References


Development and Validation of an Automated and Ultrasensitive Immunoturbidimetric Assay for C-Reactive Protein, Luis Borque, Laura Bellod, Antonio Rius, M. Luisa Seco, and Francisco Galisteo-Gonzalez (1) Laboratorio Central, Hospital “San Millán-San Pedro”, Avenida de la Autonomía de la Rioja no. 3, Logroño 26004, Spain; (2) Departamento Física Aplicada, Universidad de Granada 18071, Spain; *author for correspondence: e-mail 03673715n1@abonados.cplus.es)

C-Reactive protein (CRP) is a marker of acute phase response to inflammation that increases rapidly within hours of disease onset. CRP measurements have been used for many years in the management of a variety of clinical situations, such as bacterial infections, ischemic necrosis of tissue, and active inflammatory conditions (1). Recent studies of CRP concentrations within the conventional reference interval have suggested new clinical applications. CRP is a prognostic risk factor for coronary heart disease in both patients with angina (stable or not) and in apparently healthy subjects (2), and distinguishes rapidly destructive vs slowly progressive osteoarthritis (3). In neonates, CRP >1–2 mg/L could be associated with serious disease, usually bacterial infection (4). In most of these studies, the authors have proposed a cutoff point for CRP of 2–3 mg/L. Recent reports show median values in healthy controls of 0.58–1.72 mg/L, and 95 percentile ranges of ~0.1–6.0 mg/L (5, 6). Nevertheless, these results can be influenced by the sensitivity of the method used for CRP quantification.

Among the many commercially available assays for CRP, the most common use fluid-phase or particle-enhanced turbidimetric or nephelometric procedures (7). Such assays are suitable for the measurement of proteins at concentrations >5–10 mg/L. New methods of measuring CRP have lower limits of detection one-tenth those of earlier methods (8, 9). Some use expensive monoclonal antibodies, and others use higher sample volumes and antibody concentrations in the reaction mixtures, which can increase nonspecific reactions (10).

We describe a microparticle reagent that we use in an optimized turbidimetric procedure with immunopurified polyclonal antibodies against CRP. It allows lower antibody coverage on the microparticle reagent and provides highly sensitive and robust particle-enhanced turbidim-
ric immunoassay for serum CRP. In addition, we present a new procedure of sequential covalent coupling of IgG and bovine serum albumin (BSA) that improves the reagent colloidal stability and could eliminate most of the drawbacks of the light-scattering immunoassays, such as nonspecific agglutination. On the basis of clinical data, the aim of the present study has been the development of a highly colloidaly stable turbidimetric reagent with a detection limit <0.3 mg/L for CRP and an analytical range up to 50–100 mg/L, which may be applicable to most of the turbidimetric clinical chemistry analyzers.

An IgG fraction of a goat polyclonal antiserum (International Enzymes, Inc.) and an affinity-purified IgG (SCI-PAC) against human CRP were used. The microparticle reagents were prepared according to a previously published method by the carbodiimide [1-ethyl-3-(3-dimethylamino-propyl)carbodiimide chloride] procedure (11). The anti-human CRP immunoglobulin was covalently coupled to carboxyl-modified polystyrene microparticles, with diameters between 100 and 330 nm, purchased from Polymer Laboratories Ltd and Bangs Laboratories as a 100 g/L suspension. For the immunopurified anti-CRP antibodies, we used a modified procedure consisting of two sequential covalent coupling steps. Antibody solutions (0.08–0.9 mg/m² of latex) were incubated with 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide chloride micro-particles at 4 °C. After 60 min, free surfaces were saturated with BSA added at a final concentration of 15 mg/m², and the particles were then incubated at 4 °C for 6 h. After centrifugation, the coated microparticles were resuspended (5 g/L) in glycine-buffered saline-BSA containing 0.17 mol/L NaCl, 0.1 mol/L glycine, 3 g/L BSA, 1 g/L Tween 20, pH 8.2.

Coverage was calculated by measuring the uncoupled IgG present in the supernatants of the first centrifugation, after filtering with a Nucleopore polycarbonate filter (pore diameter, 0.1 μm), using the copper reduction/bicinchoninic acid reaction (BCA method; Pierce) and/or the measurement of the absorbance at 280 nm (E_{280}^{cm} = 1.4 kg/L). The extent of covalent coupling was determined by displacement of passively adsorbed protein with sodium dodecyl sulfate (10 g/L in 0.1 mol/L Tris, pH 8.2). After elution, the released protein was measured using the BCA procedure.

The immunoagglutination reaction was measured on a Cobas Mira (Hoffmann-La Roche) by a turbidimetric assay and on an IMMAGE™ immunochemistry system (Beckman Coulter) by a near-infrared particle immunoassay (NIPIA) method (12). The experimental details are summarized in Table 1.

