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Evaluation of the Stability of Human Erythropoietin in Samples for Radioimmunoassay

K.-U. Eckardt[1], A. Kurtz[1], P. Hirth[2], P. Scigalla[2], L. Wieczorek[2], and C. Bauer[1]

[1] Physiologisches Institut der Universität Zürich
[2] Boehringer Mannheim GmbH

Summary. Radioimmunoassays for erythropoietin are limited so far to a few specialized laboratories and this requires transport and storage of samples. We therefore tested the stability of immunoreactive erythropoietin in serum and plasma samples obtained from a uremic and a nonuremic anemic patient. No significant change in the concentration of immunoreactive erythropoietin was found in either serum or plasma samples for up to 14 days of storage. This type of stability was observed no matter whether the samples were stored at room temperature, 4° C, or −20° C. There was no difference between the estimates of erythropoietin in serum and heparinized plasma. Validity of the radioimmunoassay used in this study was demonstrated by parallelism of dilution curves of test specimens and the 2nd International Reference Preparation for erythropoietin and by a close correlation between the immunoreactivity and the bioactivity of the hormone, as assessed in the same samples by the exhypoxic polycythemic mouse bioassay.

In conclusion the data obtained clearly indicate that the necessity of storage and transport of clinical samples does not limit the practicability of the radioimmunoassay for erythropoietin.

Key words: Erythropoietin – Stability – Radioimmunoassay – Polycythemic mouse bioassay – Recombinant DNA

Since recombinant human erythropoietin (rhEPO) became available for replacement therapy in patients with certain forms of anemia [5, 9], the determination of erythropoietin (EPO) levels in body fluids has gained increasing clinical importance. However, radioimmunoassay kits are not yet commercially available and the limitation of EPO determinations to specialized laboratories necessitates storage and transport of the samples. This makes it important to know the stability of EPO in human samples. We therefore performed subsequent radioimmunological determinations of EPO for up to 14 days on serum and plasma samples which were stored at room temperature, 4° C, or −20° C. The validity of RIA estimates was secured by determination of the biological activity of the same samples using an in vivo bioassay and by comparison of the slopes of dilution curves of test samples and the 2nd International Reference Preparation (IRP) for EPO.

Abbreviations: BSA = bovine serum albumin; EPO = erythropoietin; irEPO = immunoreactive erythropoietin; IRP = International Reference Preparation; hct = hematocrit; rhEPO = recombinant human erythropoietin; RIA = radioimmunoassay

Material and Methods

Antiserum to Erythropoietin

Three New Zealand white rabbits were immunized with pure rhEPO (25 μg per animal) according to standard methods. EPO for immunization was obtained from Genetics Institute, Boston, USA. During immunization two rabbits (# 1 and # 2) became severely anemic. Figure 1 shows the binding of radiolabelled recombinant human EPO to the different rabbit sera. It is obvious that rabbit # 1 produced a high titer of antibodies against EPO. The serum of this animal was used for radioimmunoassay.

Radiolabelled Erythropoietin

Human 3-(^{125}I)iodotyrosyl-erythropoietin, prepared by iodination of rhEPO using sodium

