ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1455–1469

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

In vitro bioassay for human erythropoietin based on proliferative stimulation of an erythroid cell line and analysis of carbohydrate-dependent microheterogeneity

Ulf Hammerling[a,*], Richard Kroon[a], Tore Wilhelmsen[b], Lars Sjödin[a]

[a] *Division of Biotechnology, Medical Products Agency, P.O. Box 26, S-751 03 Uppsala, Sweden*
[b] *The Norwegian Medicines Control Authority, Sven Oftedals Vei 6, N-0950 Oslo, Norway*

Received for review 3 January 1996

Abstract

The human erythroleukemia cell line TF-1 was employed for the determination of proliferative stimulation induced by recombinant human erythropoietin (rhEpo). Potencies of various intact and sugar-trimmed rhEpo preparations were estimated using the International Standard for Human r-DNA-derived Epo (87/684) as a reference for activity. The cellular response was measured in a multi-channel photometer using a colorimetric microassay, based on the metabolism of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, by viable cells. The linear part of the log dose–response relationship encompassed 2.5–90 pM and activity of rhEpo preparations was measured at doses between 3 and 60 pM. The assay was designed as a parallel line test, using three or four concentrations for potency determinations, which fulfills pharmacopoeial requirements for assay validity. Inter-assay relative standard deviation varied between 4.1% and 12.6% and most assays revealed potencies with limits of error within 87–113%. In order to acquire an additional means for an efficient probing of physiologically relevant features of rhEpo, a luminiscence-dependent Western detection system, based on a combined isoelectric focusing/sodium dodecyl sulphate–polyacrylamide gel electrophoresis separation, was established. As opposed to conventional electrophoresis the two dimensional approach enabled the disclosure of minor truncations in the rhEpo-attached glycan moieties using picomolar quantities of the hormone. Moreover, the separated isoforms of rhEpo were quantified by computer-assisted densitometry and compared with the 87/684 standard. Accordingly, results obtained by the cellular response were balanced against the general pattern observed and the relative amounts of separated rhEpo isomers as determined by the quantitative Western analysis. The method described should be suitable for potency assessments of pharmaceutical formulations of rhEpo.

Keywords: Bioassay; Erythropoietin; Sialic acid; TF-1; Two-dimensional analysis

* Corresponding author. Tel.: (+46) 18-17-47-41; fax: (+46) 18-54-85-66.

PII S0731-7085(96)01799-2

1. Introduction

Erythropoietin (Epo) is the principal regulator of erythrocyte production in mammalian species. In the adult, Epo is mainly produced in the kidney and interacts with a specific receptor on responsive precursor cells in the spleen and bone marrow, preferably erythrocyte progenitors, but megakaryocytes are also described as targets for this hormone [1–5]. The expansion/maturation of committed precursor cells in vivo is accomplished in conjunction with other cytokine components, such as IL-3, GM-CSF and the stem cell factor [6,7]. Several studies, using erythroleukemia cell lines or immortalized erythroblasts, demonstrate that Epo can induce signals leading to both proliferation and differentiation [8–10].

Human Epo is a heavily glycosylated protein and structural characterization has demonstrated a product of 34 000–40 000 Da, of which about 18 400 Da is contributed by the unmodified polypeptide. Two intra-chain disulphide linkages in the mature 166 amino acid hormone have been delineated [11–13]. Further studies have revealed the presence of one serine-linked and three asparagine-linked carbohydrate chains on Epo [14–16]. The N-linked glycan chains are of the complex type and encompass bi- tri- and tetra-antennary configurations with the latter type as the predominant isomeric structure. Most oligosaccharide branches have terminal *N*-acetylneuraminic acid (also denoted sialic acid) residues [17,18] and extensive sialidation, at the nonreducing ends, is pivotal for persistent action of Epo in vivo [19–22]. Moreover, the efficacy of hEpo also appears to reside on a high degree of branching structure of sugar chains [23]. When determined by conventional in vitro systems this important aspect escapes appropriate detection; the stimulative potency is rather enhanced upon release of terminal sialic acid residues [24–26]. However, Epo molecules devoid of glycan modification exhibit an increased propensity to polymerize which results in reduced activity [23,26–28].

The anaemic malady in patients with end-stage renal desease, as a result of a deficient endogenous Epo production, can be corrected by administration of rhEpo [29–31]. The isolation of hEpo-encoding cDNA clones and their subsequent expression in eukaryotic cells has enabled the production of unlimited amounts of this hormone [32–35]. Recombinant human Epo is most commonly obtained from transfected clones of Chinese hamster ovary (CHO) cells but other sources, such as recombinant baby hamster kidney (BHK) cells, also exist. The glycan structures of such molecules generally show good agreement with that of native hEpo but certain dissimilarities may occur [18,36,37]. Moreover, differences and variations in manufacturing procedures could also result in a suboptimally modified hormone [38]. Hence, the reliable determination of hEpo activity, with respect to the efficacy in vivo, is of great importance for the control of this substance.

The present authors have developed a combined biological/physicochemical assay for human Epo. The cellular system is based on non-isotopic recording of proliferative stimulation using the human erythroleukemia cell line TF-1 as an indicator [39,40]. It is designed as a parallel line assay using three or four concentrations for potency determinations which fulfil pharmacopoeial requirements for assay validity. The data obtained prove a capacity to reveal small differences in hEpo potency. Moreover, the determination of physiologically relevant hEpo molecules, an aspect that is not readily appreciated in previously described in vitro bioassays for this substance, has been addressed. A luminiscence-based two-dimensional Western method, that could reproducibly identify several isomeric forms of rhEpo, was established. Potency readings, obtained by cellular stimulation with rhEpo, were thus balanced against quantitative densitometry analyses of the separated glycoprotein isoforms. In a panel of such assays, using sugar-trimmed hormone, evidence was furnished that the bioassay can discriminate between functionally satisfactory and suboptimal rhEpo preparations as regards structural features pivotal for the pharmacokinetic properties. The method presented has been evaluated with regard to specificity, precision and reproducibility. Both parts of the assay system—cell culture stimulation and physicochemical characterization—operate in the picomolar range, which enables potency determinations of limited amounts of hEpo.

2. Materials and methods

2.1. Cells and culture conditions

The human erythroleukemia cell line TF-1 [39] was obtained from Dr. T. Kitamura, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA. TF-1 was maintained in RPMI 1640 medium (The National Veterinary Institute, Uppsala, Sweden), supplemented with 10% foetal calf serum (FCS), 4 mM L-glutamine, 100 IU ml^{-1} benzylpenicillin, 100 $\mu g\ ml^{-1}$ streptomycin (Labassco AB, Partille, Sweden), 20 $\mu g\ ml^{-1}$ ferro-saturated transferrin and 10 μM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). In addition, 12 ng ml^{-1} rhGM-CSF and 3 IU ml^{-1} rhEpo were included in the culture medium. Cells were maintained in a humid atmosphere, with a composition of 5% CO_2/95% air in 50 ml (25 mm^2) polystyrene tissue culture flasks (Nunc A/S, Roskilde, Denmark) and split three times per week. Before use in an experiment the cells were washed three times in ice-cold phosphate-buffered saline (PBS) (The National Veterinary Institute, Uppsala, Sweden) and resuspended in assay medium, which is equivalent to culture medium with the following modifications: FCS and β-mercaptoethanol excluded; Fe^{2+}-saturated transferrin and protease-free bovine serum albumin (BSA) (Sigma Chemical Co.) added to concentrations of 100 $\mu g\ ml^{-1}$ and 2 mg ml^{-1} respectively. The aforementioned cytokine cocktail (rhGM-CSF/rhEpo) was not included.

2.2. Reagents

A 6.0 mg ml^{-1} sterile stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) was prepared by dissolving MTT in PBS and passing it through a 0.2 μm membrane. This solution was stored for up to 1 month in a light-protected container at +4°C.

