A new turbidimetric method for assaying serum C-reactive protein based on phosphocholine interaction

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Abstract

Background: C-reactive protein (CRP) is able to bind phospholipids (mainly phosphocholine) in the presence of calcium ions. We investigated the use of this property for developing an affordable turbidimetrical CRP assay based on diluted soy oil.
Methods: Serum (or heparinized plasma) was mixed with Intralipid® 20% in Tris-calcium buffer (pH 7.5). After 30 min of incubation at 37°C, the CRP-phospholipids complexes were measured by turbidimetry (660 nm/700 nm) with a Cobas 6000 analyzer (Roche). Results were compared with those obtained using a typical immunoturbidimetric method (Roche).
Results: Good correlation ($r^2 = 0.931$) was obtained between the functional and the immunoturbidimetric CRP assay. Within-run and between-run %CV values for the functional assay were 2.4% (100 mg/L); 6.0% (50 mg/L); 10% (20 mg/L), and 3.6% (100 mg/L); 8.0% (50 mg/L); 11% (20 mg/L), respectively. The limit of detection was 7 mg/L. Results were not affected by serum calcium, triglyceride, or phospholipid concentrations.
Conclusions: The functional CRP assay allowed measurement of CRP in serum and plasma in the range of 7 mg/L–400 mg/L. The assay is particularly suited in conditions where resources are restricted. Since the assay is species independent, the described functional CRP assay could be used for veterinary purposes as well.
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Keywords: C-reactive protein; immunoturbidimetry; L-index; phospholipids; soy oil.

Introduction

C-reactive protein (CRP) is an acute phase protein found in humans and most other animals (1). Following infection or tissue injury resulting from a variety of inflammatory causes, trauma or burns, serum CRP concentrations rise rapidly and markedly and may attain levels more than 1000 times greater than normal concentrations. The magnitude of the serum CRP response reflects the extent of tissue injury and may predict the course of ensuing illness (2, 3). Recently, an association between minor increases in CRP (after excluding active inflammatory conditions) and future major cardiovascular events has been recognized. This has led the Centers for Disease Control and the American Heart Association to recommend measurement of high sensitivity CRP in patients at intermediate risk of coronary heart disease (4, 5).

In the routine clinical laboratory, serum CRP is usually measured using turbidimetric or nephelometric immunoassays (5). However, these techniques are often not affordable in low-income countries, particularly in sub-Saharan Africa. In the district hospitals of these countries, due to resource-limited health care systems, patients suffering from fever are often treated empirically with antibiotics based on clinical suspicion only and without any benefit of laboratory evidence for bacterial infection. Since responses to CRP occur more frequently in bacterial compared with viral infections (2, 6–8), and values drop rapidly after recovery or treatment, measuring and charting serum CRP may contribute to the diagnosis and management of infectious diseases and other inflammatory conditions.

In addition, the use of immunoassay methods for the measurement of CRP in veterinary medicine does not readily detect CRP from unrelated species due to the absence of common antigenic sites. Therefore, the development of an affordable non-immunological method for CRP assay could also be applied for veterinary purposes (9, 10).

Calcium-mediated CRP binding to immobilized phosphocholine, in association with enzyme-labeled antibody detection of captured CRP, has been proposed as an alternative method for measuring CRP (9). Heegaard et al. (10) described an ELISA for the determination of pig and human CRP based on the affinity of CRP for phosphocholine using synthetic globular polymers as scaffolds for the multivalent display of phosphocholine molecules. Similarly, preliminary studies have demonstrated the potential use of soya oil emulsions as a functional CRP reagent (11). Both in vitro and in vivo, CRP has been reported to produce agglutination ('creaming') of the intravenously administered lipid suspension Intralipid® (12, 13). Similarly, CRP also produced agglutination of isolated normal very low-density lipoproteins (VLDL) (14, 15). Intralipid® is a fat emulsion; the size distribution of the lipid particles corresponds to a diameter of 0.78 ± 0.21 μm (16, 17). The dimensions of these particles are in the same magnitude as the wavelength of visible light (18).

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The observations dealing with CRP and phosphocholine tempted us to explore the possibilities of developing an affordable functional method for assaying CRP in serum using soy oil emulsions containing phosphocholine (Intralipid®) as a natural and cheap turbidimetric reagent.

