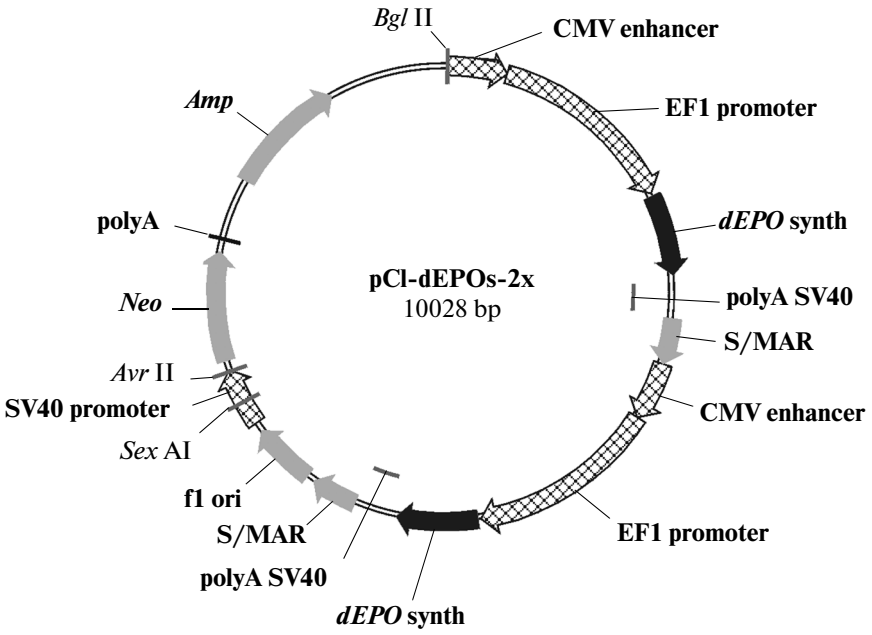

Fig. 1. pCI-dEPOs-2x construction containing a double pool of the *dEPOs* gene. *dEPO synth*-synthetic DARB gene with optimized codon composition; S/MAR-S/MAR-element of 5'-end of human β-interferon gene; CMV enhancer, enhancer of human cytomegalovirus immediate early genes; EF1 promoter, promoter of the alpha subunit of rat translation elongation factor 1.

0.001%. The probability was about 1.0% for the dilution of 300 cells/well and 22% for the dilution of 1000 cells/well. For further studies, we decided to use dilutions with a probability of clone absence of less than 5%, i.e. 100 and 300 cells/well. Based on the calculated evaluation criteria, 380 clones were selected for further analysis.

CCF from the 96-well plates in which the selection was performed was used for the primary screening of clones. Analysis of the productivity of clones for DARB was performed by ELISA. Thirty clones with maximal expression levels were selected based on ELISA results (data not shown).

Clones with the best growth characteristics were transferred to 24-well plates for a more precise analysis of the productivity. Productivity of clones was measured by ELISA and HPLC analysis after three days of culture in plates (data not shown). Then, the 17 best of the analyzed clones were transferred to a 125 mL flask and productivity was estimated for each clone after 4 days of cultivation in a closed volume. At this stage, based on several characteristics (growth rate and productivity) the best clones were 13, 17, 23, 28, and 29 (Table 1).

A repeat experiment for determining the productivity of the 5 best clones was performed by culturing in 125 mL flasks for 7 days in triplicate. The result of the first measurement, which indicated that clone 28 was the best based on growth and productivity, was confirmed. The concentration of DARB in CCF reached 101.7 mg, and the specific productivity of the clone was 2.14 pg/cell/day (Table 2).

Thus, stable clones producing DARB were produced during the first stage of the study, and the best clones were selected based on their growth characteristics and productivity. The next task was characterization of the target protein produced by cells of the selected clones via different methods.

Characterization of isoform composition of recombinant DARB. CCF samples selected on the 7th day of cultivation in 125 mL flasks were analyzed by Western blotting for additional quantitative determination of DARB and preliminary evaluation of the quality of the product synthesized by five clone-producers. The European standard sample EPO-BRP and final dosage form of DARB- drug Aranesp were used as control samples (Fig. 2).

It is known that DARB has five binding sites of the oligosaccharide chains and that the molecular weight of the protein may vary from 18.3 to 37.0 kDa depending on the completeness of glycosylation reactions in *CHO* cells [2]. Western blotting allows for only a preliminary analysis of the glycosylation degree of protein synthesized by clone-producers because of the large differences in the molecular weight range of DARB glycoforms [20].