The International Standard for Human Erythropoietin, rDNA-derived (87/684), was provided by the National Institute for Biological Standards and Control (Potters Bar, Herts, UK). This preparation was stored in sealed ampoules at −20°C and transferred to −70°C in small aliquots following reconstitution in assay medium. Purified preparations of rhEpo, expressed in CHO cells, were provided by Cilag AB (Sollentuna, Sweden), Boehringer-Mannheim Scandinavia AB (Bromma, Sweden) and from a local pharmacy. Enzyme digestions were conducted on concentrated preparations of rhEpo, henceforth referred to as AI and BI.

Neuraminidase from Vibrio cholerae, *N*-glycosidase F (recombinant Flavobacterium meningosepticum) and recombinant human interleukin-6 were purchased from Boehringer-Mannheim GmbH (Mannheim, Germany). Insulin (Actrapid®) and somatotropin (Norditropin®), both of human type, were from Novo Nordisk A/S (Bagsvaerd, Denmark). Interleukin-2 and interleukin-3 (both recombinant, human) were gifts from EuroCetus B.V. (Amsterdam, The Netherlands) and from Sandoz Pharma Ltd. (Basle, Switzerland) respectively. Human interleukin-4 was obtained from Promega Co. (Madison, WI). Becton Dickinson Labware (Bedford, MA) and Genzyme Co. (Cambridge, MA) were suppliers of rabbit anti-hIL-3 and rabbit anti-hGM-CSF respectively. Peroxidase-conjugated goat anti-rabbit immunoglobulins were obtained from Dako A/S. Substances and antibodies were reconstituted and employed according to specifications by the respective supplier.

High titer polyclonal antibodies against hEpo were raised in rabbits, by immunization with a highly purified antigen, according to standard procedures. One of the sera obtained was used in cellular experiments (24- to 1200-fold dilutions) and Western analyses (1/5000).

2.3. Enzymatic digestions

2.3.1. Neuraminidase

Samples of rhEpo, 1 nmol each, were exposed to either 1.0×10^{-3} or 10.0×10^{-3} units of neuraminidase (Boehringer-Mannheim GmbH) and incubated at 37°C for various time periods of up to 15 min in a buffer solution recommended by the enzyme vendor. The reaction mixtures were subsequently frozen separately in small aliquots (−70°C), following a ten-fold dilution in assay

medium. The digested products corresponded to rhEpo molecules with minor, intermediate and complete removal of terminal sialic acid residues, referred to as BIpl, BIp2 and BIa or AIa respectively.

2.3.2. N-glycosidase

rhEpo (2 nmoles) was partially digested with peptide-*N*-glycosidase F in a solution composed as suggested by the supplier. Following treatment for 6 h at 37°C the sample was stored at −70°C, subsequent to a ten-fold dilution in assay medium.

2.4. Western analysis

The two-dimensional Western analyses were conducted on a Mini-Protean II® system (Bio-Rad Laboratories, Inc., Richmond, CA) using electrotransfer [41,42] essentially as outlined [43]. Pivotal details were as follows. Samples (1–3 pmoles) of rhEpo were loaded on 55 mm capillaries containing polyacrylamide and carrier ampholytes (Pharmalyte®; Pharmacia AB, Uppsala, Sweden). After a prefocusing sequence, the samples were separated at 400 V for 18 h. The cylindrical gels were placed on a 12% sodium dedecyl sulphate (SDS)–polyacrylamide gel (1.0 mm thickness) and the electrophoresis was run at 175 V for 60 min subsequent to 15 min at 100 V. Proteins were transferred to 0.45 μm nitrocellulose membranes (BA 85; Schleicher and Schuell, Dassel, Germany) using a trans-blot cell (Bio-Rad Laboratories, Inc.). Membranes were blocked in PBS containing 0.1% Tween-20 and 5% nonfat dry milk [44,45], exposed to a rabbit anti-hEpo serum and subsequently to purified horseradish peroxide-conjugated anti-rabbit Ig Fc-domain antibodies (Dako A/S, Copenhagen, Denmark), with several washes in between. Spots, corresponding to isomeric forms of rhEpo, were identified on exposed Kodak-XAR film (Eastman Kodak Inc, Rochester, NY) subsequent to luminescence activation (ECL, Amersham International plc, Bucks, UK). Prestained marker proteins (low range; Bio-Rad Laboratories, Inc.) were employed as standards for molecular weight. The pH gradients, across both types of isoelectric focusing (IEF) gels used, were recorded with a miniaturized pH-sensitive electrode (Microelectrodes, Inc., Londonderry, NH).

Single-dimension sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) separations, including Western transfer and protein identification, were preformed as described above after the IEF step, using 1 pmole of rhEpo in each lane.

2.5. Assay conditions

2.5.1. General outline

Solutions of rhEpo were subjected to serial dilutions in polypropylene tubes, using assay medium, transferred in quintuplicate (90 μl each) to flat-bottomed microtiter plates (Nunclon Delta SI, A/S Nunc) and mixed with an equal volume of prewashed cell suspension (2.0×10^5 cells per ml). Plates were then incubated at 37°C, as described above, for 48 h before the addition of 20 μl per microwell of the MTT chromogene substrate stock solution. Reduction of MTT is proportional to the number of viable cells [46,47]. Following incubation, in the same environment, for another 4 h, reactions were terminated with 70 μl of the acidified SDS solution (pH 2.7) per microwell [47]. Formazan crystals were dissolved by an overnight incubation at room temperature, protected from light exposure. Following a brief agitation, the absorbance values were determined on a multi-channel spectrophotometer (SLT 340 ATTC, SLT-Labinstruments, Salzburg, Austria). The plates were read at 570 and 620 nm as test and reference wavelengths respectively. The arithmetic mean values of three readings (OD_{RES}), according to the following formula: $OD_{RES} = -(OD_{570} - OD_{620}) - (OD_{570BLANK} - OD_{620BLANK})$, were used for further processing.

2.5.2. Dose–response analysis

Two-fold dilutions of Epo were prepared, transferred to microtiter plates and mixed with TF-1 cells for stimulative response analysis, as described above. When antibodies directed against epitopes on hEpo or other cytokines were included in the experiment, a 30 min preincubation step was included before the addition of indicator cells.

2.5.3. Potency determination

The International Standard for Human Recombinant Erythropoietin (WHO 87/684) was employed as a reference for potency. The cytokine preparations (test and reference) were diluted in assay medium in two separate series, each comprising five different concentrations of Epo (two-fold dilutions). These concentrations were selected from the linear range of the log dose–response curve. Quintuplicate samples were loaded on microtiter plates and treated as delineated in Section 2.5.1.

2.6. Densitometry

Captured signals on X-ray films were read on an XRS-6cx flat bed scanner (X-Ray Scanner Corp., Torrance, CA). Digitized images were processed using a SPARC Classic workstation (SUN Microsystems Inc., Mountain View, CA) and the Bioimage software modules for 1-D and 2-D gel analysis (Bio Image Inc., Ann Arbor, MI).