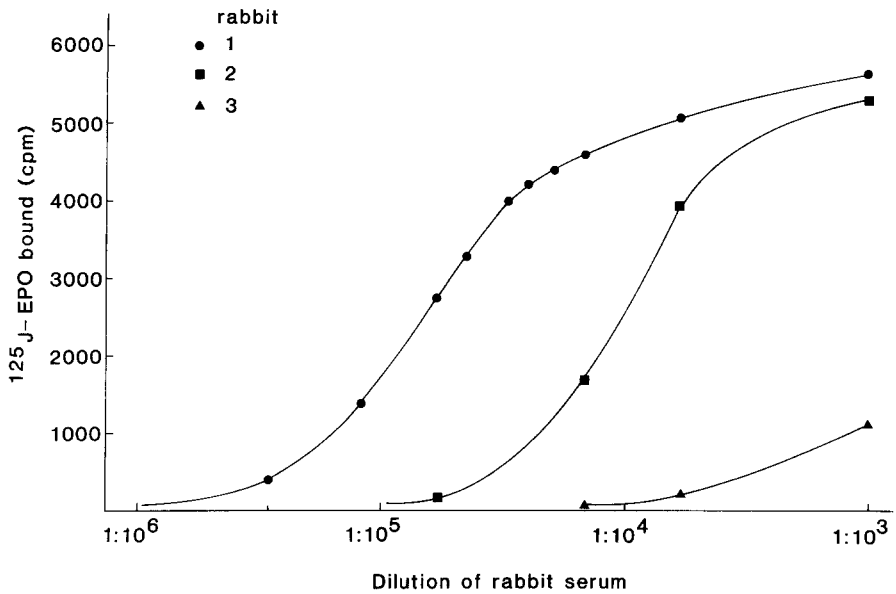

Fig. 1. Binding of recombinant human ^{125}I-EPO to sera of rabbits immunized against EPO. 100 µl serum at different dilutions was incubated with 100 µl ^{125}I-EPO (containing 6000 cpm, i.e., 14 fmol EPO) at 4° C overnight. EPO bound to antibodies was precipitated using 100 µl (1 unit) of goat-anti-rabbit gammaglobulin

(^{125}I)iodide and chloramine T, with a specific activity of 320 to 620 Ci/mmol was purchased from Amersham International (Amersham, England). A dilution containing 8×10^{-11} mol radiolabelled EPO/l, corresponding to 6000–9000 cpm/100 µl, was prepared for use in the RIA.

Erythropoietin Standard

The 2nd International Reference Preparation (IRP) of human urinary EPO was obtained from the Bureau of Biological Standards, National Institute of Medical Research, London, England, and was used as standard in the RIA and the bioassay.

Radioimmunoassay for Erythropoietin

Phosphate buffered saline, pH 7.5, containing 0.1% bovine serum albumin (BSA) was used as diluent buffer for all reagents. All incubations were carried out at 4° C.

Test Procedure. Aliquots of the following were combined in Eppendorf reaction cups: (a) 100 µl of plasma, serum, or standard solutions (10–500 mU EPO/ml), (b) 100 µl of diluted EPO antiserum (1:60000), and (c) 20 µl of 30% BSA in PBS. After preincubation for 24 h, 100 µl of radiolabelled EPO (8×10^{-15} mol) was added and tubes were incubated for another 24 h. This delayed addition of the tracer resulted in increased steepness and a leftward shift of the log dose-response curve (see below) compared to the simultaneous addition of tracer with sample and antiserum (50% binding at 65 mU/ml vs 250 mU/ml EPO) and thereby markedly increased sensitivity of the assay. This effect has been attributed to the formation of multivalent antigen-antibody complexes which are less likely to dissociate than univalent complexes [1]. After the incubation, separation of bound vs free ligand was accomplished using a secondary antibody technique. For this purpose 100 µl (1 unit) of goat-anti-rabbit-gammaglobulin (Calbiochem) and 100 µl rabbit gammaglobulin (0.03 mg; Calbiochem) were added to the Eppendorf reaction cups to precipitate bound ligand. After incubation for 4 h the tubes were centrifuged at 9500 *g* for 15 min. The supernatant was aspirated and the pellet counted for ^{125}I radioactivity.

Data Analysis. Data were expressed as percent of binding in the absence of unlabelled EPO. Calculations of unknowns were made on the basis of a standard log dose-response curve derived by the "spline-function" method (LKB Wallac Compu Gamma RIA program 1282–114); (Fig. 2, right panel).

Quality Controls. Protein bound radioactivity (as determined by adding 1 ml 10% TCA to a control tube before the last incubation) was about 90% of the radioactivity added. The maximum amount of antibody-precipitable ^{125}I EPO (as determined