According to the results of western blotting of CCF (see Fig. 2), all 5 clone producers secreted several DARB alpha isoforms; however, protein glycosylation is very different. Clones 23 and 29 produced a lower amount of the target protein (see Fig. 2, bands *7* and *9*). Cells of clone 17 had a productivity comparable to clone 28 (see Fig. 2, bands *6* and *8*, respectively), but they produced more light and less glycosylated iso-

Table 1. DARB productivity of best initial clones cultured in flasks for 4 days

Clone number*	Concentration of DAR, mg/mL	The number of live cells, $\times 10^6$ cells/mL	Doubling time, h
5	13.4	0.40	96.0
8	2.6	0.84	46.4
9	17.0	0.50	72.6
11	7.7	0.44	84.4
13	28.3	2.03	28.7
15	7.7	0.71	52.5
17	16.0	0.40	96.0
18	8.2	0.81	47.6
20	11.5	0.71	52.5
21	5.4	1.00	41.3
23	30.1	2.12	28.2
28	43.0	3.14	24.2
29	17.7	1.90	29.6
3	2.7	0.12	–
6	2.6	0.07	–
14	2.6	0.20	–
22	5.6	0.20	–

* The best clone, based on growth characteristics and productivity, is marked in gray; (–) indicates the absence of division.

Table 2. Repeat determination of the productivity of the five best initial clones cultured in flasks for 7 days

Clone number	Concentration of DAR, mg/mL ($x \pm \sigma$)	The number of live cells, $\times 10^6$ cells/mL ($x \pm \sigma$)	Doubling time, h ($x \pm \sigma$)	Specific productivity, pg/cell/day ($x \pm \sigma$)
13	44.9 ± 2.5	9.3 ± 0.3	17.3 ±1.1	1.38 ± 0.35
17	48.2 ± 3.1	7.9 ± 0.5	18.1 ±1.2	1.74 ± 0.24
23	27.9 ± 2.4	6.1 ± 0.4	19.5 ±1.6	1.31 ± 0.33
28	101.7 ± 9.4	13.6 ± 0.7	15.8 ±1.4	2.14 ± 0.10
29	22.2 ± 1 .3	5.8 ± 0.3	19.8 ±1.6	1.09 ± 0.11

* The best clone, based on growth characteristics and productivity, is marked in gray.

forms. The product secreted by clone 13 is close to the product of clone 28 by molecular weight, but it had significantly lower productivity (see Fig. 2, bands *5* and *8*, respectively). Clone 28 was selected for further study based on the obtained data for productivity and glycosylation degree of the product.

The western blot method, used during the initial characterization of clones, does not provide information about quantitative proportion and the ratio of the different glycosylated isoforms; at the same time, an accurate determination of the glycosylation profile of the recombinant DARB was needed.

Isoelectric focusing (IEF) in SDS-PAGE, followed by transfer to the nitrocellulose membrane and staining with antibodies specific for the desired protein (IEF combined with western blot) [21], is often used

for estimation of the glycosylation profile of the recombinant proteins in CCF. The method of capillary zone electrophoresis (CE) [22], which requires preparation of CCF samples for purification of the targeted protein, for example, by immunoaffinity chromatography used for quantitative measurement of each of the isoforms of the recombinant protein. The selection of these methods to control N-bounded glycosylation of DARB in this work was also due to the fact that these methods are recommended by the European Pharmacopoeia (5th, 6th, and 7th Editions) for analysis of all types of DARB glycoforms [22].

The investigation of the isoform composition of DARB was performed in CCF obtained by culturing clone 28 in flasks in an enclosed volume. Samples were taken on the 7th day of cultivation and analyzed by the IEF method via membrane staining with antibodies specific to EPO (Fig. 3).

According to IEF, the CCF of clone 28 contains at least 10 isoforms of DARB, which differ by their degree of sialylation and, hence, by the charge; this is a prerequisite for separation by the used method of analysis (see Figs. 3, *1*). As can be seen from Fig. 3, the investigated CCF contains the target highly sialylated DARB isoform present in the comparison sample. Consequently, the cells of the obtained clone-producer performed correct glycosylation of the produced recombinant DARB.

A further task was to determine the ratio of DAR isoforms in CCF of clone 28. For this purpose we used the CZE method. Before performing this analysis, CCF samples were purified by immunoaffinity chromatography and then concentrated up to a final protein content of at least 1 mg/mL. The results of the analysis of chromatograms obtained by the separation of DARB isoforms using CZE are shown in Fig. 4.