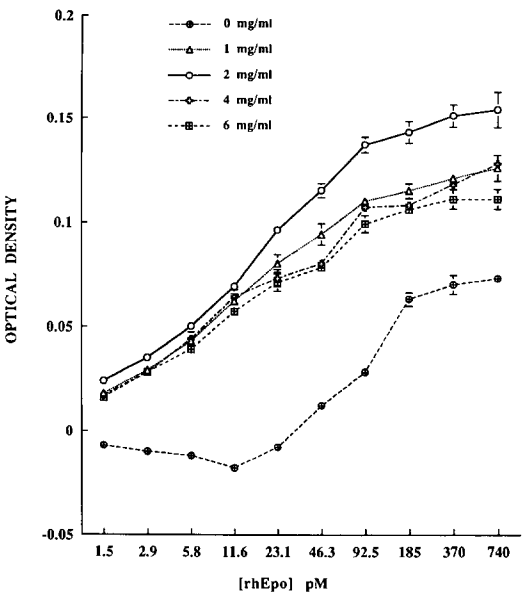

Fig. 1. Stimulation of TF-1 in the presence of various concentrations of BSA. Effect of increasing amounts of BSA, in the assay medium, on the rhEpo-stimulated dose–response relationship. Growth was measured by the MTT colorimetric microassay. Each point shows the mean of five replicates and bars represent ± standard error of the mean (S.E.M.). Cells were allowed to grow for 48 h, following addition of indicated concentrations of rhEpo, before the addition of MTT solution and a subsequent arrest of the enzymatic reaction 4 h later. Optical absorbance values were calculated as outlined in Section 2.

2.7. Data processing

The potency of each test preparation was calculated by analysis of variation for a three- or four-dose assay according to principles described in the European Pharmacopoeia [48]. The statistical weight is defined as the reciprocal value of the variance of the $\log_{10}$ potency estimate [48]. The index of precision was obtained by dividing the standard deviation of the responses by the negative slope of the $\log_{10}$ dose–response relationship [49]. After testing for homogeneity, the weighted log potency estimates were employed for combination of results from separate assays. The RSD (relative standard deviation) is defined as the ratio between the standard deviation of responses and the arithmetic mean value of these responses [48].

3. Results

3.1. Cellular proliferation studies

Growth of the erythroleukemia cell line TF-1 is dependent on either IL-3 or hGM-CSF and these factors have synergistic effects on proliferation, whereas viability can be sustained by Epo alone for a limited period only [39]. During the course of studies on stimulation with rhEpo it was found that reproducible cellular responses to this hormone, in the present assay system, required the presence of both hCM-CSF and hEpo in continuously growing cultures.

Erythroleukemic primary cells and cell lines, at the stage of haemoglobin synthesis, are known to express surface transferrin receptors and to require the cognate factor for proliferation [35,50]. Several tests revealed an optimum concentration of 0.1 mg ml^{-1} Fe^{2+}-saturated transferrin (not shown) which was consistently employed in subsequent tests and assays. Furthermore, although the TF-1 cells generally reacted with a proportional growth upon exposure to rhEpo, when FCS was

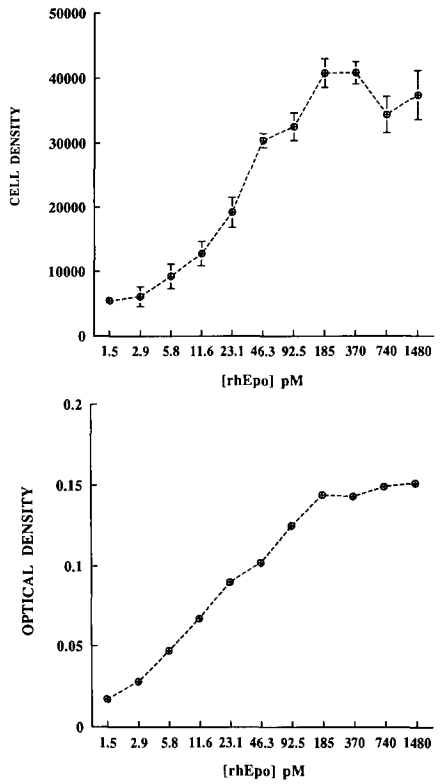

Fig. 2. Comparison between cellular count and photometric formazan reading upon exposure of TF-1 cells to rhEpo. Points on the curves represent the arithmetic mean of five replicates and vertical bars indicate ±S.E.M. values. Final values were processed as described in Section 2. Upper panel: growth was measured using an electronic cell counter. Numbers on the ordinate refer to cells per microwell. Lower panel: cellular stimulation was recorded by the MTT colorimetric method.

included in the test medium, the responses tended to vary between separate but identically conducted experiments. Moreover, the presence of a poorly defined component during potency determinations could possibly interfere with the cytokine resulting in underestimated activities. However, a considerable loss of biological activity upon dilution of polypeptides to low concentrations in protein-poor solutions, due to adsorbance to plastic surfaces, has been reported [51,52]. Therefore, various concentrations of protease-free BSA in the assay medium were tested. The most obvious finding was that the addition of BSA provided an efficient competitor for non-specific binding sites for rhEpo (Fig. 1). A concentration of 2 mg ml^{-1}, which resulted in the best index of stimulation, was adopted henceforth.

Various other parameters including length of incubation and cell density, were also tested using rhEpo as a stimulator of growth (not shown). Reproducible responses, that discriminated between small dose increments, were obtained by a 48 h incubation period using 100 000 TF-1 cells ml^{-1}. The threshold concentration of rhEpo for stimulation of cell proliferation, using the specified conditions (see Section 2), was below 2 pM and the proliferative stimulation displayed linearity with rhEpo concentrations in the range 3–90 pM, equivalent to about 10–400 mIU ml^{-1} (Figs. 1, 2 and 5).

Stimulation of cellular growth, upon exposure to rhEpo, was assessed by cell counting. The dose–response relationship, recorded with this method, showed a high resemblance to that obtained with MTT microcolorimetry, thus confirming that the metabolic MTT reduction is proportional to the TF-1 cell number (Fig. 2). The dynamic range (relative response), obtained

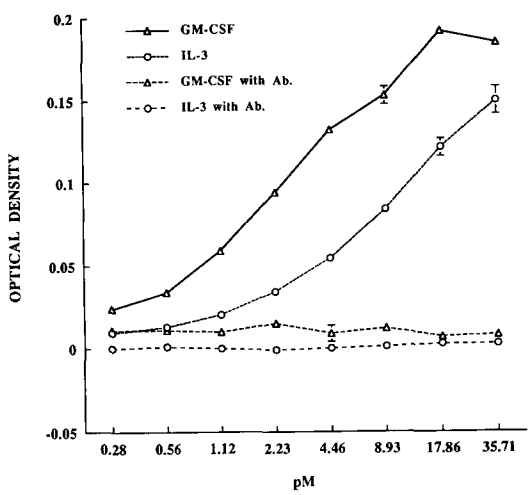

Fig. 3. Response of TF-1 to GM-CSF and IL-3 and suppression of stimulation with specific antibodies. Proliferation was determined by reading formazan absorbance. Doses were tested in quintuplicate and ±S.E.M. values are indicated by vertical bars. Serial dilutions of indicated cytokines in the absence or presnece of specific antibodies (0.15 μg of α-GM-CSF and 0.30 μg of α-IL-3 per microchamber respectively). Optical readings wer calculated as delineated in Section 2.

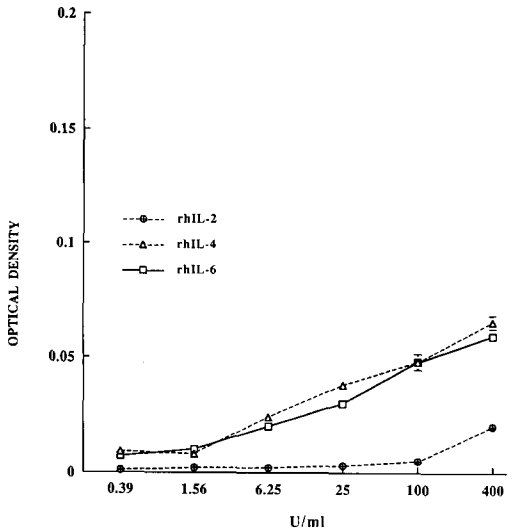

Fig. 4. Effect of rhIL-2, rhIL-4 and rhIL-6 on TF-1. Bioactivity of the indicated cytokines was measured with the MTT colorimetric assay. Vertical bars denote ± S.E.M. of quintuplicate samples. Recorded data were processed according to outlines in Section 2.

by cell number counting, was comparable with that of the formazan absorption values but the latter end-point described a wider linearity, which was especially marked at low hormone concentrations.