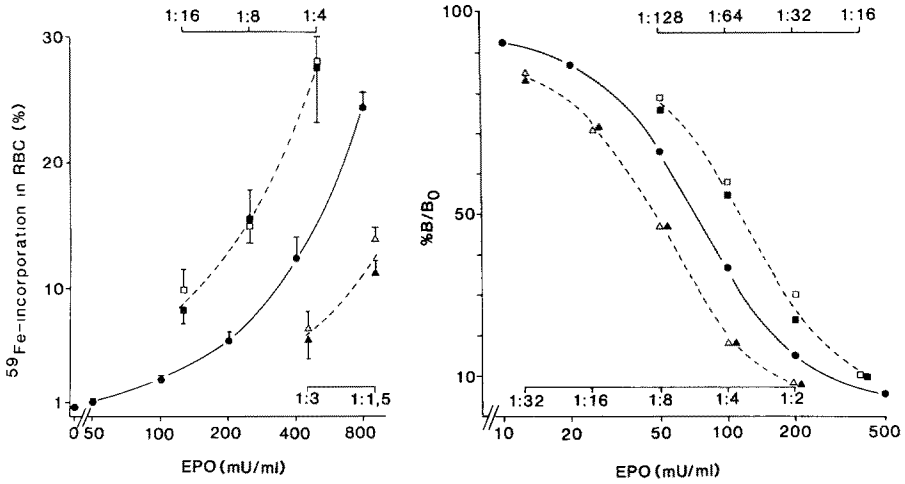

Fig. 2. Parallelism of erythropoietin-standard (2nd IRP) dose-response curves and serial dilution curves of heparinized plasma (*open symbols*) and serum (*closed symbols*) from patient 1 (□:■) and patient 2 (△:▲) in the exhypoxic polycythemic mouse bioassay (*left panel*) and radioimmunoassay (*right panel*). Data in the bioassay indicate mean ±S.E. of five animals each

using 100 µl of diluent buffer instead of EPO standard) was approximately 55% of the total radioactivity added. Nonspecific binding (as determined using 100 µl of diluent buffer instead of antiserum) was about 1% of the total radioactivity added.

The intraassay coefficient of variation (assessed by tenfold determination of a diluted serum sample with a mean of 70.4 mU/ml) was 1.9%. The interassay coefficient of variation of 20 assays was 7.5% and 6.8% for samples containing, respectively, a mean of 43.8 mU and 168.5 mU rhEPO.

Bioassay for Erythropoietin

The biological activity of EPO was measured by a modification of the exhypoxic polycythemic mouse bioassay [3]. Female ICR-strain mice (25–31 g) were exposed to intermittent (20–22 h/day) normobaric hypoxia for 14 days, using a chamber that was gassed with a mixture of nitrogen and normal atmospheric air. The resulting oxygen concentration in the chamber was 7%–8%. This regime renders animals severely polycythemic (hematocrit 80% on average) and leads to a subsequent inhibition of endogenous erythropoiesis.

On days 5 and 6 after removal from the chamber, assay animals were injected subcutaneously with 0.5 ml of divided doses of standards or samples dissolved in 0.9% NaCl. On day 7 they were injected with 0.1 µCi ^{59}Fe i.p. Two days later heparinized blood was obtained by heart puncture for determination of hematocrit (hct) and radioactive iron incorporation, which was calculated on the assumption of a blood volume of 7.5% body weight. The mean hct in the assay described was 66.4% ±5.46% (mean ±SD; $n=105$). Only mice having a hct of 55% or higher were used for the calculations. EPO content of samples was determined on the basis of a standard log dose-response curve (Fig. 2, left panel), using the mean of five animals per point. In red blood cells of animals not receiving EPO ^{59}Fe incorporation averaged 0.47%.

Sample Collection and Storage

Two male anemic patients served as blood donors. Patient 1 (hct 21%) was suffering from aplastic anemia, patient 2 (hct 40%) was on hemodialysis because of chronic renal failure due to polycystic kidney disease. Venous blood samples of these two patients were collected into either plain or heparin-coated glass tubes and immediately kept on ice. Serum and plasma were separated in a refrigerated centrifuge and aliquotted under sterile conditions. The first RIA and estimation of EPO activity in the bioassay were performed within 2 h after collection of samples. The remaining aliquots were stored either at room temperature, at 4° C, or frozen (−20° C), and allowed to thaw only on the day of assay. Subsequent RIAs on serum and plasma stored under these different conditions were then performed daily up to day 6 and on days 9 and 14 after collection.

Results

The serum levels of EPO determined in the bioassay immediately after collection of the blood samples were 4200 mU/ml in patient 1 and 546 mU/ml

