diabetic nephropathy (17), polycystic kidney disease (9), and other or nonspecified kidney disease (38)] and 12 healthy individuals (of those, 2 were excluded because of urinary albumin >30 mg/24-h) in a cross-sectional study approved by the local ethics committee (No. 20020055). Plasma and urine samples (24-h urine and second-void morning urine) were stored immediately at −80 °C and, without thawing, moved to a −20 °C freezer before measurement. We measured plasma Gc by our immunonephelometric method on a Behring Nephelometer 2 (Dade Behring) (9) and albumin and creatinine on an Integra 700 (Roche). We used Kruskal–Wallis and Mann–Whitney tests for comparisons between groups and the Spearman nonparametric method to look for correlations. We performed the calculations with SPSS 10.0.5 and set 0.05 as the significance level.

In the 99 patients, the median 24-h excretions of Gc and albumin did not differ significantly between patients in the various kidney disease groups but were significantly (P < 0.001) higher in patients than in healthy controls (Table 1). Measurement of Gc in second-void morning urine gave a good estimate of the 24-h urinary excretion, equivalent to that given by the Gc/creatinine ratio, as seen from the high correlations in Table 1. Urinary Gc did not correlate with plasma Gc, but correlated with markers of kidney disease, particularly with urinary albumin excretion (Table 1). Linear regression with Gc as the dependent and albumin as the independent variable (after In transformation to obtain approximate gaussian distributions of residuals) showed a highly significant relationship in urine [r = 0.86; P < 0.001; mean (95% confidence interval) slope, 1.2 (1.04–1.3); mean (95% confidence interval) intercept, −4.6 (−5.5 to −3.7)], but not in plasma (P = 0.23). The median urinary Gc/albumin ratio was significantly lower in patients with glomerulonephritis than in patients with polycystic, other, or nonspecified kidney diseases. The correlation between creatinine clearance and the Gc/albumin ratio in 24-h urine was high (r = −0.54; P < 0.001) as was the correlation in second-void morning urine (r = −0.59; P < 0.001). Longitudinal studies, however, are needed to test the utility of the urinary Gc/albumin ratio as a marker of kidney function.

In the kidneys, both Gc and albumin are believed to be filtered in the glomerulus and subsequently reabsorbed by megalin-cubulin–mediated endocytosis in the proximal tubule (10). Because of this shared fate, the high correlation between urinary Gc and urinary albumin was expected. However, contrary to the negative correlation between plasma and urinary albumin, we found no significant correlation between plasma and urinary Gc. The missing decrease in plasma Gc concentration is in agreement with findings in megalin-knockout mice (6) and in some studies of patients with kidney diseases (11, 12), but not in others (13, 14).

In conclusion, we have developed a sensitive automated ELISA capable of measuring very low Gc concentrations with low imprecision (functional detection limit, 0.01 nmol/L). In 99 patients, the urinary loss of Gc increased with increasing severity of kidney disease, but had no relationship with plasma Gc concentration.

We thank Nyrevenningsen, the Institute of Clinical Medicine at Aarhus University, the County of Funen Research Foundation, and the Aarhus University Research Foundation for financial support and Lene Dabelstein Petersen for technical assistance.

**References**


**DOI:** 10.1373/clinchem.2004.045658

**Serum Erythropoietin Measured by Chemiluminescent Immunometric Assay: An Accurate Diagnostic Test for Absolute Erythrocytosis, Pascal Mossuz, François Girodon, Sylvie Hermouet, Irène Dobo, Eric Lippert, Magali Donnard, Veronique Latger-Carrand, Nathalie Boiret, Vincent Praloran, Jean Claude Lecron.**

DOI: 10.1373/clinchem.2004.045658

**Serum Erythropoietin Measured by Chemiluminescent Immunometric Assay: An Accurate Diagnostic Test for Absolute Erythrocytosis, Pascal Mossuz, François Girodon, Sylvie Hermouet, Irène Dobo, Eric Lippert, Magali Donnard, Veronique Latger-Carrand, Nathalie Boiret, Vincent Praloran, Jean Claude Lecron.**