3.2. Tests of assay selectivity

The TF-1 cell line is reported to react by growth upon exposure to hGM-CSF, hIL-3 and hEpo [39]. When compared on a molar basis, both rhGM-CSF and rhIL-3 stimulated proliferation at lower concentrations than rhEpo and proliferation was most pronounced for rhGM-CSF. When these cytokines were subjected to preincubation with antibodies, with specificity for the respective substance, no cellular reaction was recorded (Fig. 3). Moreover, the response to rhEpo was not suppressed by the presence of such antibodies (not shown). The TF-1 cells were found to react at fairly low doses of rhIL-4 and rhIL-6. However, the indexes of these responses, which were very similar among themselves, were considerably weaker than that of rhEpo (Fig. 4). Hence, the marked deviation from parallelism provides a prompt discriminator in this respect. Furthermore, when analysed over a broad dose range, rhIL-2 was only able to elicit a faint induction at the highest dose tested (400 IU ml^{-1}; Fig. 4).

The assay system was further tested using a panel of substances known to elicit growth stimulus in various target cells. Below a micromolar level, insulin, human growth hormone and substance P did not produce any measurable impact on TF-1 and hG-CSF was negative throughout all doses examined (not shown).

Finally, the rhEpo substance was subjected to pretreatment with various dilutions of a polyclonal antiserum against this hormone. A complete extinction of the TF-1 stimulation, over a wide rhEpo concentration range, was obtained and more diluted antibodies resulted in a gradually released suppression of the proliferative response (Fig. 5).

3.3. Potency determinations

The erythropoietin assay is conducted in 96-well microtiter plates using a multi-channel pho-

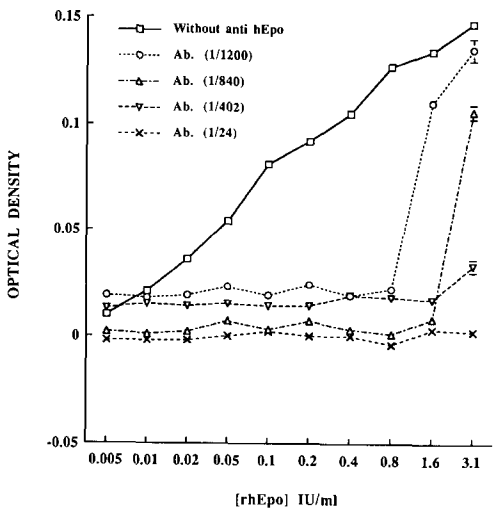

Fig. 5. Neutralization of rhEpo-mediated stimulation of TF-1 cells by preincubation with an anti-hEpo antiserum. Growth was measured by the MTT colorimetric microassay. Each point shows the mean of five replicates and bars represent ± S.E.M. rhEpo was either untreated or preincubated (30 min) with the antiserum at dilution factors indicated. Details on data processing and the anti-hEpo serum used are given in Section 2.

Table 1
Estimated relative potencies of three different dilutions of rhEpo as determined by the micro-colorimetric assay

Potency		Limits of error[a] (%), $P = 0.95$	Index of precision[a]	RSD[a]
Nominal	Estimated			
0.70	0.72	89–113	0.066	
0.70	0.73	88–113	0.083	
1.00	0.86	89–112	0.076	
1.00	0.94	85–117	0.092	
1.00	1.11	87–115	0.091	
1.00	1.00	89–112	0.065	
1.00	0.81	90–112	0.074	
1.00	1.11	89–113	0.080	
1.00	1.09	91–109	0.061	0.126
1.30	1.45	90–111	0.070	
1.30	1.12	91–110	0.065	
1.30	1.22	87–114	0.089	
1.30	1.26	91–110	0.052	0.109

[a] For definitions, see Section 2.

tometer as a recorder for the absorbance of blue formazan. This approach enables a fast processing of several separate preparations of the hormone during 72 h. The method is designed as a parallel line assay, employing five concentrations from the linear response range. In each experiment, quintuplicate samples were included. Data obtained were subjected to analysis of variance to give estimates of relative potency as well as assessments of linearity and parallelism, using the International Standard for Human Recombinant Erythropoietin (WHO 87/684) as a reference for activity [48,49,53].

In order to evaluate assay accuracy and precision, the potencies of a rhEpo preparation, diluted to known concentrations, were determined. The results are summarized in Table 1. Deviations of estimates from nominal potencies were small and the limits of error were generally very narrow. Moreover, the index of precision, which is inversely proportional to intra-assay precision, was consistently below 0.1. Typical examples of such determinations are depicted in Fig. 6. As the recordings of separate quintuplicate samples, within each dosage group, were generally very close, minute deviations from linearity or parallelism sometimes became statistically significant. When such results were obtained, the four-dose analysis of variance was replaced by a three-dose equivalent. Following tests of homogeneity the maximum number of weighted log potency estimates were combined into geometrical means and fiducial limits of these means were calculated. Estimates from each assembly, shown in Table 1, were processed accordingly and compared with arithmetic mean values of all determinations in each group (Table 2). These figures, together with those presented in Table 5, indicate that two

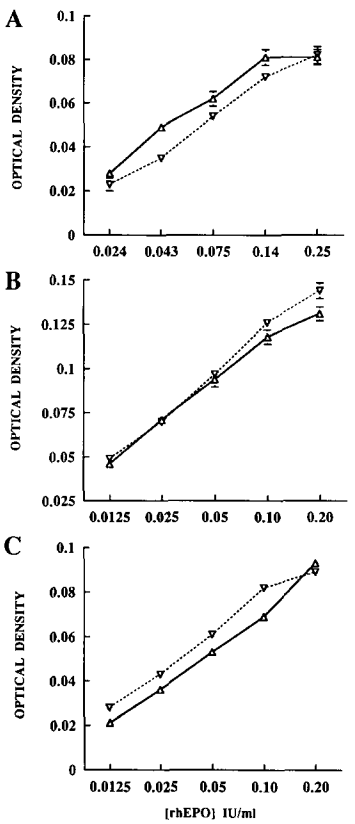

Fig. 6. Potency determinations of rhEpo. Activities of an rhEpo preparation, diluted to various known concentrations, as determined by the TF-1 in vitro bioassay. Two separate sample series were treated in parallel and five doses, from the linear response range, were selected for the MTT microcolorimetric assay (see also Tables 1 and 2). Vertical bars denote ± S.E.M. of quintuplicate samples. As a reference for hEpo bioactivity the International Standard for recombinant human erythropoietin (87/684; solid lines) was employed. (A) The test solution was diluted to 70% of the reference ($T = 0.70 \times R$; (B) $T = 1.00 \times R$; (C) $T = 1.30 \times R$.

Table 2
Weighted combinations of potency estimates presented in Table 1

Nominal potency	Estimated potency		Limits of error (%), $P = 0.95$	Statistical weight	No. of combined experiments[a]
	Arithmetic mean	Combined results			
0.70	0.73	0.72	92–109	2964	2 (2)
1.00	0.99	1.06	95–105	8134	5 (7)
1.30	1.26	1.25	93–108	3863	2 (4)

[a] The number of experiments conducted, in each group, is given in parentheses.

experiments, slightly less than average in performance, are sufficient for high accuracy potency estimates.

