Chapter 5

Comparison of two immunoassays to establish the serum ECP concentration

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Abstract

To interpret results of serum ECP concentrations between studies, it is necessary to establish the differences in results obtained with different tests. Two commercially available tests, the fluorescence immunoassay from Kabi Pharmacia and the chemiluminescent immunometric assay of Diagnostic Products Corporation, were compared. A relation between the results from these tests of $y = 0.87x + 2.7$ was established. Both tests yield comparable results, except for concentrations of ECP exceeding 80 µg/l. When measuring control samples or calibration standards from Kabi Pharmacia with the assay from Diagnostic Products Corporation, increased concentrations over the whole concentration range were obtained. Discrepancies might be due to matrix effects and recognition of different epitopes by the antibodies used in the tests.

In addition, stability of Eosinophil Cationic Protein during storage of serum samples was measured. Eosinophil Cationic Protein in serum was shown to be stable for two weeks at 4°C or for 9 months at -20°C or -80°C. Commercially available control specimens from Kabi Pharmacia were stable at 4°C for at least one year.

Introduction

It is known that inflammatory conditions and parasitic diseases are associated with eosinophilia. Evidence has accumulated that increased numbers of eosinophils may be involved in tissue damage observed in subjects with an allergic constitution (1, 2). When stimulated, eosinophils are able to secrete toxic proteins, amongst others Eosinophil Cationic Protein (ECP). ECP is stored in the granules and is a very potent cytotoxic agent (3, 4). ECP acts by causing membrane damage of parasites or tissue cells (5).

Several studies dealing with results of serum ECP concentrations have been published (6, 7). Standardized preanalytical conditions are important to reduce variations in analytical results (8, 9). To interpret results from several studies it is necessary to establish methodological deviations in results of different tests. Therefore, we studied the performance of two commercially available tests for ECP, *i.e.* the fluorescence immunoassay from Kabi Pharmacia and the chemiluminescent immunometric assay of Diagnostic Products Corporation. The stability of ECP during storage was also established.

Materials and Methods

Serum samples were obtained from outclinic patients. Blood samples were collected by venepuncture in plain tubes (Vacutainer® ref 367703 SST with clot activator, Becton Dickinson, Plymouth, UK). The blood sample was allowed to clot for 120 minutes in a waterbath at 37°C. After incubation, samples were centrifuged for 10 minutes at 1350 x g at room temperature. Serum was separated from the blood clot and stored at -20°C.

Both tests for ECP determination are immunoassays.

In the fluorescence immuno-assay (Kabi Pharmacia, Uppsala, Sweden) monoclonal anti-ECP, covalently coupled to the reaction tube, reacts with the ECP in the patient serum specimen. After washing, enzyme-labeled (β-galactosidase) monoclonal antibody against ECP are added to form a complex. After incubation, unbound enzyme-anti-ECP is washed away and the bound complex is then incubated with a developing agent (4-methylumbelliferyl-β-D-galactoside). The fluorescence is directly proportional to the concentration of ECP in the serum sample.
The ECP assay from DPC (Los Angeles, USA) is a two-site chemiluminescent immunometric assay. The solid phase is coated with a monoclonal antibody specific for ECP. Patient serum sample and alkaline phosphatase-conjugated polyclonal antibody are simultaneously incubated for approximately 30 minutes at 37°C. The added chemiluminescent substrate, a phosphate ester of adamantyldioxethane, undergoes hydrolysis to yield an unstable intermediate which forms two stable compounds, resulting in emission of light. The amount of bound phosphate-conjugated antibody and thus indirectly the amount of ECP bound to the solid phase, is proportional to the concentration of ECP in the sample.

Statistical analysis

Statistical analysis was performed with SPSS (Benelux BV, Gorinchem, The Netherlands; Windows, Release 8.0). To establish the correlation between the assays, regression analysis was performed.
A paired T-test was used for statistical evaluation of results to test the stability of ECP in time. A p-value less than 0.05 was considered to be statistically significant.

Results

In figure 1 the relationship between the Kabi Pharmacia (x axis) and the DPC (y axis) results is shown. The line reflecting the relationship between the results from both tests is calculated as $y = 0.87x + 2.7$ ($r = 0.96$, $p<0.01$). A few samples with serum ECP concentrations exceeding 80 µg/l showed discrepancies; lower results were measured with the DPC method than with the method of Kabi Pharmacia. The bias between these values was also measured when the samples were diluted.