DOI: 10.1373/clinchem.2004.045658

**Serum Erythropoietin Measured by Chemiluminescent Immunometric Assay: An Accurate Diagnostic Test for Absolute Erythrocytosis, Pascal Mossuz, François Girodon, Sylvie Hermouet, Irène Dobo, Eric Lippert, Magali Donnard, Veronique Latger-Carrand, Nathalie Boiret, Vincent Praloran, Jean Claude Lecron.**

DOI: 10.1373/clinchem.2004.045658

**Serum Erythropoietin Measured by Chemiluminescent Immunometric Assay: An Accurate Diagnostic Test for Absolute Erythrocytosis, Pascal Mossuz, François Girodon, Sylvie Hermouet, Irène Dobo, Eric Lippert, Magali Donnard, Veronique Latger-Carrand, Nathalie Boiret, Vincent Praloran, Jean Claude Lecron.**

DOI: 10.1373/clinchem.2004.045658
Absolute erythrocytosis (AE), suspected from a high hemoglobin concentration and/or hematocrit, can be confirmed by an increased red cell mass (RCM) (1). Schematically, one distinguishes three major mechanisms of AE: (a) erythropoietin (Epo)-independent proliferation of clonal erythroid precursors as found in polycythemia vera (PV) and other myeloproliferative disorders; (b) Epo-dependent polyclonal proliferation of erythroid precursors as found in secondary erythrocytoses that are secondary to production of Epo as a consequence of either a physiologic response to tissue hypoxia or of tumoral production; (c) idiopathic erythrocytoses (IEs) in patients without evidence of PV or secondary erythrocytoses (2).

The serum Epo concentration reflects its oxygen-regulated production by kidney. Thus, serum Epo is decreased in PV and increased in secondary erythrocytoses. Use of serum Epo as a diagnostic test for PV (3–5) is controversial (6–8). Indeed, until recently, the lack of standardization of the reagents and methods impeded identification of reliable thresholds. As a consequence, the diagnosis of PV is still largely based on exclusion and/or indirect clinical and biological criteria initially proposed by the Polycythemia Vera Study Group (PVSG) (9). However, the WHO guidelines (10), which are based on major criteria (e.g., splenomegaly, lack of secondary erythrocytosis) and minor criteria (e.g., modification in blood cell count, bone marrow histology), recently classified the endogenous erythroid colony assay and serum Epo measurements as major and minor PV diagnostic criteria, respectively.

We recently demonstrated in a large multicenter study (n = 241) that a commercial ELISA for serum Epo was a reliable and accurate biological diagnostic test in patients with AE (11). In this study, we determined a low Epo threshold with 65% sensitivity and 100% specificity for the diagnosis of PV and a high Epo threshold with 19.7% sensitivity and 100% specificity for secondary erythrocytoses. However, this ELISA, which is not automated, could be negatively impacted by technical and/or interindividual bias limiting interlaboratory reproducibility; in addition, it is not suitable for a short series of samples.

A fully automated chemiluminescent enzyme-labeled immunometric assay (Immulite; DPC) has been developed that is suitable for routine assay of serum Epo in individual samples as well as in small or large series of samples (12, 13). We compared the sensitivity, specificity, and predictive values of this automated method with those of the ELISA for the diagnosis of PV and secondary erythrocytoses.

From 2001 to 2004, 193 samples from patients with suspected AE (hematocrit >50% for males and >45% for females) were collected in 8 university hospitals. Recruitment was in agreement with standards of the ethics committee “Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale” (CCPPRB). AE was established by isotopic determination of RCM in 137 patients before any treatment. Patients were classified according to WHO guidelines (10) as follows: 81 PV, 53 secondary erythrocytoses, and 3 IE. The demographic and clinical characteristics of the patients are summarized in Table 1. Blood was harvested at diagnosis in dry tubes and centrifuged 15 min at 1400g after blood clotting, and the sera were frozen at −80°C.