Two separate pharmaceutical preparations of CHO-derived rhEpo (including two batches of one preparation) were subjected to analysis of potency against the International Standard (WHO 87/684). As outlined in Table 3 the estimated potencies were determined with an average index of precision of 0.071. The assay experiments using one of these preparations were additionally repeated to provide an estimate of reproducibility. The RSD value of this series was 0.041. Combined relative potencies are listed in Table 5. Furthermore, various enzymatically treated preparations of rhEpo were produced for analysis. In line with previous reports [24–26], asialo rhEpo samples showed markedly elevated cellular stimulation. Moreover, partially desialylated hormone samples (BIp1 and BIp2) had intermediate activities in the present assay, revealing 1.35 and 1.88 times the potency respectively, of the untreated rhEpo (Tables 4 and 5).

Table 3
Estimated relative potencies of different preparations of rhEpo as determined by the micro-colorimetric assay

Preparation	Potency[a]	Limits of error[b] (%), $P = 0.95$	Index of precision[b]	RSD[b]
rhEpo (A1)	0.87	84–119	0.097	
	1.13	89–113	0.067	
	1.19	90–111	0.057	
rhEpo (A2)[c]	0.86	83–121	0.107	
	0.79	86–116	0.099	
rhEpo (B)	1.01	94–106	0.034	
	1.04	90–112	0.074	
	1.06	91–109	0.060	
	1.09	90–111	0.068	
	0.98	90–111	0.072	0.41

[a] Defined here as potency referred to WHO 87/684 (1.00 equals 124600 IU mg^{-1}.
[b] For definitions, see Section 2.
[c] A1 and A2 represent separate batches of a single preparation.

3.4. Analysis of intact and carbohydrate-trimmed rhEpo

To obviate the need for extensive amounts of highly purified protein substance, required for structural oligosaccharide determination, a sensitive Western detection method for hEpo was established. Using a high-titer rabbit immune serum, serial dilutions of the hormone demonstrated a detection limit of about 0.5 pmoles (not shown). SDS–PAGE analysis revealed relatively broad bands of intact as well as sialidase-digested rhEpo, corresponding to about 39 000 Da and 37 500 Da respectively (Fig. 7A, lanes a–c; Fig. 7B, lanes a and d). A more marked change apppeared upon a partial digestion with *N*-glycanase (Fig. 7A, lane d). To investigate if rhEpo molecules, with limited disturbances in terminal sugar occupancy, could be discriminated from untreated samples, preparations of rhEpo enzymatically treated to retain the major part (BIp1) and about half of the sialic acid residues (BIp2) respectively, were analysed. Whereas a shift in M_r was evident following complete removal of terminal Neu5Ac moieties the partially digested samples were less clearly resolved from intact hormone (Fig. 7B). Because of the acidic properties conferred on glycoproteins by Neu5Ac residues, it was decided to include a charge-dependent separation into the

Table 4
Estimated relative potencies of enzyme-treated preparations of rhEpo as determined by the micro-colorimetric assay

Preparation	Potency[a]	Limits of error[b] (%), $P = 0.95$	Index of precision[b]
Asialo-rhEpo (AIa)	3.86	92–109	0.044
	4.12	89–113	0.058
	3.26	89–113	0.077
N-glycosidase-treated rhEpo(AIn)	0.97	81–123	0.101
	1.08	85–117	0.076
	0.98	90–111	0.052
Partially de-sialylated rhEpo (BIp1)	1.43	86–116	0.097
	1.31	89–112	0.076
Partially de-sialylated rhEpo (BIp2)	2.19	80–124	0.124
	1.87	90–111	0.057
Asialo-rhEpo (BIa)	3.81	80–125	0.113
	3.25	81–123	0.109

[a] Defined here as potency referred to untreated rhEpo.
[b] For definitions, see Section 2.

Western system. Using a two-dimensional analysis approach [41,42] the 87/684 reference was compared with two CHO-derived rhEpo preparations from other sources. As delineated in Fig. 8 a very typical pattern was seen in all three preparations. Optimal exposures of intact rhEpo samples revealed six discernible isomeric groups with isoelectric points (pI) between pH 4.3 and 5.5. However, the most acidic spot was not consistently visualized due to a minute fraction of the hormone at this pI. Limited release of the sialic acid residues resulted in considerably changed patterns: the most gently treated rhEpo sample (BIp1) revealed a marked reallocation of material from the most acidic spots to more neutral positions, whereas the slightly more enzyme-exposed preparation (BIp2) became almost invisible on this ampholyte system. Using a gradient, encompassing a neutral region, this sample was resolved as three distinct spots (Fig. 10). Furthermore, neither asialo- nor partially *N*-glycanase-digested rhEpo preparations (see Fig. 7 for SDS–PAGE) were resolved on the acidic gradient but appeared as largely homogeneous materials, at pI 7.6–7.7 on the neutral IEF system (Figs. 9 and 10).

3.5. Quantification of separated rhEpo isoforms

Isomers of rhEpo, separated by 2-D electrophoresis, were processed for quantitative densitometry. When intact samples of various commercial rhEpo preparations were analysed, the recordings were very consistent. More than 90% of the material in the test samples was identified in four spots (e2–e5) around pI 4.5–5.0, whereas the remaining isoforms (e1 and e6) were distributed in two flank-migrating spots (Table 6). The 87/684 rhEpo reference displayed densitometry recordings that shared the general typical features of the aforementioned samples, i.e. essentially superimposable spots with similar distribution patterns. However, the most acidic group did not appear and the e6 fraction (pI 5.5), which is likely to represent the least sialylated rhEpo isomer(s), was slightly more prominent, compared with the test samples.

The density distribution of the mildly carbohydrate-digested rhEpo sample (BIp1) showed a clear shift towards less acidic positions. About two-thirds of the total amount was found at pI

Table 5
Weighted combinations of potency determinations shown in Tables 3 and 4

Preparation[a]	Estimated potency		Limits of error (%), $P = 0.95$	Statistical weight	No. of combined experiments[b]
	Arithmetic mean	Combined results			
A1	1.06	1.17	93–108	3846	2 (3)
A2	0.82	0.82	89–112	1643	2 (2)
B	1.04	1.03	96–104	15193	5 (5)
AIa	3.75	3.95	93–107	4489	2 (3)
AIn	1.01	1.00	92–108	3443	3 (3)
BIp1	1.37	1.35	91–109	2670	2 (2)
BIp2	2.03	1.88	91–110	2337	2 (2)
BIa	3.53	3.49	86–116	975	2 (2)

[a] A1, A2 and B are intact preparations of rhEpo. AIa and BIa refer to asialo-rhEpo. AIn is *N*-glycosidase F treated rhEpo. BIp1 and BIp2 represent rhEpo following partial sialic acid release.
[b] The number of experiments conducted, in each group, is given in parentheses.

5.0 and pI 5.5, whereas only 21–34% of each of the intact rhEpo preparations migrated to these positions (Table 6). Because of entirely changed appearances on the 2-D images, the less sialylated samples did not warrant quantitative analysis.

4. Discussion

The advantage of cell-based detection systems for the determination of biomolecule activity, as compared to immunometric quantification, has been extensively reported. In recent years cultured cell lines have been increasingly utilized for such purposes [54,55]. In this study the human erythroleukemia cell line TF-1 [39,40] has been employed as a cellular indicator system for Epo bioactivity. This study has shown that TF-1 can be used for accurate and precise potency determinations of hEpo. Hormone activity was measured using the International Standard for Human rDNA-derived Erythropoietin (87/684) as a reference and assay validity was evidenced by parallelism of test and standard rhEpo response dilution lines. Moreover, the approach presented is dependent on the metabolic conversion of a tetrazolium salt to blue formazan which obviates the need for radionuclides.