Materials and methods

Samples

Serum was obtained by collecting blood into blood tubes and centrifuging at 2000 g for 20 min. One hundred and three randomly selected serum samples with a broad range of CRP concentrations from 1 to 400 mg/L were obtained from the routine laboratory, or stored at 4°C for up to 1 week prior to testing. The suitability of heparinized plasma for use with the assay was investigated by analyzing heparinized plasma obtained in parallel from 10 patients with serum CRP concentration between 10 and 250 mg/L. Sera from patients suffering from hyperlipoproteinemia type IV with high CRP concentrations were used as a source for VLDL-CRP complexes.

Immunoturbidimetric assay

The latex-enhanced immunoturbidimetric assay for CRP, regarded as a standard technique, was carried out using Tina-quant CRP reagent (Roche, Mannheim, Germany), with a Modular P analyzer (Roche, Mannheim, Germany). Standardization was performed using a commercial standard traceable to CRM 470 (19–21) (Roche, Mannheim, Germany).

Functional CRP assay

Hydroxymethyl aminomethane (Tris) and calcium chloride were purchased from Merck (Darmstadt, Germany): 10 μL of serum was diluted in 960 μL of Tris-calcium chloride buffer (Tris 0.1 mol/L; 0.1 mol/L calcium chloride; pH was adjusted to 7.5 by hydrochloric acid 1 mol/L). Next, 20 μL of Intralipid® 20% was added. Following preincubation for 30 min in a small laboratory oven at 37°C, samples were thoroughly shaken. The L-index of the mixture was measured turbidimetrically with a bichromatic technique at 660 and 700 nm with a Cobas 6000 analyzer (22, 23). The functional assay was standardized using the same standard as the immunoturbidimetric method.

Intralipid® 20% (Fresenius Kabi, Uppsala, Sweden) is a fat emulsion for intravenous infusion containing, per liter, 200 mL of soybean oil, 12 mL of egg phospholipids, and 22 mL of glycerin, with the balance made up by water.

Study of the agglutination reaction

Following incubation of serum samples (CRP ranging from 1 to 190 mg/L) with the buffered fat emulsion for 30 min (37°C), the mixtures were diluted 3 times in phosphate buffered saline (pH 7.4, 0.1 mol/L). Forward and side scatter of particles was evaluated using a Cytomics FC500 flow cytometer (Beckman Coulter, 92835 Fullerton, CA, USA). This instrument is equipped with an argon-ion laser (wavelength 488 nm). Side scatter is detected at an angle of 90°, forward scatter is measured at an angle of 1–19° to the axis of the laser beam.

The kinetics and stability of the interaction between CRP and phosphocholine were investigated by turbidimetric monitoring (700 nm) of the incubated mixture (at 25°C and 37°C) in serum samples (CRP concentration: 50 mg/L) with Tris-calcium buffer and Intralipid®, after a tenfold dilution with the same phosphate buffered saline in a 1-cm cuvette using a Beckman DU-70 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Sensitivity-linearity

The lower limit of detection (24) was calculated as the mean value +3 standard deviation (SD) for a blank sample. Linearity was evaluated in the concentration range between 10 and 400 mg/L.

Specificity

The calcium dependency of the turbidimetric reaction was evaluated following the addition of EDTA (final concentration 0.34 mol/L) to the Tris-calcium chloride buffer.

In order to investigate the possible effects of confounding factors, serum calcium, cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, phospholipids, and total and direct bilirubin were measured in parallel using standard methods.

Reproducibility – analytical performance

Three human serum pools with CRP concentrations of ~20 mg/L, 50 mg/L and 100 mg/L were used to assess the precision of the assay. The within-run coefficient of variation (CV) was determined as the average of 10 measurements obtained each day. The between-run variance was calculated from the variance of the means obtained each day over a 10-day period, which was also used to determine the within-day variance component.

VLDL-associated CRP

Ultracentrifugation of sera (n=6) containing high concentrations of VLDL particles (triglycerides >5 mmol/L) and CRP (>20 mg/L) was performed using the adjusted Esterbauer method according to Terpstra et al. (25, 26). Subsequently measurement of CRP concentration and the L-index was performed on the obtained VLDL-fractions.

Statistics

Results are expressed as the mean (SD). Agreement between functional methods and immunonephelometry was evaluated using Passing-Bablok regression. Multiple regression analysis was used to investigate potential confounding factors; p < 0.05 were considered significant.

Results

Formation and stability of CRP-phosphocholine complexes

After mixing serum containing CRP with the buffer solution containing the soy oil emulsion, a complex was formed between CRP and the phosphocholine. Figure 1 shows the flow cytometric analysis of the formed CRP-lipid complexes following a 30-min incubation time (37°C). At high CRP concentrations, increased forward scatter is observed, indicating the formation of large CRP-phospholipid aggregates.