In both procedures, sample CRP values were automatically calculated from a multipoint calibration curve using a log/logit calculation procedure. As calibrator, we used a CRP reference standard containing 180 mg/L CRP (CRP T Standard, ref. 0737224; Hoffmann-La Roche), according to IFCC reference material CRM 470.

The covalently coupled isotherms of IgG anti-CRP on carboxyl-modified polystyrene particles (100-nm diameter) are shown in Fig. 1A. The maximum adsorption plateau is achieved at a polyclonal antibody concentrations of 3.6 mg/m². The immunopurified antibody showed a similar trend, having a covalently coupled adsorption isotherm according to that of the nonimmuno-purified antibody in the range studied (0.08–0.9 mg/m²). The covalent coupling efficiency rate was always >92% (IgG chemically bound/IgG chemically plus passively adsorbed). The microparticle reagent response of the different antibodies to increasing CRP concentrations can be seen in Fig. 1B, where immunopurified antibodies show the best results. The most striking feature in Fig. 1B is that the curve for the immunopurified antibody at 0.3 mg/m² was equivalent to that obtained for nonimmuno-purified antibody at 3.6 mg/m². No further improvement was obtained when IgG loadings for immunopurified antibody concentrations >0.3 mg/m² were tested.

We found that at any given working dilution of microparticles studied, the initial absorbance decreases with increasing wavelength and increases with microparticle diameter, according to the light-scattering theory. The effect of varying the particle size on a reagent with a constant charge of 0.3 mg/m² anti-CRP immunopurified antibody indicates that the analytical sensitivity increases with size and decreases with wavelength, whereas the upper measuring limit shows an opposite behavior (Fig. 1C). Thus, a compromise between sensitivity and measurement range is demanded. A microparticle size of 150 nm and a wavelength of 600 nm were selected as the best choice because of the high slope and wide dynamic range showed. We chose this microparticle reagent, with a coverage of 0.3 mg/m² anti-CRP immunopurified antibody, for method validation.

Polyethylene glycol (PEG) is commonly used to increase the immunoagglutination kinetics. However, a negative influence on reagent stability was clearly visible at PEG concentrations ≥30 g/L in the reaction buffer because of nonspecific aggregation of the reagent. Because PEG can also produce nonspecific precipitation of endogenous and exogenous serum components in the reaction medium, we fixed the final PEG concentration in the reaction buffer at 25 g/L. We monitored the reaction kinetics at pH values of 6.5–9.5, observing that immunoagglutination decreased with increasing pH (data not shown); no significant differences were observed when several nonionic detergents were used at concentrations up to 1 mL/L, although the addition of nonionic detergent increases the

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<th>Table 1. Conditions for CRP determination.</th>
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<td><strong>Assay mode</strong></td>
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<td>Sample, μL</td>
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<tr>
<td>Diluent (water), μL</td>
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<td>Reaction buffer, μL</td>
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microparticle stability by decreasing the nonspecific binding. We then selected 0.1 mol/L Tris (pH 6.5) containing 0.15 mol/L NaCl, 3 g/L BSA, 25 g/L PEG, and 1 g/L Tween 20 as the reagent buffer for method validation.

The intra- and interassay CVs (n = 20) for three pooled samples studied were 2.5–6% and 1.8–5.2% for the turbidimetric and NIPIA measurements, respectively. The turbidimetric method affords a measuring range of 0.3–45 mg/L for CRP in human serum on the Cobas Mira analyzer. In the Beckman IMMAGE instrument, the upper assay range was extended to 100 mg/L, with a substantial increase in the dynamic range, by means of the near-infrared wavelength capability of the system (940 nm reading). Samples with CRP values above the upper limit of the calibration curve can be reanalyzed after automated or manual sample dilution. The prozone effect did not occur for CRP concentrations up to 260 mg/L. The detection limits for CRP, defined as the mean plus 3 SD of the zero signal (saline solution), were 0.25 and 0.14 mg/L on the Cobas Mira and Beckman IMMAGE analyzers, respectively. Rheumatoid factor at concentrations up to 650 kIU/L did not interfere with the assay (CRP recovery within 100% ± 5%).

The comparison study between the turbidimetric and NIPIA assays (y) and a monoclonal latex-enhanced nephelometric method (x; Dade-Behring N-Latex mono CRP) gave correlation coefficients >0.99, with no significant differences between regression parameters (P >0.05), indicating a close relationship between these procedures even at low CRP values (<5 mg/L). Parameter values (a, b), correlation coefficients (r), and confidence regions were as follows: Cobas Mira, b = 0.99 (range, 0.96–1.01); a = 0.25 (–0.11 to 0.32); r = 0.997; n = 68; and IMMAGE, b = 0.95 (0.92–1.05); a = 0.10 (–0.17 to 0.15); r = 0.99; n = 68.

