

Original Research Article

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Improvement of Colloidal and Temperature Stability of hsCRP Latex Reagents with Stabilizers

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ABSTRACT

The role of high sensitivity C-reactive protein (hsCRP) level elevation in low-grade systemic inflammation was proved with the confirmation of cardiovascular risk assessment. Hence the measurement of hsCRP has got importance to predict the cardiovascular risk among the suspected population. The current market is still looking for a latex based reagent assay which requires minimum instrument setting and technical expertise to perform the test. Hence we have tried to develop a latex based test for hsCRP. The present study was conducted to test the colloidal and temperature stability of prepared hs-CRP latex reagents with different stabilizers. Trial reagent was tested for its colloidal stability using different stabilizing agents at a different temperature. A trial to improve colloidal stability of Bovine serum albumin (BSA) at higher temperature was studied using higher percentage w/v of BSA in the final reaction buffer. Latex reagent was tested for real-time temperature stability studies at three different temperatures and tested for colloidal stability and agglutination reaction with the positive control, with calibration concentration 1mg/L and 10 mg/L. The colloidal stabilising agent BSA showed higher colloidal stability, which has higher availability and cheaper in cost compared to other agents tested. Addition of higher concentration BSA causes inhibition of agglutination reaction with the CRP calibrator. The agglutination reaction with calibrator is showing a reduced reaction on reagent bottles kept at higher temperature. The colloidal stability is also seen reduced at a higher temperature. The concentration of BSA that can be used for further trials is estimated as 1.25% w/v, which gives better colloidal stability and visual agglutination reaction with the Hs CRP calibrators.

Keywords

Hs CRP, Stabilising agent, Colloidal stability, BSA, Temperature stability

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Introduction

C-reactive protein (CRP) is a biomarker of inflammation. Plasma CRP concentrations increase rapidly and dramatically (100-fold or more) in response to tissue injury or inflammation (Aziz *et al.*, 2003). It is believed

that CRP binds to and precipitates soluble ligand, aggregates particulate ligand, and activates the classical complement pathway, thus contributing to both host defence against infection and enhancing inflammatory tissue damage (Shrivastava *et al.*, 2015). Conventional CRP assays include qualitative,

semi-quantitative and quantitative assays, with indications for use for evaluation of infection, tissue injury, and inflammatory disorders.

These assays provide information for the diagnosis, therapy, and monitoring of inflammatory diseases. The conventional methods are reproducible, fully automated, and capable of measuring CRP with a lower detection limit of 5–10 mg/L (Algarra *et al.*, 2013). This detection limit is adequate for the traditional clinical utility of CRP in monitoring infection.

More sensitive CRP assay with a measurement range of 0-5 mg/L is introduced and named as Hs CRP useful in assessing and predicting the risk of coronary and cerebrovascular disease in diabetic and apparently healthy populations. High-sensitivity CRP (hs-CRP) is more precise than standard CRP when measuring a baseline (i.e., normal) concentrations and enables a measure of chronic inflammation. Atherosclerosis is an inflammatory disease, and hs-CRP has been endorsed by various guidelines as a biomarker of atherosclerotic cardiovascular disease risk (Montecucco *et al.*, 2009).

A large prospective clinical trial demonstrated significantly less cardiovascular risk for patients with hs-CRP less than 2.0 mg/L. (Ridker *et al.*, 2003) CRP levels increase 6 hours after an acute stimulus and peak within 48 hours. The half-life of CRP being relatively long and remain stable allows it to be used as a useful marker of inflammation. However, the utility of Hs CRP estimation for the CVD risk assessment in the Indian population is very low. This can be due to the lack of awareness among the population or the high cost of the test. In India, only very few laboratories in the rural sector are carrying out Hs CRP assessment, which is again a reason that Hs CRP estimation is not popular among the diabetic and non-diabetic population.

The currently available reagent systems used for the detection of Hs CRP are imported and requires highly sophisticated instruments for the detection (Turner *et al.*, 2013). No available market kits are of Indian origin, and the present study focuses on the indigenous development of a latex based reagent assay which requires no instrument and minimum technical expertise to perform the test.