A large panel of substances were employed in tests of assay selectivity. Four of those were either negative or weakly stimulatory at the highest level tested, corresponding to five or six magnitudes higher molar concentrations than those used for rhEpo potency readings. Other compounds probed for action on TF-1, as measured by MTT reduction, could either be distinguished from hEpo by extensive dissimilarities in dosage–response relationship (rhIL-2, rhIl-4 and rhIL-6) or by including neutralizing immunoglobulins with specifity for the respective cytokine (rhIL-3 and rhGM-CSF). However, an abrogation of the rhEpo-dependent response, as was shown in the presence of neutralizing anti-hEpo antibodies, could be considered as a more ultimate demonstration of selectivity of the presented bioassay system.

Apart from being essential for normal cellular secretion, intact carbohydrate moieties protect Epo from a rapid degradation in vivo [16,19–22]. Limited deviations from the native structure, such as a reduced occupancy of the penultimate galactose residues with *N*-acetylneuraminic acid, has been shown to severely suppress the physiological action of this hormone [36,56]. However, certain sugar-trimmed Epo products have been demonstrated to display enhanced activity in vitro [24–26], which is also confirmed in this study. Accordingly, a correlation of an in vitro stimulatory effect of Epo to its potency in vivo requires information about the carbohydrate configuration. Notably, the sialic acid residues provide a limited proportion of the hEpo molecular weight but confer acidic properties and a considerable charge heterogeneity on this hor-

mone. To enable determination of subtle changes as well as more drastic truncations in the rhEpo glycan structure in a single analytical step, a quantitative Western detection system was established based on two-dimensional glycoprotein separation. This system provides detection of changes in charge distribution as well as molecular weight and accordingly reveals features of the protein that could escape detection in a 1-D IEF analysis. Hence, with regard to rhEpo, the 2-D approach provides a multi-faceted identification protocol. Extensively trimmed hormone samples, following a protracted

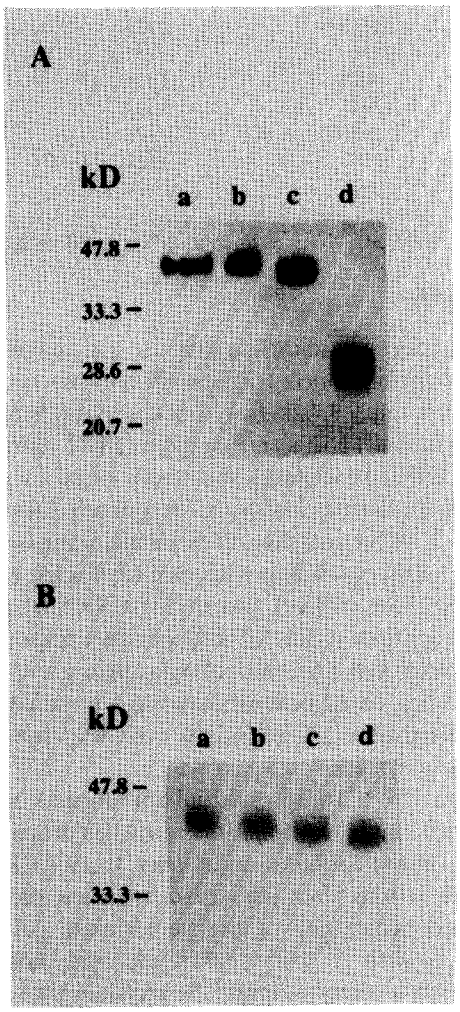

Fig. 7. Western immunoblot of rhEpo. An equal amount (1 pmole) of rhEpo was loaded in each lane. Samples were separated by SDS–PAGE (12%) and detected with a polyclonal antiserum against hEpo, followed by an HRP-conjugated anti-rabbit IgG ab. Membranes were subsequently treated with luminol and peracid reagents and exposed to Kodak XAR-5 films. (A) Lanes a and b depict untreated 87/684 and AI respectively, whereas c and d contain asialo-rhEpo (AIa) and *N*-glycosidase-treated hormone (AIn) respectively. (B) Lane a contains untreated hormone (BI). Lanes b–d show rhEpo molecules with gradually decreasing sialic acid modification. See Section 2 for details.

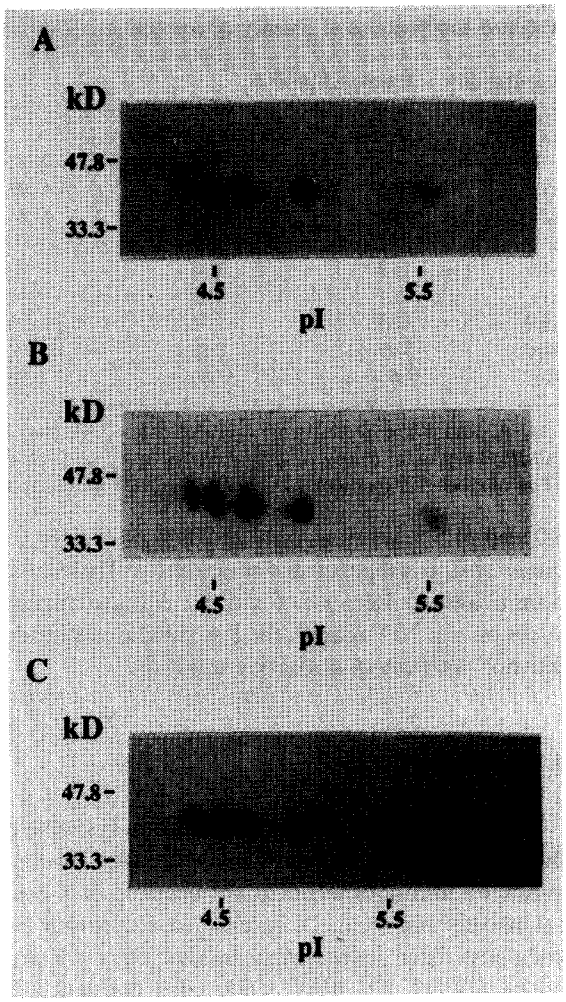

Fig. 8. Separation of various intact preparations of rhEpo by two-dimensional gel electrophoresis. Aliquots (1–3 pmoles) of rhEpo were separated by 2-D PAGE using Pharmalyte® broad range (acidic) ampholines for IEF and 12% polyacrylamide for SDS–PAGE. Gels were subjected to Western analysis and visualized on Kodak XAR-5 films, employing HRP-conjugated secondary antibodies and peracid/luminol reagents (see Section 2). (A)–(C): untreated samples of rhEpo, corresponding to preparations 87/684, A and B respectively (see also Tables 3 and 5).

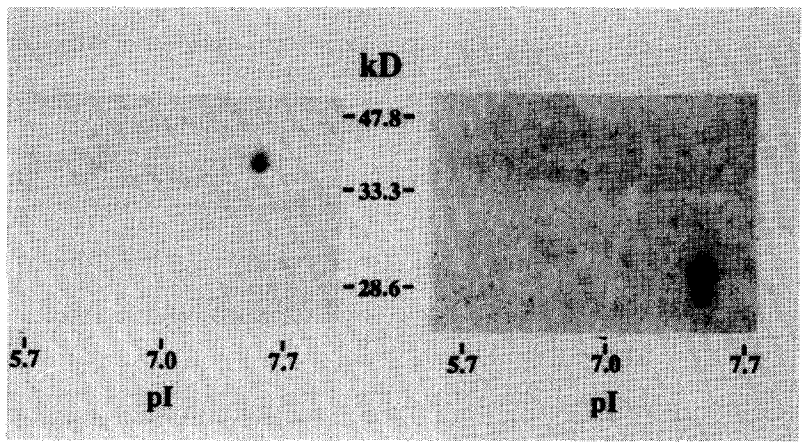

Fig. 9. Two-dimensional electrophoresis separation of glycan-trimmed rhEpo samples. The Pharmalyte® broad range (neutral) ampholytes were employed for the IEF step. SDS–PAGE was conducted on a 12% linear polyacrylamide gel. Following transfer to membranes, spots were visualized by exposure of luminiscence to Kodak XAR-5 film. Left panel: asialo-rhEpo (AIa; identical with the sample shown in Fig. 7A, lane c). Right panel: rhEpo subjected to digestion with *N*-glycanase (AIn; also shown in Fig. 7A, lane d).

treatment with either *N*-glycosidase or neuraminidase, were discernible from intact rhEpo using either of the SDS–PAGE or 2-D methods. However, more subtle changes in the carbohydrate configuration, such as a limited release of *N*-acetylneuraminic acid residues, required the enhanced separation approach for optimum resolution. It was also shown that such minor glycan derangements can be expressed quantitatively.