The sera from the 137 AE patients were assayed for Epo by trained technicians in a blind manner in two indepen-

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>PV</th>
<th>SE*</th>
<th>IE</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/F</td>
<td>81</td>
<td>53</td>
<td>3</td>
</tr>
<tr>
<td>Median (range) age, years</td>
<td>67 (34–91)</td>
<td>59 (25–88)</td>
<td>55 (33–82)</td>
</tr>
<tr>
<td>Mean (SD) RBC, × 10^12/L</td>
<td>6.8 (0.97)</td>
<td>5.7 (0.55)</td>
<td>5.4 (0.88)</td>
</tr>
<tr>
<td>Mean (SD) Hb, g/L</td>
<td>18.8 (1.6)</td>
<td>17.7 (1.6)</td>
<td>16.4 (2.9)</td>
</tr>
<tr>
<td>Mean (SD) Ht, %</td>
<td>58 (6)</td>
<td>53 (5)</td>
<td>50 (9)</td>
</tr>
<tr>
<td>Mean (SD) platelets, × 10^9/L</td>
<td>446 (198)</td>
<td>209 (56)</td>
<td>286 (107)</td>
</tr>
<tr>
<td>Mean (SD) WBC, × 10^9/L</td>
<td>12.8 (1.7)</td>
<td>9.4 (1.4)</td>
<td>8.0 (0.5)</td>
</tr>
</tbody>
</table>

ELISA Epo, IU/L

| Mean (SD) | 1.69 (2.19) | 10.40 (6.80) | 8.33 (2.7) |
| Median (range) | 0.8 (0.6–13.7) | 8.2 (1.45–33.9) | 7.7 (6–11.3) |

Immulite Epo, IU/L

| Mean (SD) | 2.38 (1.60) | 13.07 (7.95) | 10.86 (2.63) |
| Median (range) | 1.6 (0.25–13.0) | 11.7 (2.8–40.1) | 10.1 (8.7–13.8) |

Correlation coefficient (r)/slope

| 0.79/1.11 | 0.87/1.14 | 0.99/1.07 |

*a Patients with AE were classified into 3 groups according to the WHO classification of tumors of hematopoietic and lymphoid tissues as PV, secondary erythrocytosis, and IE. Serum Epo concentrations were measured by ELISA and chemiluminescent immunometric (Immulite) methods according to the procedures described in the text and in the manufacturers’ instructions. Regression analysis was performed using Deming regression method.

*b SE, secondary erythrocytosis; WBC, leukocytes; RBC, erythrocytes; Hb, hemoglobin; Ht, hematocrit.

*c Pearson correlation.
dent laboratories. The assays used, the Epo ELISA (Quantikine IVD Erythropoietin ELISA; R & D Systems Inc.) and a two-site sandwich immunoassay with chemiluminescent detection on an automated random access immunoassay analyzer (Immulite; DPC), were performed according to the manufacturers’ instructions. Values were expressed as IU/L. Manufacturer reference intervals for serum Epo were 3.3–16.6 IU/L for the ELISA and 4.1–20.1 IU/L for the chemiluminescence immunoassay. We performed statistical analysis of the sensitivity and specificity of the Epo ELISA and chemiluminescence assay with Statview software, Deming regression and correlation analysis with Analyze-It software, and ROC curve analysis and predictive values with Stata, Ver. 7.0.

We first evaluated the performance characteristics of the automated chemiluminescent immunoassay for Epo (Immulite). The within-run imprecision (CV) was 6.8% and 10% for sera at 28.2 and 0.95 IU/L, respectively (n = 8). The interassay CV was 9.2%, as determined by 12 independent measurements of a sample containing 28.2 IU/L Epo. The apparent recovery of Epo added in a 1:1 ratio to sera containing 13 and 0.5 IU/L Epo was 105%. To assess assay linearity, a serum containing 124 IU/L Epo was diluted 1:5, 1:10, 1:100, and 1:1000 in the sample diluent. Compared with theoretical values, the measured concentrations were, respectively, 107%, 106%, 110%, and undetectable (for the 1:1000-diluted sample; theoretical value = 0.124 IU/L) in 1 representative experiment of 2. As reported previously (12, 13), all of these tests confirmed that the Immulite Epo assay is reproducible and accurate. We tested Epo stability in a serum containing 9.6 IU/L. Measured concentrations after 1, 2, and 7 days were, respectively, 104%, 96%, and 93% of initial values in samples stored at 4°C and 105%, 95%, and 98% in samples stored at 20°C (1 representative experiment of 3).