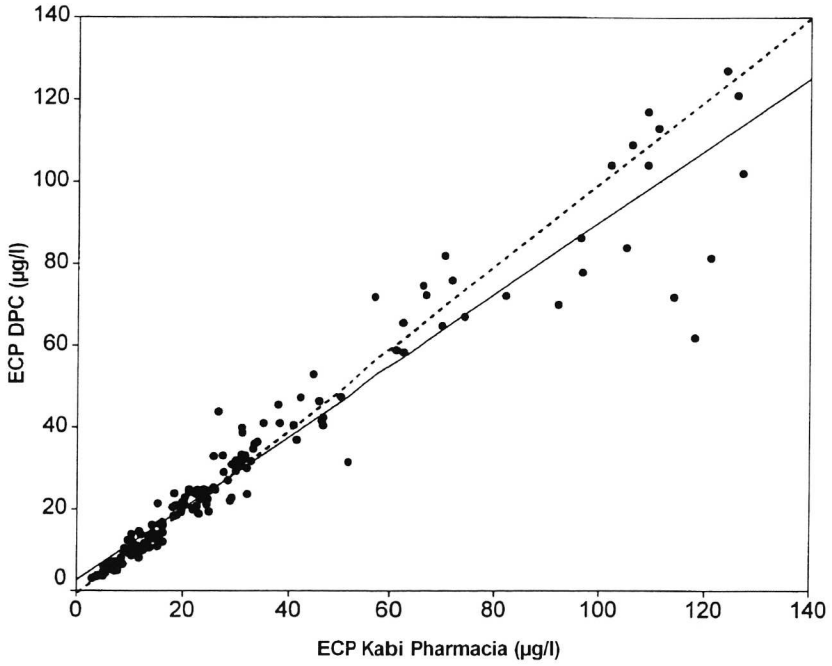

Figure 1: Correlation between ECP values measured with Kabi Pharmacia and DPC assay. Solid line, $y = 0.87x + 2.7$ ($r = 0.96$, $p<0.01$). Broken line, $y = x$.

When serum samples were diluted with the diluent provided by the Kabi Pharmacia test, three times higher results in the DPC assay were measured, while in the Kabi Pharmacia assay no influence on the results was measured in several dilutions with this diluent. The standards of the Kabi Pharmacia assay also yielded deviating results in the DPC assay and vice versa (table I).

Table I: Results of control samples and calibration standards measured with the two tests (Kabi Pharmacia and Diagnostic Products Corporation (DPC)). Mean data of duplicate assays are shown.

Sample	ECP (µg/l) measured with Kabi Pharmacia	ECP (µg/l) measured with DPC
control Kabi Pharmacia	65	> 200
cal. standard Kabi Pharmacia	2	5
cal. standard Kabi Pharmacia	5	13.1
cal. standard Kabi Pharmacia	15	48.6
cal. standard Kabi Pharmacia	100	>200
cal. standard Kabi Pharmacia	200	>200
control DPC	2.6	3.7
control DPC	20.7	29.4
adjustor DPC	<2	2
adjustor DPC	23.2	33.8

ECP in serum, frozen for 12 months at -20°C or -80°C, showed appropriate stability (table II). A slight increase of about 15% was found during storage, but this increase was not statistically significant. One day storage at room temperature did not show deterioration. Stability of ECP was found for two weeks at 4°C. Storage for more than two weeks at 4°C gave a significant decrease in ECP concentration. Control specimens, commercially available from Kabi Pharmacia, stored at 4°C, showed stability for one year. The control sample, measured twice a month, demonstrated a mean value of 67 μg/l together with a coefficient of variation amounting to 6.5% (n = 25). However, when a control sample was mixed with a serum sample, a decrease of 14% was found after a storage period of one day at 4°C and a decrease of 36% after 5 weeks at 4°C.

Table II: ECP recovery percentages (mean ± sd) with respect to day 0 during the mentioned time interval at mentioned temperatures, regarding 11 patient samples. The ECP concentrations in these patients ranged from 5 to 115 µg/l, with a mean of 35 µg/l.
** statistically significant deviation compared with day 0.*

storage interval	ECP in serum samples				ECP in control samples
	room temperature	4°C	-20°C	-80°C	4°C
day 0	100	100	100	100	100
day 1	106 ± 11	105 ± 13			106
week 1		108 ± 11			117
week 2		98 ± 21			122
week 4		71 ± 14*	117 ± 21	125 ± 20	106
week 6		53 ± 15*			106
month 2			117 ± 23	116 ± 20	99
month 4			117 ± 22	116 ± 16	106
month 7			113 ± 22	113 ± 16	107
month 9			124 ± 29	120 ± 21	96
month 12			107 ± 25	111 ± 18	94

Discussion

The Kabi Pharmacia and DPC immuno-assay methodologies revealed uniform reference ranges. Linear regression analysis with respect to these two methodologies, as provided in the DPC manufacturer's guide showed another correlation ($y = 1.16x + 1.46$; $r = 0.988$) than we have determined. At higher ECP concentrations we found lower levels with the DPC method. The bias between these values was also measured when the samples were diluted. This may be explained by the fact that both methods use different antibodies to detect the amount of ECP. During release of higher amounts of ECP other epitopes can be formed, for example due to different stages of glycosylation. Possibly the polyclonal antiserum detects "neo-epitopes" of ECP when samples are diluted in a dilution solution of Kabi Pharmacia.
Control samples showed deviating results with both tests. When measuring the control samples or calibration standards from Kabi Pharmacia with the test assay from Diagnostic Products Corporation considerably higher results were found than those given by Kabi Pharmacia. We assume that these results can be explained by the fact that the matrix of control specimens is different from patients' sera. Binding of antibodies to the epitope may be influenced by the matrix.
To explain the deviation of a few patient samples and the control samples, the stability of ECP should be taken into consideration. Storage of samples is allowed for one day at room temperature, two weeks at 4°C or at least 9 months at -20°C or -80°C. A remarkable finding is the control stability of one year at 4°C of specimens, while mixing these control samples with patients' serum give a rapid decrease in results during storage. Proteases present in serum may lead to ECP degradation in the standard solution.

In conclusion, within the reference range both tests yielded comparable results with patients' samples. Therefore, it is allowed to compare the results from studies that apply either of these two different immuno assays. The differences between both methods,

measured in control samples or in patient samples with higher ECP concentrations, cannot be explained by sample instability. It is more likely that the discrepancies are due to detection of different epitopes of ECP, when present in a higher serum concentration, or to matrix effects.

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