The identity of the separated rhEpo isomeric forms has not been specifically addressed. However, extensive studies on released rhEpo glycans by ion-exchange chromatography reveal isoform separation largely according to sialic acid content [17,18,23,37,57]. Hence the observed glycoprotein redistribution to more alkaline pIs following limited digestions with neuraminidase, is in accordance with the appearance of poorly sialylated rhEpo glycans of asparagine-linked type in other systems, based on separation by charge.

Several in vitro bioassays for human Epo have been described [31]. However, only in vivo assays in rodents [58–60] monitor the ultimate physiological aspect of Epo bioactivity, including glomerular filtration, lectin entrapment and metabolism in organs. Such assays, however, are dependent on extensive facilities, require an extended experimental time and are comparatively imprecise. The approach presented here provides accurate and specific potency determinations of hEpo with improved physiological relevance, as compared to earlier in vitro methods, by combining quantitative physicochemical analysis with readings of Epo in vitro activity. The overall sensitivity of the TF-1/2-D protocol renders the assay operable with minute amounts that would be insufficient for traditional in vivo estimation and vastly underscored to provide a more detailed structural oligosaccharide analysis. Finally, the assay presented has been extensively evaluated and is designed to fulfil pharmacopoeial requirements for potency assessments of pharmaceutical cytokine preparations.

Acknowledgements

The expert technical assistance of Ms. Eila Viitanen and Ms. Pia Grönhed and the excellent secretarial assistance of Ms. Jenny Brenner are greatly appreciated. We thank Dr. Toshio Kitamura for providing the TF-1 cell line. The National Institute for Biological Standards and Control (NIBSC), Potters Bar, Herts, UK is acknowledged for providing the International Standard for Human rDNA-derived Epo. We thank

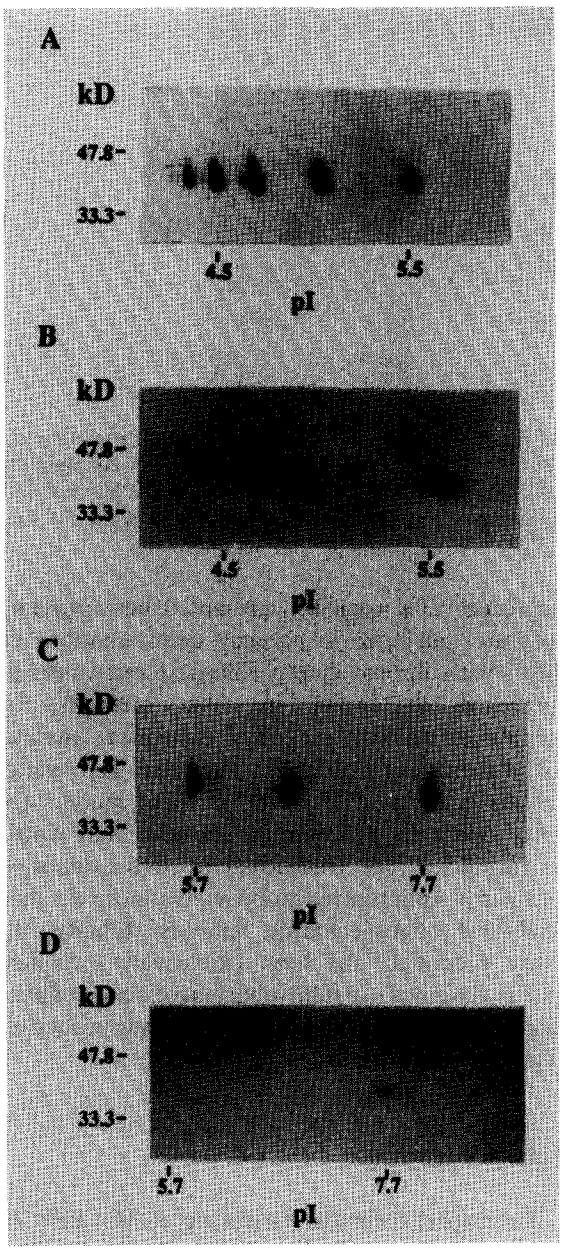

Fig. 10. 2-D separation of intact and variously sialic acid released rhEpo preparations. Intact and neuraminidase-treated rhEpo were separated using the 2-D approach followed by Western transfer and luminiscence detection (see Section 2). (A), (B): Intact (BI) and partially de-sialylated (BIp1) rhEpo respectively. IEF was conducted on an acidic Pharmalyte® gradient. (C), (D): Incompletely sialyl-liberated (BIp2) and asialo-rhEpo (BIa) using the neutral Pharmalyte® ampholytes in the first dimension.

Table 6
Densitometry recordings of rhEpo isomers, separated by 2-D electrophoresis

rhEpo isomers[a]	T.I.D. (%)[b]				
	87/684	A1	B	BI	BIp1
e1	–	0.5	1.4	3.3	–
e2	9.3	12.1	13.8	14.9	2.0
e3	23.4	24.0	32.9	24.0	11.7
e4	33.2	30.1	30.7	36.1	19.1
e5	22.3	26.7	19.5	18.2	34.2
e6	11.8	6.7	1.7	3.5	33.0

[a] e1–e6 represent isomeric groups of rhEpo within the pH range 4.3–5.5.
[b] Total integrated optical density. Each column represents mean values of two separate IEF/PAGE experiments.

Cilag AB, Sollentuna, Sweden and Boehringer-Mannheim Scandinavia AB, Bromma, Sweden for the supply of purified rhEpo material. This work was supported by the National Board for Laboratory Animals (CFN No 91-37).

References

[1] A. Erslev, Blood, 8 (1953) 349–357.
[2] S.B. Krantz, O. Gallien-Lartigue and E. Goldwasser, J. Biol. Chem., 238 (1963) 4085–4090.
[3] D.L. McLeod, M.M. Shreeve and A.A. Axelrad, Nature, 261 (1976) 492–494.
[4] V.C. Broudy, N.Lin, J. Egrie, C. De Haën, T. Weiss, T. Papayannopoulou and J.W. Adamson, Proc. Natl. Acad. Sci. U.S.A, 85 (1988) 6513–6517.
[5] S.B. Krantz, Blood, 77 (1991) 419–434.
[6] A.L. Jones and J.L. Millar, Baillièr's Clin. Haematol., 2 (1989) 83–111.
[7] K. Nocka, J. Buck, E. Levi and P. Besmer, EMBO J., 91 (1990) 3287–3294.
[8] V.C. Broudy, B. Nakamoto, N. Lin and T. Papayannopoulous, Blood, 75 (1990) 1622–1626.
[9] H. Tsuda, N. Aso, T. Sawada H. Hata, M. Kawakita, K. J. Mori and K. Takatsuki, Int. J. Cell Cloning, 9 (1991) 123–133.
[10] P. Steinlein, E. Deiner, A. Leutz and H. Beug, Growth Factors, 10 (1994) 1–16.
[11] S.-I. Yanagawa, K. Hirade, H. Ohnota, R. Sasaki, H. Chiba, M. Ueda and M. Goto, J. Biol. Chem., 259 (1984) 2707–2710.
[12] F.F. Wang, C.K.-H. Kung and E. Goldwasser, Endocrinology, 116 (1985) 2286–2292.