A typical first order reaction was observed. At 37°C, turbidimetric monitoring showed that the reaction was completed in about 45 min. The complex that
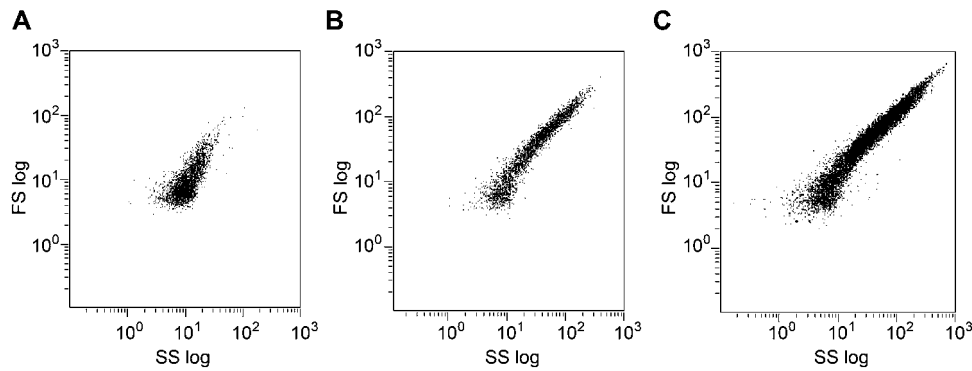
Figure 1 Flow cytometric analysis of CRP-phosphocholine complexes illuminated by the argon-ion laser beam at 488 nm (x-axis: side scatter signal; y-axis: forward scatter signal) following incubation of serum containing 1 mg/L (A); 100 mg/L (B) and 190 mg/L of CRP (C) with soy oil emulsion (30 min, 37°C).

formed was found to be stable for more than 2 h. Figure 2 depicts the kinetics and stability of the CRP-phosphocholine complex process at 25°C and 37°C.

Assay performance

The lower limit of detection of the functional CRP assay was 7 mg/L. The assay was found to be linear in the range between 7 mg/L and 400 mg/L. When serum CRP values exceeded 400 mg/L, a high-dose hook effect was observed. The sensitivity of the method could be considerably improved by increasing two- or three-fold the amount of serum used, but this leads to a more pronounced high-dose hook effect at CRP values exceeding 100 mg/L (results not shown). Within-run CVs ranged from 2.4% (100 mg/L); 6.0% (50 mg/L) and 10% (20 mg/L). Between-run CVs were found between 3.6% (100 mg/L); 8.0% (50 mg/L); 11% (20 mg/L). Table 1 summarizes the reproducibility data.

Serum/plasma comparison

The suitability of heparinized plasma was tested in comparison to serum. Passing-Bablok regression using 10 pairs of samples yielded the following equation: Y (CRP, plasma; mg/L) = 1.01X (CRP, serum; mg/L) −4.91; $r^2 = 0.996$.

Specificity

Addition of EDTA to the reaction mixture resulted in complete absence of the reaction.

Neither hemolysis (free hemoglobin up to 0.24 mmol/L) or icterus (bilirubin up to 400 μmol/L) affected test results. No significant effect of serum phospholipids (up to 345 mg/dL), total cholesterol (up to 7.22 mmol/L), HDL-cholesterol (up to 2 mmol/L), triglycerides (up to 40 mmol/L) and calcium (up to 2.5 mmol/L) was observed on the correlation between the immunological and functional CRP assay.

