Performance of an Immunoradiometric Assay of Erythropoietin and Results for Specimens from Anemic and Polycytemic Patients

Marie Andre,1 Alice Ferster,2 Michèle Toppet,2 Pierre Fondu,3 Max Dratwa,4 and Pierre Bergmann1,5

We evaluated a new commercially available two-site immunoradiometric assay (IRMA; BioMérieux 125I-EPO CoatRIA) for erythropoietin (EPO) in human serum. The precision (CV) was 4.1% intra-assay and 8% interassay for a serum pool with an EPO concentration of 17.1 units/L; the detection limit was 0.5 int. unit/L, one order of magnitude lower than by classical radioimmunoassay (RIA), although standardization of IRMA and RIA were similar. Results by both IRMA and RIA are compared for normal subjects, patients with nonrenal noninflammatory anemias, patients with β-thalassemia major, hemodialysis patients, and patients with primary or secondary polycythemia. Values by IRMA compared well with those by RIA in the upper range; IRMA and RIA values for EPO show parallel expected variations with the degree of anemia. However, because of its greater sensitivity and specificity, we consider the IRMA more appropriate than RIA for investigating patients with sub-normal EPO concentrations.

Erythropoietin (EPO) has a major role in the stimulation of erythrocyte production.6 Since the development of radioimmunoassays for EPO, it has become easier to assess the possible role of abnormal EPO concentrations in anemia and polycythemia. Although such radioimmunoassays (RIA) have a much lower detection limit than available bioassays conducted in vivo (2,3) or in vitro (4), they are still too insensitive to detect low concentrations of EPO. Here we evaluated a new immunoradiometric assay (IRMA) of EPO, both for its performance characteristics and for its possible usefulness in several clinical situations. We compared the results obtained with the two-site IRMA with those obtained with a radioimmunoassay.

Patients and Methods

Patients

Blood was collected from 17 male and 10 female patients, ages 1 to 72 years, who had noninflammatory, nonrenal anemia (blood Hb < 120 g/L); 5 male and 3 female patients, ages 7 to 29 years, with β-thalassemia major (pre-transfusion Hb: 74 to 116 g/L); 18 male and 11 female hemodialysis patients, ages 34 to 75 years, with terminal renal insufficiency who had never been treated with EPO (Hb: 53 to 123 g/L); 9 hypocromic patients with chronic obstructive pulmonary disease and secondary polycythemia (pO2 50–70 mmHg; Hb: 155–175 g/L; hematocrit: 47–56%); and 7 patients with untreated polycythemia vera (pO2 > 70 mmHg, Hb: 170–200 g/L, hematocrit: 53–61%). No anticoagulant was added. Thalassemic patients were investigated on at least five occasions to cover a complete transfusion cycle: before transfusion (time n), immediately after the end of this transfusion, 5–14 days later (median: 9 days), and before and after transfusion (time n + 1). For other anemic patients, care was taken to exclude those who had a recent transfusion. Healthy subjects working in the laboratory, 13 men and 13 women, ages 26 to 53 years, served as controls. Informed consent was obtained from all subjects.

Serum Pools

Pooled specimens of human serum were used to determine assay performance. These were a low-concentration pool (serum from normal subjects and patients with chronic renal failure), a medium-concentration pool (serum from patients with mild nonrenal anemia), and a high-concentration pool (serum from patients with nonrenal severe anemia).

Procedures

IRMA. The assay 125I-EPO CoatRIA (supplied by BioMérieux, Marcy-L’Etoile, Lyon, France) includes two monoclonal anti-EPO antibodies (mouse) raised against human recombinant EPO. As described previously (5), these antibodies bind with two different epitopes of the peptide sequence, both involved in the interaction of EPO with its receptor. Their binding is independent of the presence of the sugars. One antibody, Ac1, is fixed on the test tube, while the other, Ac2, is labeled with 125I. Standards of urinary EPO were prepared in Tris HCl (100 mmol/L, pH 7.3) containing bovine serum albumin (Cohn Fraction V), 50 g/L, and merthiolate, 0.1 g/L. The assay was calibrated against the Second MRC Reference Preparation 67/343 (urine EPO; National Institute for Biological Standards and Control, Potters Bar, U.K.).

A one-step procedure was used, with EPO and Ac2 added simultaneously to the coated tube. After a 3-h incubation at room temperature, with constant mixing, the tube was washed and its radioactivity was counted. EPO that had bound both Ac1 and 125I-labeled Ac2 was measured.