After 1–4 freeze–thaw cycles, values for 1 sample were 100%, 103%, 98%, and 89% of initial values, respectively. Thus, sera can be stored up to 1 week at 20°C or frozen/thawed up to 3 times before being assayed. In 17 paired serum and EDTA-plasma (Vacutainer; Becton Dickinson) samples, Epo concentrations were 33% lower in plasma, with a correlation coefficient of 0.99, and SD of the residuals of 2.25 IU/L. Taking into account this difference and according to manufacturer’s instructions, we recommend avoiding EDTA-plasma for measurements of Epo by the Immulite method.

The mean (SD) difference between the Immulite assay and the ELISA was 1.54 (2.61) IU/L with one major outlier (13.6 IU/L for the ELISA and 36.9 IU/L for the Immulite assay). As shown in Table 1, the measured Epo concentrations for all groups of patients were significantly higher with the Immulite assay than with the ELISA (P = 0.017), but the results were highly correlated as evidenced by regression analysis [n = 137; r = 0.93; slope = 1.17 (95% confidence interval, 1.09–1.25); intercept = 0.44 (0.23–1.13) IU/L]. Correlations between the two methods remained excellent and highly significant when results for each group of patients were considered separately: PV, n = 81, r = 0.79 (P < 0.001); secondary erythrocytoses, n = 53, r = 0.87 (P < 0.001); IE, n = 3, r = 0.99 (P < 0.001).

A cutoff of 2.8 IU/L provided 100% specificity (95% confidence interval, 95%–100%) and 78% sensitivity (68%–85%) for the diagnosis of PV, and a cutoff of 13.8 IU/L provided 100% specificity (95%–100%) and 34% sensitivity (23%–47%) for the diagnosis of secondary erythrocytoses. For 59% of the 137 untreated AE patients, the results were outside these 2 thresholds. By comparison, the Epo ELISA thresholds of 1.4 and 13.7 IU/L (also defined by ROC curve analysis as giving 100% specificity and 100% positive predictive value) allowed the direct diagnosis of 49% of the 137 untreated patients with AE. Sera with Epo concentrations lower than the detection limit of the ELISA (<0.6 IU/L) were measurable by the Immulite assay [mean (SD), 1.37 (0.75) IU/L; median (range), 1.1 (0.25–3.3) IU/L]. The Epo concentration was below the detection limit of the Immulite assay (0.25 IU/L) in only 1 patient. The better differentiation of low Epo concentrations by the Immulite assay could be of prognostic interest in PV patients because low serum Epo values correlate with a high risk of thrombosis (5, 14). Indeed, we found significant inverse correlations between Epo concentrations and RCM, hematocrit, and hemoglobin (n = 81). For RCM, r = −0.33 (P = 0.01); for hematocrit, r = −0.31 (P = 0.004); and for hemoglobin, r = −0.37 (P = 0.006).

In conclusion, this study performed with an automated chemiluminescent immunometric method on a large group of polycythemic patients defined low and high serum Epo thresholds (2.8 and 13.8 IU/L) that allowed the presumptive diagnosis of 78% of PV and 34% of secondary erythrocytoses, without further investigation. A low Epo concentration having been validated as a criterion for PV diagnosis by the WHO classification (10), our study demonstrates that serum Epo is a major biological criterion for the diagnosis of PV and secondary erythrocytoses in patients with confirmed AE and supports development of a new diagnostic strategy based on serum Epo. The ability to measure serum Epo in small series and in individual samples (because of a random-access immunoassay analyzer) should facilitate and increase use of this assay as a first-line test.

Hence, in accordance with the new WHO PV diagnostic criteria, we propose use of the Epo assay in a first step with determination of the RCM in patients with suspicion of AE. We suggest that more complex, time-consuming, or costly tests (e.g., clonogenic cultures, PRV-1 determination, and/or bone marrow histology) should be performed in a second step for patients whose serum Epo results are inconclusive.