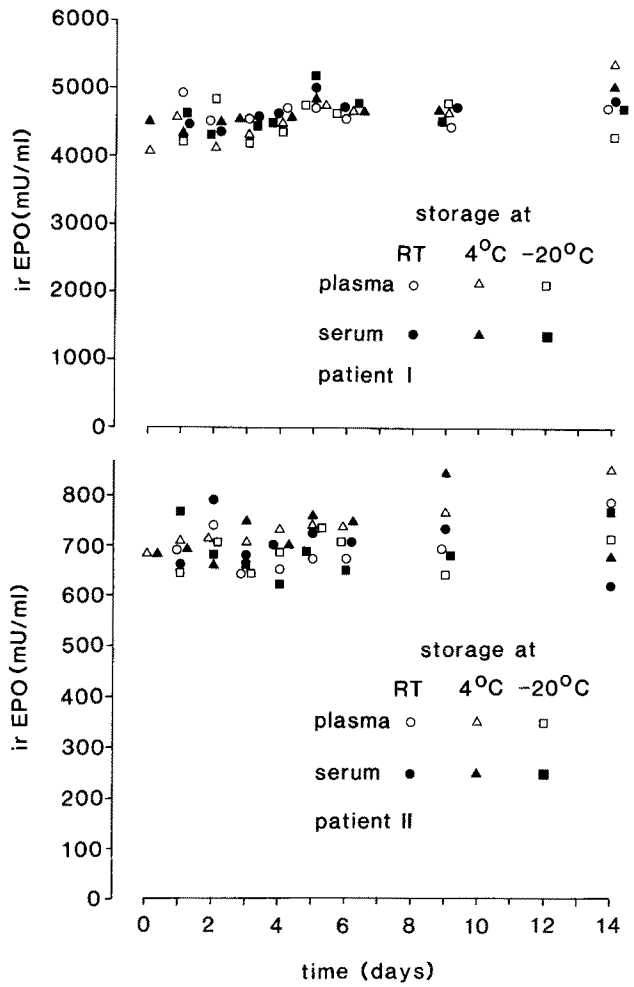

Fig. 3. Results of subsequent RIA determinations of EPO on serum and plasma samples stored under different conditions

in patient 2, the corresponding estimates in plasma were 4000 mU/ml (patient 1) and 618 mU/ml (patient 2). Thus there was no significant difference between the biological activity of EPO in serum or plasma.

The results of the first radioimmunological estimations of the serum and plasma samples, which were performed shortly after collection, were 4520 mU/ml and 4068 mU/ml respectively in patient 1 and 677 mU/ml and 682 mU/ml respectively in patient 2. The comparison between these results and the bioassay results shows a close correlation between the biological activity of the hormone and the immunoreactivity as assessed in the RIA, providing good evidence for the validity of the latter. As documented in Fig. 2, high specificity of both the bioassay and the RIA was further reflected by identical slopes of dilution curves of the samples and the 2nd IRP of EPO, used as standard.

Figure 3 shows the results of the subsequent RIAs, performed on aliquots of serum or plasma stored under sterile conditions for various time intervals at different temperatures. It is obvious, that in samples from both patients there was no significant change of the estimates of immunoreactive (ir) EPO up to 14 days, no matter whether the samples were stored at room temperature, 4° C, or −20°. Furthermore, there was no significant difference between the estimates of irEPO in serum and plasma, either in individual assays, or comparing the means. The coefficient of variation of all determinations was 5.6% in patient 1 and 8.0% in patient 2 and this was within the normal limits of the assay.

Discussion

In order to determine the conditions that allow storage of human samples for valid determinations of irEPO, we tested the in vitro stability of irEPO in serum and plasma samples from a uremic and a nonuremic blood donor by subsequent RIAs.

First we found no significant difference between estimates of EPO in serum or heparinized plasma. This agrees with other investigators, who also compared RIA estimates of serum and plasma and found either equally no difference [4] or a minor reduction of the mean recovery in heparinized plasma [2] or EDTA plasma [7]. For clinical purposes it is important that heparinized plasma and serum can be used interchangeably in the EPO-RIA, as many candidates for EPO determinations are uremic patients, in whom blood samples are often collected at the occasion of hemodialysis and may contain various amounts of heparin.

Second, we found that storage of the samples for up to 14 days even at room temperature did not significantly affect the amount of immunoreactive EPO. This finding does, of course, not exclude any minor changes in the molecular structure of the hormone. However, it demonstrates, that EPO is certainly not subjected to rapid proteolysis, as has been shown for some polypeptide hormones in biological fluids [10]. This complements previous observations of a considerable stability of EPO under various other conditions, e.g., in response to heating [8], change in pH, or exposure to various chemicals [6].

We conclude from the high stability of irEPO in serum and plasma, that no special precautions such as addition of protease inhibitors are required for the preservation of the hormone content and that samples do not necessarily have to be frozen, as long as assaying is guaranteed within 2 weeks of collection.

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Dr. Kai-Uwe Eckardt
Physiologisches Institut
Universität Zürich
Winterthurerstrasse 190
CH-8057 Zürich/Switzerland