RIA. The EPO-Trac procedure (Incstar, Stillwater, MN) was a competitive-binding nonequilibrium radioimmunoassay involving a rabbit polyclonal antiserum.
directed against human EPO. The complete procedure has been described elsewhere (6). Recently, a goat antiserum was substituted for the rabbit antiserum, but this modification did not significantly change the results. Recombinant EPO in buffered saline was used as tracer and standard. The RIA was calibrated against the Second MRC Reference Preparation 67/343 (urine EPO). After an overnight incubation, bound radioactivity was separated by using an accelerated double-antibody reagent (goat anti-rabbit or donkey anti-goat precipitating complex).

**Calibration.** For both kits, calibration was verified by measuring the EPO concentration observed after addition of different amounts of MRC 67/343 to the zero standard and to a pool of human serum with a low EPO concentration. Calibration of the IRMA system was also verified against recombinant EPO (MRC 87/684). Also, the standards of each kit were measured with the other kit.

**Results**

**Assay Performance**

**IRMA.** Figure 1A shows the entire standard curve and sequential dilutions of two patients' sera. The standard curve had a steeper positive slope and was linear in a log scale after subtraction of the zero standard counts per minute; dilution curves of the sera were parallel to those of the reference preparation. In Figure 2, standard curves were constructed by adding the two MRC reference preparations (67/343 and 87/684) either to the zero standard or pooled plasma from patients with a low EPO content; these curves paralleled that obtained with the kit standards (standard CoatRIA) down to a concentration <1.0 unit/L. Analytical recovery of added MRC 67/343 varied between 71% and 94% in serum, and from 92% to 112% in the zero standard (Table 1). Results for the standards of the RIA measured with the IRMA were between 88% and 100% (93 ± 5%) of their nominal values (Table 2).

The detection limit, determined by the simultaneous determination of 22 zero standards was 0.500.1 unit/L (radioactivity was counted for 3 min, modified from the recommended method of counting for at least 1 min.). Figure 1B shows results of parallel dilutions of the 4.7 int. units/L standard and of a pool containing EPO at 3.9 int. units/L down to a final concentration of 0.50 int. unit/L. All dilutions were done with the zero standard. The results obtained with these low concentrations were linearly distributed.

The precision was evaluated by repeated testing of the three pools (10 times intra-assay; 9 times interassay). The intra-assay and interassay coefficients of variation (Table 3) were 2.7% and 4%, respectively, when the EPO concentration was 40 int. units/L, and 10% and 12%, respectively, when the EPO concentration was 4 int. units/L. Figure 3 gives the profile of CVs for paired determinations.

**RIA.** A logit-log transformation of the standard curve (Figure 4) was used for evaluation of the RIA data. Reference curves obtained with MRC 67/343 were parallel and generally superimposable on those obtained with the kit standards, down to 4 int. units/L. Analytical recovery of the added MRC 67/343 varied between 77% and 95% in serum, and from 70% to 125%

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**Fig. 1.** (A) Measured radioactivity of coated IRMA tubes as a function of EPO concentration, for the standard preparation (●) and for sequential dilutions of two sera with a previously determined EPO concentration of 5.4 (●) and of 602 (●) int. units/L; (B) IRMA results for sequential dilutions of the lower standard (4.7 int. units/L) (●) and of the low-concentration pool (3.9 int. units/L) (●)

**Fig. 2.** Measured radioactivity of coated IRMA tubes as a function of EPO concentration (int. units/L)

Solutions were prepared with two EPO reference preparations (MRC 67/343 or 87/684) either in the kit zero standard (●) or in a serum pool with a low EPO concentration (○, ●). The reference curve of the kit (●) Std CoatRIA) is given for comparison.
Table 1. Analytical Recovery of MRC 67/343 EPO

<table>
<thead>
<tr>
<th>Added to pooled serum*</th>
<th>EPO-CotRIA</th>
<th>RIA-Trac</th>
</tr>
</thead>
<tbody>
<tr>
<td>value</td>
<td>Measured</td>
<td>Recovery, %</td>
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<tr>
<td>Added to zero standard</td>
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<td></td>
</tr>
<tr>
<td>1.5</td>
<td>7.2</td>
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<td>3.12</td>
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<td>97</td>
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<tr>
<td>6.25</td>
<td>13.70</td>
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<tr>
<td>12.50</td>
<td>19.7</td>
<td>109</td>
</tr>
<tr>
<td>25.0</td>
<td>33.8</td>
<td>108</td>
</tr>
<tr>
<td>50.0</td>
<td>60.1</td>
<td>107</td>
</tr>
<tr>
<td>100.0</td>
<td>111.9</td>
<td>105</td>
</tr>
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</table>

*Original serum content of EPO: 6.3 int. units/L for IRMA study, 9.5 int. units/L for RIA study.