Hence we have developed a reagent system that is rapid, cost-effective and can be performed at a primary health care centre. They also formulate a latex-enhanced reagent format, inert microscopic latex particles enlarge the immune complexes, amplifying the reaction and significantly increasing the sensitivity of the reaction. Here the assay reagent is Latex particles coated with the Anti-CRP antibodies. This method is expected to be more rapid, cost-effective and requires minimum technical expertise to perform. Present work was carried to confirm the colloidal stability and sensitivity setting of the prepared reagent.

Materials and Methods

Colloidal stability studies with stabilising agents

The trial reagent was checked for colloidal stability at different temperature using different stabilising agents in the final reaction buffer. The final reaction buffer used is Tris buffer (tris (hydroxymethyl) aminomethane buffer).

The pH range for tris buffer used is pH 7.0. One percent of the trial stabilising agents such as BSA, PEG 600, Tween 80, Fish skin gelatin, Tween 60 was used in the buffer, and the latex kept at three different temperature for 7 days. The latex assay was then checked for auto-agglutination in saline after the seven days of incubation.

Trials for improvement in colloidal stability and temperature stability

Trial to improve colloidal stability at higher temperature was studied using higher percentage w/v of BSA in the final reaction buffer. The concentration used is 1%, 1.25%, 1.5%, 2%, 2.5% w/v BSA to the final Tris Buffer at pH 7.5.

The trial latex reagent was kept at room temperature (22-25°C), 37°C and elevated temperature as 45°C for 14 days and observed for agglutination with normal saline. The same trial reagents with different BSA concentration were tested for agglutination with positive control serum and calibrator concentration at 1mg/L and 10mg/L after 14 days of incubation at a different temperature.

Real-time stability trials at different temperature

The trial latex reagent assay was suspended in tris buffer with pH 7.5 with 1.25% BSA, 5µl/ml NaCl and 0.1% NaN₃. The latex reagent was tested for real-time temperature stability studies. The trial latex reagent was kept at three different temperature, at room temperature, 37°C, and at 45°C for 30 days. The reagent was tested for colloidal stability and agglutination reaction with the positive control, with calibrator concentration 1mg/L and 10 mg/L.

Results and Discussion

The trial reagent was checked for colloidal stability at a different temperature like room temperature, 37°C, and at 45°C for using stabilising agents like BSA, PEG 600, Tween 80, Fish skin gelatin, Tween 60 in the final reaction buffer Tris buffer at pH 7.0. The latex assay was then checked for auto-agglutination in saline after the seven days of incubation (Table 1).

Comparing the different stabilising agents used, BSA 1% w/v, Tween 80, and Fish skin gelatin showed good colloidal stability at room temperature and 37°C.

Tween 60 showed good colloidal stability at room temperature but not at 37°C. PEG 600 showed agglutination reaction at all temperature. Higher temperature showed agglutination reaction with all agents used. Probably, BSA as plasma protein showed a strong affinity for Tris buffer so that Tris buffer molecules could bind with proteins and amino acids present in BSA attaching with thiol groups providing steric stabilisation due to the large protein molecules (Basle *et al.*, 2010).

The BSA has higher colloidal stability, easy availability and cheaper compared to other agents. Hence BSA was selected for further studies. Trials to improve colloidal stability at higher temperature were studied using higher percentage w/v of BSA in the final reaction buffer (Table 2).

The initial trials showed the use of higher concentration of BSA gives better stabilisation even at a higher temperature. Initial trials showed that 1% BSA exhibited better colloidal stability compared to the other agents used in the trial at room temperature (22-25°C) and 37°C. At 45°C all agents showed auto agglutination when suspended in saline.

In other words, increased thermal stability corresponds to a reduced conformational flexibility. Several authors have observed a correlation between protein thermostability with protein flexibility detected by H/D exchange (Celej *et al.*, 2003). The same trial reagents with different BSA concentration were tested for agglutination with positive control serum and calibrator concentration at 1mg/L and 10mg/L after 14 days of incubation at different temperature (Table 3).