We are grateful to our colleagues in the various clinical departments for providing clinical data. This work was financed by a grant from the French Ministry of Health (PHRC région Bourgogne). We also greatly thank Dr. J.L. Bosson from the clinical center of investigation (CHU-Grenoble, France) for expert assistance in the statistical study.
New Enzymatic Assay Using Phospholipase D to Measure Total Calcium in Serum, Mitsutoshi Sugano, Kazuyoshi Yamauchi, Keiko Sugano, Kenji Kawasaki, Minoru Tozuka, Tsutomu Katsuyama, Haruyo Soya, Tatsuhiko Tanaka, Shigeyuki Imamura, and Shozo Nomoto (1) Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan; 2 Clinical Laboratory Center, The University of Tokyo Hospital, Tokyo, Japan; 3 Department of Laboratory Medicine, Shinshu University School of Medicine, Matsumoto, Japan; 4 Shino-test Corporation, Kanagawa, Japan; 5 Asahi Kasei Pharma Corporation, Shizuoka, Japan; * address correspondence to this author at: Department of Laboratory Medicine, Shinshu University Hospital, 3-1-1 Asashi, Matsumoto 390-8621, Japan; fax 81-263-34-5316, e-mail yamauchi@hsp.md.shinshu-u.ac.jp

Various methods have been used to measure calcium in body fluids. Atomic absorption spectrophotometry (AAS) is the most reliable (1), but it requires special instrumentation. The most widely used method involves colorimetric detection of calcium complexes by o-cresolphthalein complexone (o-CPC) (2, 3) or arsenazo. With the o-CPC method, reagent stability and recoveries at low concentrations are poor, and magnesium interferes in the reaction (4). Newer methods using o-CPC (5–7) or other colorimetric agents (8–10) are not entirely satisfactory.

Various enzymatic methods have been described, including methods using porcine pancreatic α-amylase (EC 3.2.1.1) (11), phospholipase D (PL-D; EC 3.1.4.4) (12, 13), and urea amidolyase (14). The first two are based on activation of enzymes by calcium, whereas the third is based on inhibition of the enzyme by calcium. The α-amylase method is reportedly inaccurate for patients with hyperamylasemia (11), and the other 2 methods each require 2 reaction steps (12). In this report, we describe a new, simple, specific enzymatic assay based on activation of PL-D. We investigated the assay characteristics and its suitability for use in routine laboratory tests.

We obtained 126 serum samples from patients admitted to Shinshu University Hospital after receiving informed consent from the patients and approval by our institutional ethics committee.

We obtained PL-D from Streptomyces chromofuscus (15); Asahi Kasei Pharma, bis(p-nitrophenyl) phosphate (BNPP) from Kanto Chemical Co., Good’s buffer from Doujin Laboratories, and SRM 915 and 909a from NIST. The reagent sets for the o-CPC and α-amylase methods were from Serotec Co. Ltd. and Ono Pharmaceutical Co. Ltd., respectively. Other reagents were analytical grade (Wako Pure Chemical).

The new method is based on increased PL-D-catalyzed hydrolysis of BNPP by calcium ions, as follows. The p-nitrophenol released by the reaction is detected at 405 nm (Scheme 1).

The assay is a 2-point fixed-rate assay performed at 37 °C; for our experiments we used a Hitachi 7170 analyzer. Briefly, 9.5 μL of sample was mixed with 160 μL of solution containing 750 U/L PL-D and 0.275 mmol/L calcium acetate in 60 mmol/L Good’s buffer (pH 7.5). After incubating the mixture for 5 min at 37 °C, we added 160 μL of reagent containing 1.5 mmol/L BNPP in 60 mmol/L Good’s buffer (pH 7.5) and measured the absorbance at time points 23–34 (6.76–10.00 min). We performed the o-CPC and α-amylase methods on the same analyzer according to the manufacturer’s instructions.

For the AAS method (1), we used a 0.1-mL sample on a Hitachi Z-5000 Atomic Absorption Spectrophotometer (flame-type analyzer) equipped with an autosampler and a multirange micropipetting device (Gilson Inc.) with triple rinsing of the inside of the tip. We increased sample

References


Previously published online at DOI: 10.1373/clinchem.2004.047365