Table 2. Comparisons of EPO Kit Standards

<table>
<thead>
<tr>
<th>EPO-CotRIA</th>
<th>RIA-Trac</th>
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<tr>
<td>RIA nominal value</td>
<td>Measured value</td>
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<tr>
<td>0</td>
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<tr>
<td>11</td>
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<tr>
<td>78</td>
<td>68.6</td>
</tr>
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<td>295</td>
<td>288.4</td>
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</table>

Table 3. EPO-CotRIA Performance

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay (n = 10)</th>
<th>Interassay (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD) EPO, int. units/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>Low pool*</td>
<td>3.7 (0.37)</td>
<td>10</td>
</tr>
<tr>
<td>Medium poolb</td>
<td>17 (0.71)</td>
<td>4.1</td>
</tr>
<tr>
<td>High poolc</td>
<td>44 (1.2)</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Sera from normal subjects and patients with chronic renal failure.

b Sera from patients with mild, nonrenal anemia.

c Sera from patients with severe, nonrenal anemia.

in the zero standard (Table 1). The standards samples of EPO CoatRIA measured by RIA were 67% to 95% (82 ± 11%) of their nominal values (Table 2). The lower detection limit, deduced from the mean – 2 SD observed upon repetitive testing of the zero standard (n = 15), was 4.0 int. units/L. The dilution with the EPO-Trac sample diluent of a serum having an EPO value of 35 int. units/L yielded a curve that was acceptably linear

Fig. 3. IRMA CV profile of paired determinations according to EPO concentration (int. units/L)

Fig. 4. RIA tracer displacement by increasing concentrations of EPO (int. units/L)

Solutions were prepared with EPO reference preparation MRC 67/343, either in the kit zero standard (a) or in a serum pool with low EPO concentration (b). The reference curve of the kit (c) is given for comparison.

Fig. 5. RIA results of sequential dilutions of a serum with an initial EPO value of 35 int. units/L, showing a linear dilution down to the lower detection limit (4 int. units/L).

Equation of the regression line: \( y = 1.434x + 0.9686x \)
down to this lower detection limit (Figure 5). The intra-assay CV, calculated from 10 repeated measurements of two serum pools, was 9.1% for an EPO concentration of 15 int. units/L and 10.9% for an EPO concentration of 51 int. units/L. The interassay CV (mean CV of five paired determinations) was 16.9% for values <10 int. units/L and 8.8% for higher values. These performance characteristics are similar to those described by Schlageter et al. (6).

Clinical Results

Comparison of the EPO values observed with both methods. For all subjects, there was a highly significant correlation between EPO by RIA (x) and EPO by IRMA (y); \( y = 0.712x - 9.862 \) (\( r^2 = 0.939, n = 120, S_{yy} = 15.6 \)). A significant correlation between the two methods was found for each patient's group (Figure 6) except for the seven patients with polycythemia vera. The EPO values measured by IRMA were lower than those measured by RIA. The discrepancy between RIA and IRMA values was greater in the lower part of the range.

Normal range. The mean (SD) EPO concentration measured by the IRMA in the control group was 4.7 (1.9) int. units/L (mean ± 2 SD range: 0.9–8.5). There was no influence of sex or age on the results. These values were lower than those observed with RIA (mean 18, SD 5 int. units/L).

Anemic patients without renal failure and patients with \( \beta \)-thalassemia before transfusion. EPO was increased in these patients, and log EPO was negatively correlated with Hb concentration (Figure 7). As previously shown with the RIA (7), when EPO is measured by IRMA, EPO concentrations relative to the Hb concentrations in thalassemic patients and in patients with another form of anemia were similar; pretransfusion values from thalassemic patients were thus pooled with others in the subsequent comparisons.

Evolution of EPO with transfusion. As has been shown previously with RIA (7), the EPO concentration by IRMA decreased after transfusion and reached a nadir after 5 to 14 days; by this time, the EPO concentration was no
Fig. 7. Relation between Hb and EPO concentrations measured by IRMA (A) and RIA (B) in thalassemic patients (○) and in patients with nonrenal anemia (□).

Results for the thalassemic patients are only for pretransfusion samples. The relations calculated in the whole group of patients were as follows: IRMA: \( y = 3.454 - 0.210x (r = 0.49) \); RIA: \( y = 3.453 - 0.198x (r = 0.52) \).