Table.1 Auto-agglutination reactions on different temperature with different stabiliser

Stabilizer in Buffer (1% w/v)	At RT	37°C	45°C
BSA	-	-	+
PEG 600	-/+	+	++
Tween 80	-	-	+
Fish skin gelatin	-	-	+
Tween 60	-	-/+	++

Grading Interpretation: '-' No agglutination, '+/-' Doubtful agglutination, '+' Fine agglutination

Table.2 Auto-agglutination reactions at different temperature with different % stabilizer

Concentration of BSA Stabilizer in Buffer (% w/v)	At RT (22- 25°C)	37°C	45°C
1	-	-	+
1.25	-	-	+/-
1.5	-	-	-
2	-	-	-
2.5	-	-	-

Grading Interpretation: '-' No agglutination, '+/-' Doubtful agglutination, '+' Fine agglutination

Table.3 Reactions of trial latex reagent with different BSA concentration

	Trial Latex reagent in Tris Buffer with different BSA conc (%w/v)				
	1%	1.25%	1.5%	2%	2.5%
Saline	-	-	-	-	-
Positive control	+++	+++	+++	++	++
CRP Calibrator 1mg/L	+	+	+/-	-	-
CRP Calibrator 10mg/L	+++	+++	++	++	+

Grading Interpretation: '-' - No agglutination, '+/-'- Doubtful agglutination, '+' - Fine agglutination, '++'- Coarse agglutination without central clearing, '+++'- Coarse agglutination with central clearing.

Table.4 Real-time stability studies at a different temperature

	Normal Saline			Positive Control			Calibrator 1mg/L			Calibrator 10mg/L		
	RT	37°C	45°C	RT	37°C	45°C	RT	37°C	45°C	RT	37°C	45°C
5 th day	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
10 th day	-	-	-	+++	+++	+++	+++	++	++	+++	++	++
15 th day	-	-	+/-	+++	+++	+++	++	++	++	++	++	++
30 th day	-	+/-	++	+++	+++	+++	++	+	+	++	+	+

Grading Interpretation: '-' - No agglutination, '+/-'- Doubtful agglutination, '+' - Fine agglutination, '++'- Coarse agglutination without central clearing, '+++'- Coarse agglutination with central clearing.

From the trials, it was noted that the addition of higher concentration BSA causes inhibition of agglutination reaction with the CRP calibrator. So the concentration of BSA that can be used

for the further trials was estimated as 1.25% w/v, which gives better colloidal stability and visual agglutination reaction with the CRP calibrators. The latex reagent was tested for

real-time temperature stability studies at 3 different temperatures viz. room temperature, 37°C, and at 45°C for 30 days and tested for colloidal stability and agglutination reaction with the positive control, with calibrator concentration 1mg/L and 10 mg/L (Table 3).

From the above reaction, it was seen that the agglutination reaction with calibrator is showing a reduced reaction on reagent bottles kept at higher temperature. The colloidal stability was also seen reduced at a higher temperature.

We have prepared a Trial reagent for estimating the Hs CRP. The latex assay was checked for auto-agglutination in saline after the seven days of incubation with stabilising agents. The colloidal stabilising agents BSA showed higher colloidal stability, which has higher availability and cheaper in cost compared to other agents tested. Trials to improve colloidal stability of BSA at higher temperature were studied using higher percentage w/v of BSA in the final reaction buffer. The initial trials showed the use of higher concentration of BSA gives better stabilisation even at a higher temperature. The same trial reagents with different BSA concentration were tested for agglutination with positive control serum and calibrator concentration after 14 days of incubation at a different temperature, the addition of higher concentration BSA causes inhibition of agglutination reaction with the CRP calibrator (Table 4). So the concentration of BSA that can be used for further trials was estimated as 1.25% w/v, which gives better colloidal stability and visual agglutination reaction with the CRP calibrators. However, the latex reagent was tested for real-time temperature stability studies in 3 different temperatures, Viz. room temperature, 37°C, and at 45°C for 30 days and tested for colloidal stability and agglutination

reaction with the positive control, with calibrator concentration 1mg/L and 10 mg/L. Results demonstrated that the agglutination reaction with calibrator was showing a reduced reaction on reagent bottles kept at higher temperature. The colloidal stability was also seen reduced at a higher temperature.

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