[13] P.-H. Lai, R. Everett, F.-F. Wang, T. Arakawa and E. Goldwasser, J. Biol. Chem., 261 (1986) 3116–3121.
[14] H. Sasaki, B. Bothner, A. Dell and M. Fukuda, J. Biol. Chem., 262 (1987) 12059–12076.
[15] V. Broudy, J.F. Tait and J.S. Powell, Arch. Biochem. Biophys., 265 (1988) 329–336.
[16] S. Dubé, J.W. Fisher and J.S. Powell, J. Biol. Chem., 263 (1988) 17516–17521.
[17] K.G. Rice, N. Takahashi, Y. Namiki, A.D. Tran, P.J. Lisi and Y.C. Lee, Anal. Biochem., 206 (1992) 278–287.
[18] E. Watson, A. Bhide and H. van Halbeek, Glycobiology, 4 (1994) 227–237.
[19] P.H. Lowy, G. Keighley and H. Borsook, Nature, 185 (1960) 102–103.
[20] D.W. Briggs, J.W. Fisher and W.J. George, Am. J. Physiol., 227 (1974) 1385–1388.
[21] M.N. Fukuda, H. Sasaki, L. Lopez and M. Fukuda, Blood, 73 (1989) 84–89.
[22] J.L. Spivak and B.B. Hogans, Blood, 73 (1989) 90–99.
[23] M. Takeuchi, N. Inoue, T.W. Strickland, M. Kubota, M. Wada, R. Shimizu, S. Hoshi, H. Kozutsumi, S. Takasaki and A. Kobata, Proc. Natl. Acad. Sci. U.S.A., 86 (1989) 7819–7822.
[24] E. Goldwasser, G.K.-H. Kung and J. Eliason, J. Biol. Chem., 249 (1974) 4202–4206.
[25] M. Takeuchi, S. Takasaki, M. Shimada and A. Kobata, J. Biol. Chem., 265 (1990) 12127–12130.
[26] E. Tsuda, G. Kawanishi, M. Ueda, S. Matsuda and R. Sasaki, Eur. J. Biochem., 188 (1990) 405–411.
[27] M.S. Dordal, F.F. Wang and E. Goldwasser, Endocrinology, 116 (1985) 2293–2299.
[28] D.M. Wojchowski, S.H. Orkin and A.J. Sytkowski, Biochem. Biophys. Acta, 910 (1987) 224–232
[29] J.W. Eschbach, J.C. Egrie, M.R. Downing, J.K. Browne and J.W. Adamson, N. Engl. J. Med., 316 (1987) 73–78.
[30] A. Urabe, F. Takaku, H. Mizoguchi, K. Kubo, K. Ota, N. Shimizu, K. Tanaka, N. Nimura, H. Nihei, S. Koshikawa, T. Akizawa, N. Akiyama, O. Otubo, Y. Kawaguchi and T. Maeda, Int. J. Cell Cloning, 6 (1988) 179–191.
[31] For a review, see: W. Jelkmann, Physiol. Rev., 72 (1992) 449–489.
[32] S. Lee-Huang, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 2708–2712.
[33] K. Jacobs, C. Shoemaker, R. Rudersdorf, S.D. Neill, R.J. Kaufman, A. Mufson, J. Seehra, S.S. Jones, R. Hewick, E.F. Fritsch, M. Kawakita, T. Shimizu and T. Miyake, Nature, 313 (1985) 806–810.
[34] F.-K. Lin, S. Suggs, C.-H. Lin, J.K. Browne, R. Smalling, J.C. Egrie, K.K. Chen, G.M. Fox, F. Martin, Z. Stabinsky, S.M. Badrawi, P.-H. Lai and E. Goldwasser, Proc. Natl. Acad. Sci. U.S.A., 82 (1985) 7580–7584.
[35] J.K. Browne, A.M. Cohen, J.C. Egrie, P.H. Lai, F.-K. Lin, T. Strickland, E. Watson and N. Stebbing, Cold Spring Harbor Symp. Quant. Biol., 51 (1986) 693–702.
[36] M. Goto, K. Akai, A. Murakami, C. Hashimoto, E. Tsuda, M. Ueda, G. Kawanishi, N. Takahashi, A. Ishimoto, H. Chiba and R. Sasaki, Biotechnology, 6 (1988) 67–71.
[37] M. Nimtz, W. Martin, V. Wray, K.-D. Klöppel, J. Augustin and H.S. Conradt, Eur. J. Biochem., 213 (1993) 39–56.
[38] C.F. Goochee and T. Monica, Bio/Technology, 8 (1990) 421–427.
[39] T. Kitamura, T. Tange, T. Terasawa, S. Chiba, T. Kuwaki, K. Miyagawa, Y.-F. Piao, K. Miyazono, A. Urabe and F. Takaku, J. Cell Physiol., 140 (1989) 323–334.
[40] T. Kitamura, A. Tojo, T. Kuwaki, S. Chiba, K. Miyazono, A. Urabe and F. Takaku, Blood, 73 (1989) 375–380.
[41] P.H. O'Farrell, J. Biol. Chem., 250 (1975) 4007–4021.
[42] H. Towbin, T. Staehelin and J. Gordon, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 4350–4354.
[43] D.M. Bollag and S.J. Edelstein, Protein Methods, Wiley-Liss, New York, 1991, pp. 95–211.
[44] B. Batteiger, W.J. Newhall V and R.B. Jones, J. Immunol. Methods, 55 (1982) 297–307.
[45] D.A. Johnson, J.W. Gautsch, J.R. Sportsman and J.H. Elder, Gene Anal. Tech., 1 (1984) 3–8.
[46] T. Mosmann, J. Immunol. Methods, 65 (1983) 55–63.
[47] H. Tada, O. Shiho, K.-i. Kuroshima, M. Koyama and K. Tsukamoto, J. Immunol. Methods, 93 (1986) 157–165.
[48] European Pharmacopoeia, 1st edn., Vol. II, Sainte-Ruffine, Maisonneuve, France, 1971, pp. 441–498.
[49] J.A. Lorraine and E.T. Bell, Hormone Assays and their Clinical Applications, Livingstone, London, 1966, pp. 12–14.
[50] T.R.J. Lappin, G.E. Elder, S.H. McKibbin, P.T. McNamee, M.G. McGeown and J.M. Bridges, Exp. Hematol., 13 (1985) 1007–1013.
[51] R. Cecil and G.B. Robinson, Biochim. Biophys. Acta, 404 (1975) 164–168.
[52] L. Sjödin, T. Nederman, P.A. Olsson and E. Viitanen, J. Pharm. Pharmacol., 41 (1989) 402–406.
[53] WHO Expert Committee on Biological Standardization, Tech. Rep. Ser., 814 (1991) 10.
[54] R.T. Abraham, S.N. Ho and D.J. McKean, J. Tissue Culture Methods, 10 (1987) 93–99.
[55] R. Thorpe, M. Wadhwa, C.R. Bird and A.R. Mire-Sluis, Blood Rev., 6 (1992) 133–148.
[56] N. Imai, M. Higuchi, A. Kawamura, K. Tomonoh, M. Oheda, M. Fujiwara, Y. Shimonaka and N. Ochi, Eur. J. Biochem., 194 (1990) 457–462.
[57] E. Watson and A. Bhide, LC–GC, 11 (1993) 216–220.
[58] P.M. Cotes and D.R. Bangham, Nature, 191 (1961) 1065–1067.
[59] A.H. Weintraub, A.S. Gordon and J.F. Camiscoli, J. Lab. Clin. Med., 62 (1963) 743–752.
[60] T. Hayakawa, M. Wada, K. Mizuno, S. Abe, M. Miyashita and M. Ueda, Biologicals, 20 (1992) 243–251.