Graphs in Fig. 4 show 11 peaks corresponding to the various DARB glycoforms and were detected in the affine eluate obtained from CCF clone 28. The comparative drug, Aranesp, contains 6 glycosylated forms of the protein (the corresponding peaks are numbered in Fig. 4a and 4b.) The investigated compound contains the same DARB isoforms as Aranesp and the same ratio of these isoforms. Additional glycosylated forms of DARB that are present in the affine eluate with different sialylation levels can be removed in subsequent stages of chromatographic purification. At this stage of research, we confirmed that the isoform composition of recombinant DARB produced by cells of the producer clone fully matches the reference drug.

Thus, as a result of the studies, a stable (for more than 100 passages) cell line that produces recombinant DARB was selected based on a previously produced genetic construct with a double dose of a synthetic gene with optimized codon composition and *CHO-S* cells. A clone with optimal growth characteristics and a productivity of at least 100 mg target protein per 1 liter medium was selected by the limiting dilution

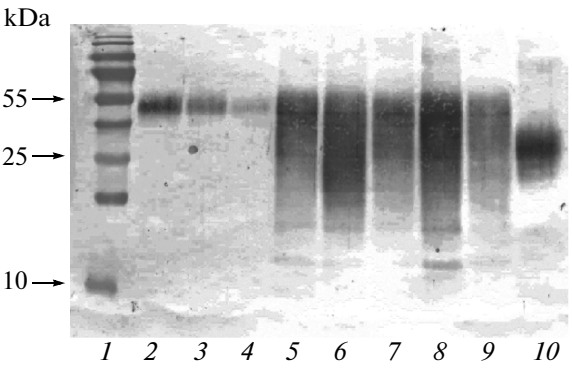

Fig. 2. Western blot of CCF samples containing DARB: band *1* shows the molecular weight markers (Fermentas); bands *2*, *3*, and *4* show the drug Aranesp at 100, 50, and 10 ng, respectively; bands 5, 6, 7, 8, and 9 show the CCF of clones *13*, *17*, *23*, *28*, and *29*, respectively; band *10* shows EPO-BRPat 50 ng.

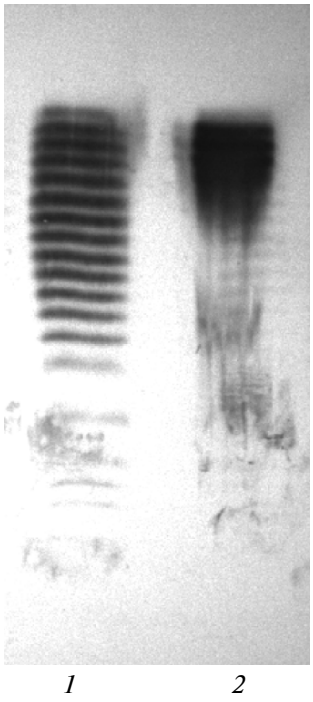

Fig. 3. Isoelectrohoretic separation of DARB isoforms (staining with antibodies to EPO): band *1* shows the CCF of clone 28, obtained on the 7th day of cultivation in a flask; band *2* shows the drug Aranesp at 100 ng.

method. The isoform composition of recombinant DARB was characterized using IEF and CZE methods, and the correspondence of the target protein and purified DARB preparation (Aranesp) by the glycosylation profile was established. The produced cell line corresponds to all modern requirements of producers of biopharmaceuticals based on recombinant proteins; in particular, it can be cultured in suspension mode on a medium of certain chemical compositions without

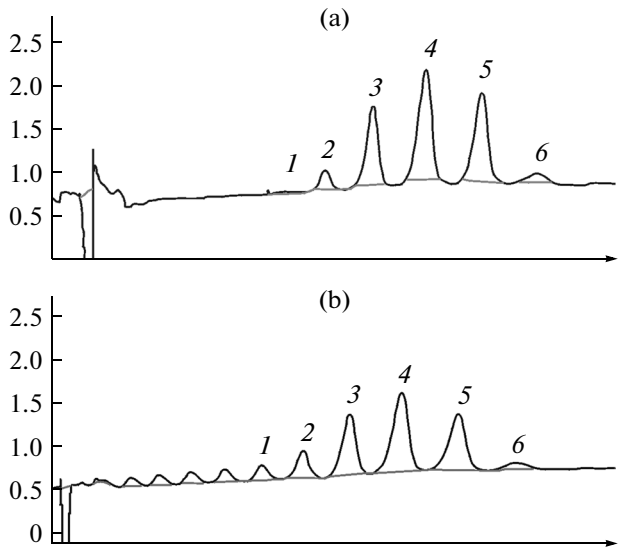

Fig. 4. Analysis of samples containing DARB by CZE: a, Aranesp; b, affinity eluate obtained from CCF of clone 28; *1–6*, DARB forms with various degrees of sialylation.

components of animal origin. This cell line can be used for designing an industrial cultivation scheme for the production and purification of recombinant DARB.

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