The most important features of the method presented here are high analytical sensitivity (detection limit <0.3 mg/L CRP) and good precision (CVs <7%) in the measuring range up to 45 mg/L. This range is wide enough to determine the CRP values found in 72% of our hospitalized patients and >90% of our outpatient population. Nevertheless, a further increase of the dynamic range up to 100 mg/L may be gained on the IMMAGE analyzer by use of a 940 nm wavelength. Both procedures display a prozone phenomenon at CRP concentrations >260 mg/L, with 99.4% of our patients included. The results obtained by these methods were well correlated (r >0.99) with those determined by a nephelometric ultrasensitive
monoclonal CRP immunoassay, opening the door to a less expensive automated method with similar sensitivity and a wide measuring range.

For practical use, it is essential to obtain microparticle reagents colloidally stable under reaction and storage conditions, with the stability often depending on protein coverage. Colloidal particles coated with polyclonal IgG usually are not very stable, mainly because of the high antibody coverage needed to obtain good sensitivities (3.6 mg/m² in our case). Posttreatment with additives (BSA, surfactants) has been proposed (13, 14), although it reduces IgG charge and reactivity. We suggest an alternative approach that uses a small amount of immunopurified antibody and stabilizes the reagent by saturation of free surface with BSA in a two-step sequential covalent procedure. In this way, the inactive IgG molecules are replaced with molecules of BSA, remarkably increasing procedure. In this way, the inactive IgG molecules are replaced with molecules of BSA, remarkably increasing the latex stability by electrostatic repulsion. Moreover, both IgG and BSA could be added in only one step if the right conditions are found (14).

In conclusion, a low coverage (0.3 mg/m²) of immunopurified IgG provides a reagent with immunoreactivity similar to or better than that of microparticles totally covered (at saturation) by a nonimmunopurified IgG antibody (3.6 mg/m²), but with higher colloidal stability (the suspension remained stable for more than 3 months when it was stored at 4 °C). This approach to antibody immunopurification could be extended to obtain reagents useful for the measurement of several other proteins at low concentrations (15).

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References


Flexibility of Melting Temperature Assay for Rapid Detection of Insertions, Deletions, and Single-Point Mutations of the AGXT Gene Responsible for Type 1 Primary Hyperoxaluria, Doroti Pirulli, Michele Bontiolo, Daniela Puzzer, Andrea Spanò, Antonio Amoroso, and Sergio Crovella. SERVIZIO DI GENETICA MEDICA, IRCCS BURLO GAROFOLO, 34137 TRIESTE, ITALY; SEZIONE DI GENETICA MEDICA, DIPARTIMENTO SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO, UNIVERSITÀ DI TRIESTE, 34137 TRIESTE, ITALY; ADDRESS CORRESPONDENCE TO THIS AUTHOR AT: SERVIZIO DI GENETICA, IRCCS BURLO GAROFOLO, VIA DELL’ISTRIA 65/1, 34137 TRIESTE, ITALY; FAX 39-040-3785210, E-MAIL crovella@burlo.trieste.it

Primary hyperoxaluria type 1 (PH1; OMIM 259900) is a rare autosomal recessive disorder characterized by impaired hepatic detoxification of glyoxylate. PH1 is caused by a deficiency of alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44), which catalyzes the transamination of glyoxylate to glycine. This defect leads to endogenous overproduction of oxalate and glycolate, producing oxalic and glycolic hyperacidurias, which are the hallmarks of the disease (1).

The AGT enzyme is encoded by a single-copy gene (AGXT), which consists of 11 exons ranging from 65 to 407 bp and spanning a 10-kb DNA segment in the 2q37.3 human region. AGT is a 392-amino acid protein with a molecular mass of 43 kDa (2).

Several technical approaches have been used to identify 7 polymorphisms and 26 mutations in the AGXT gene (3–6). Here we describe a rapid, flexible, and inexpensive method for detection of the different types of mutations (insertions, deletions, point mutations) of the AGXT gene. Our method is based on the ability to distinguish between PCR amplification products by their melting temperatures (Tm) (7–9).

Nine PH1 patients, whose mutations had first been analyzed by the single-strand conformation polymorphism (SSCP) technique and then by sequencing of abnormal mobility bands of four AGXT exons (5), were studied comparatively by the melting temperature assay (MTA). Heterozygous relatives of three patients were also included in this study. Five healthy Italian subjects served

Peula JM, Hidalgo-Alvarez R, de las Nieves FJ. Coadsorption of IgG and BSA onto sulfonated polystyrene latex. II. Colloidal stability and immunoreactiv-

In this way, the inactive IgG molecules are replaced with molecules of BSA, remarkably increasing procedure. In this way, the inactive IgG molecules are replaced with molecules of BSA, remarkably increasing the latex stability by electrostatic repulsion. Moreover, both IgG and BSA could be added in only one step if the right conditions are found (14).

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References