Fig. 8. Evolution of Hb and EPO measured by RIA (□) or IRMA (○) during a transfusion cycle (n = transfusion) in eight thalassemic patients.

Fig. 9. Relation between Hb concentration and EPO measured by IRMA (A) and by RIA (B) in hemodialyzed patients (○) and in patients with nonrenal anemia (□). Results obtained in the thalassemic patients 6-14 days after transfusion were included. The relations were, for nonrenal anemia: \( y = 3.316 - 0.194x (r = 0.68) \); hemodialyzed patients: \( y = 0.353 + 0.088x (r = 0.36, P < 0.05) \); for RIA, nonrenal anemia: \( y = 3.216 - 0.149x (r = 0.66) \); hemodialyzed patients: \( y = 1.196 + 0.0005x (r = 0.05, P > 0.05) \).

longer significantly different from normal in the hypertransfused thalassemic patients (Figure 8). The profile of EPO evolution was parallel to that observed in RIA, although absolute EPO values were lower at all times of the transfusion cycle when measured with IRMA.

Anemia of renal failure. Mean EPO concentrations were not significantly different from normal in terminal renal failure (Figure 9). However, hemodialyzed patients had an EPO concentration lower than other patients with the same degree of anemia. The discrimination between patients with anemia of renal failure and other anemic subjects was better when IRMA was used for EPO determinations. Moreover, there was a positive correlation between Hb and EPO measured by IRMA. This correlation was not observed in the same group of patients when EPO was measured by RIA.

Polycythemia. As Figure 10 shows, two of the nine patients with secondary polycythemia had a high EPO value; the others were in the normal range. All patients with untreated polycythemia vera and a high Hb concentration had undetectable EPO by IRMA, except for one who had 1.9 int. units/L. In these patients, EPO by RIA varied from 20.9 to 68 int. units/L. When the data
were analyzed by ANOVA (TADPOLE III program; Biosoft Corp.), RIA-measured EPO was significantly lower in patients with untreated polycythemia vera than in normal subjects \( (P < 0.05) \) but did not differ between the two groups of polycythemic patients. Conversely, EPO by IRMA was significantly lower in polycythemia vera than in normal subjects and in subjects with secondary polycythemia.

Discussion

The two-site IRMA for measurement of EPO concentrations in serum performed well in comparison with the RIA, although EPO concentrations measured by IRMA were always lower than those measured by RIA. As did RIA-measured EPO, IRMA values of EPO correlated negatively with Hb concentrations in anemic patients, and both IRMA and RIA showed similar profiles after transfusion, with lower absolute IRMA values. This difference in EPO values measured in patients is not caused by differences in standardization between the two kits. Indeed, we have shown that the recoveries by the two methods of MRC 67/343, both in zero standard and in plasma, were similar. The fact that the difference between values observed by the two methods was larger for low EPO values indicates that one of the factors responsible could be the detection by RIA of a nonspecific immunoreactivity in serum. However, even for higher EPO values, the IRMA and RIA measurements of EPO differed by a factor of two; this suggests that the methods recognize different forms of circulating EPO.

The CVs of the IRMA were quite small, indicating better precision than the RIA. The lowest concentration that could be reliably measured was about 10-fold less for IRMA than for RIA: 0.5 int. unit/L. In this range, the IRMA showed satisfactory parallelism between determination of EPO in sera and in standards. Again, this difference in sensitivity is not the result of a difference in standardization, as shown by the studies with the international reference preparation.

Its greater sensitivity makes the IRMA particularly suitable for studying low EPO values. The EPO concentrations of hemodialyzed patients, when plotted against the corresponding Hb concentrations, were indeed better discriminated from those of nonrenal anemia when EPO was measured by the IRMA. Moreover, a positive correlation between Hb and EPO in hemodialyzed patients became apparent when EPO was measured by the IRMA. This extends to the normal range of EPO the correlation demonstrated with EPO measured by RIA in patients with polycystic renal disease and high EPO concentration \( (8) \).

This low sensitivity could potentially be useful in the differential diagnosis of polycythemia. Our preliminary results in a small group of patients with polycythemia vera are encouraging from this point of view, because their EPO values were distinguishably lower than those in secondary polycythemia and also lower than the normal range.

In conclusion, this IRMA of serum EPO is rapid and highly reproducible. Its main advantage is its high sensitivity, which allows one to explore EPO concentration in the normal and subnormal ranges.

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References


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