VLDL-associated CRP

Following ultracentrifugation of serum containing high CRP concentrations ($>$100 mg/L) from patients with hyperlipoproteinemia type IV, the functional CRP method could detect VLDL-associated CRP. A median CRP value of 1.7 mg CRP/mmol triglycerides (range: 0–4 mg/mmol) could be measured.
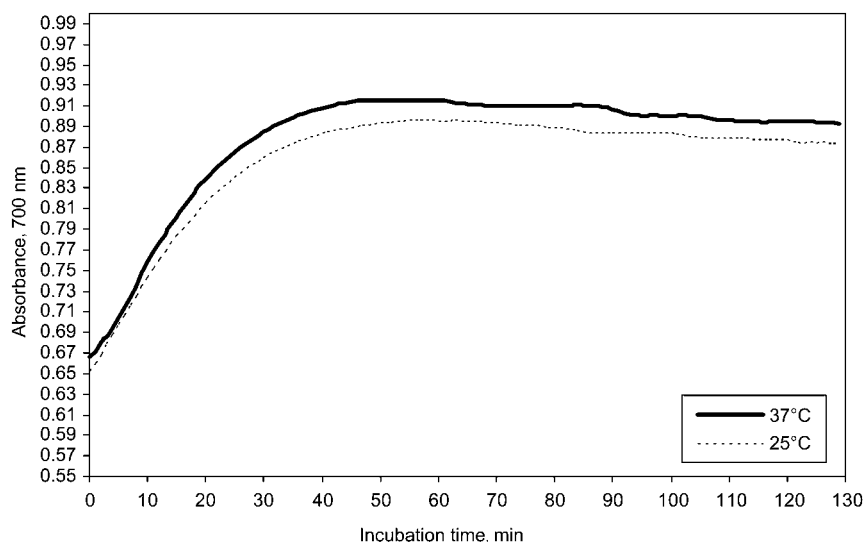
Figure 2 Kinetics and stability of the CRP-phosphocholine based agglutination reaction at 25°C and 37°C. In presence of sera containing 50 mg/L, the change in turbidity (700 nm) is depicted as a function of time.

Table 1   Precision data for the functional CRP assay method.

CRP pools assayed by immunoturbidimetry, mg/L	Within-run[a] (n = 10)			Between-run[a] (n = 10)		
	Mean, mg/L	SD, mg/L	CV, %	Mean, mg/L	SD, mg/L	CV, %
20	17.0	1.7	10	16.5	1.8	11
50	43.4	2.6	6.0	40.0	3.2	8.0
100	99.1	2.3	2.4	108	3.9	3.6

[a]Mean, standard deviation (SD), within- and between-run coefficient of variation (CV) for the functional C-reactive protein (CRP) method.

Method comparison

We found good agreement between the values (mg/L) obtained by the immunoturbidimetric (X) and the functional CRP (Y) methods (n = 103; Y = 1.033X– 6.747; r^2 = 0.932; p < 0.001). Figure 3 shows Passing-Bablok regression between the functional and the immunoturbidimetric CRP methods. Figure 4 shows the difference plot between the immunoturbidimetric and the functional methods. Both CRP methods correlate well over a broad concentration range.

Discussion

Flow cytometric analysis revealed that calcium-dependent agglutination of Intralipid® particles occurs due to the presence of CRP: the size of the formed complexes increase and the size of the aggregates largely exceeds the incident wavelength. Following formation of the complex, Rayleigh scattering loses importance and Mie scattering predominates (18) resulting in a marked increase in the turbidimetric signal. Turbidimetric monitoring of the reaction kinetics confirmed the presence of a calcium-dependent aggregation between CRP and the phosphocholine containing particles.

The assay we developed allows measurement of CRP in serum or heparin plasma in the standard clinical range. Neither pronounced hemolysis, icterus or pre-existing lipemia can significantly affect the resulting turbidity. The effect of sample turbidity using the functional CRP method is very limited because of the high dilution factor of 1:100 used in the assay. In addition, the wavelengths (700/660 nm) chosen for the turbidimetric reading strongly limits optical interference by hemolysis and icterus. Next to free CRP, small amounts of VLDL associated CRP (12, 15) could be detected with the functional assay.

The observed imprecision CV of the described functional CRP assay exceeds the one described for the immunoturbidimetric assay (27). However, the functional CRP assay fulfills the imprecision criteria based on the biological variability of the analyte (28). Although not suited as a high sensitivity CRP assay, the functional soy oil emulsion based CRP assay allows measurement of concentrations needed for monitoring infection and other inflammatory conditions (2). The differences in the CRP results generated by the two assays can be partly explained by the genetic polymorphism of CRP (29). A functional CRP

assay has the advantage of not being affected by epitopic variation. The absence of negative CRP values in the comparison graph makes it clear that the intercept of the regression line does not play a major role in the lower concentration range. The lower limit of detection (7 mg/L) of the functional CRP assay is higher than that for the immunoturbidimetric CRP method
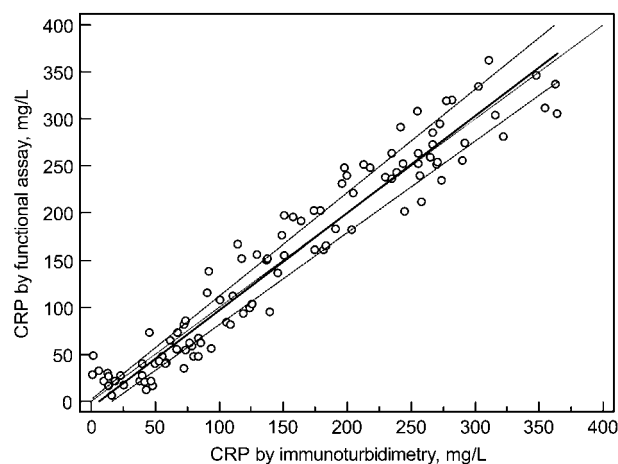
Figure 3   Comparison between CRP values obtained with the functional assay and the immunoturbidimetric method. Passing-Bablok regression line (r^2 = 0.932, p < 0.001) with 95% CI (dashed lines) for 103 serum samples: Y (CRP, functional assay; mg/L) = 1.033X (CRP, immunoturbidimetric assay; mg/L)–6.747; r^2 = 0.932. Dotted line: Y = X.
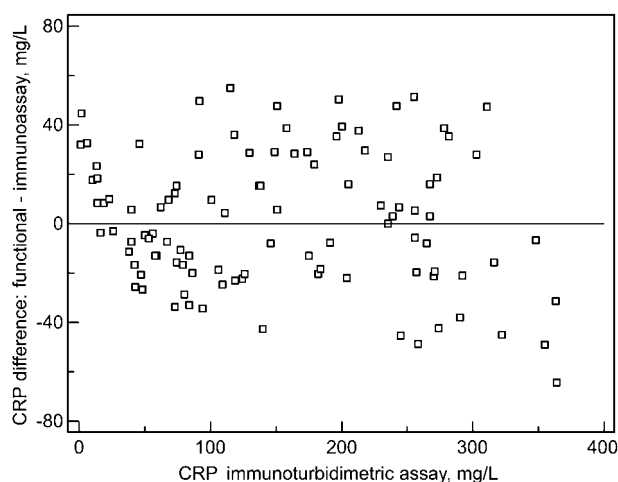
Figure 4   Difference plot between CRP concentrations measured with the functional assay and the immunoturbidimetric method.

(<1 mg/L), and is slightly above the reference range (<5 mg/L). However, most of the studies on reference values have been performed in wealthy countries (30) where health care systems can afford the CRP test. Also, it has been recognized that demographic factors (including age, gender and race), environmental variables, patient behaviors and traits, such as smoking and infections, contribute to variations in baseline CRP concentrations (31, 32). Therefore, in many developing countries, the reference range for CRP is widened due to the presence of chronic parasitic infections, tuberculosis, hepatitis, etc. The described method has been particularly designed to assess and monitor the presence, severity, and course of the inflammatory response to infectious disorders, particularly in sub-Saharan Africa where there is the highest incidence and the highest mortality rates due to infection. In these countries, there are few microbiology laboratory facilities. Clinicians need a low cost biomarker to guide the identification of patients likely to be infected and to monitor their response to treatment. The use of CRP may allow clinicians to discontinue empirical antibiotic treatment, thereby limiting unnecessary exposure to antibiotics. Although the limit of detection of the functional assay does not permit detection of low values of CRP, the analytical sensitivity is sufficient to detect the upper reference range (31).

Since the phosphocholine binding functional CRP assay requires inexpensive reagents (Intralipid®), this method can be used in resource-limited health care systems. In these settings, measurements can be performed using a simple photometer. As the method is technically simple and does not require the use of antibodies, the cost of the assay is very low. Based on actual prices for the African market, the estimated cost of the described functional CRP assay would be as low as ±0.03 €/test, vs. ±3 € for an immunological test. Although the required pre-incubation period does not allow the method to be fully automated, the reaction rate between phosphocholine and CRP is affected only minimally by the ambient temperature and provides a stable reading. Furthermore, the reagents do not require cool storage temperatures. This combination of properties makes the developed assay suitable for district hospital laboratories of tropical, third world countries.

Next to Intralipid® fat emulsions, similar fat emulsions rich in phosphocholine (e.g., dairy products like buttermilk) (33) theoretically could fulfill the basic requirements for a home-developed functional CRP assay. However, the size distribution of the Intralipid® lipid particles (diameter: 0.78±0.21 μm) (12) makes these particles ideally suited for functional turbidimetric analysis. Due to the multimeric structure of CRP, the natural lipid droplets act as scaffolds for the multivalent display of phosphocholine molecules in the new developed turbidimetric method. Since the described reaction between phosphocholine and CRP is species independent, the same functional assay can potentially be used in veterinary medicine for measuring CRP.

Conflict of interest statement

Acknowledgements

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