

Research paper

Heat-mediated enzyme-linked immunosorbent assay procedure on a photoactivated surface[☆]

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Abstract

The human burden of infectious and allergic disease has necessitated rapid screening and measurement of antibodies in sera. Here, we report an enhancement of speed and sensitivity of an enzyme-linked immunosorbent assay (ELISA) technique by performing it at elevated temperature on an activated surface. The activated polymer was able to bind covalently anti-human IgG at 50 °C over 40 min to form a solid phase. The covalently bound solid phase was stable enough to withstand subsequent ELISA steps at elevated temperature. Thus, when blocking, human IgG and antibody–enzyme conjugate binding were performed on this solid phase at 40 °C in 40 min, 50 °C in 45 min and 50 °C in 40 min, respectively. The ELISA readings obtained were 1.5-fold higher than those obtained at 37 °C over similar incubation times. Total IgE was also determined by the heat-mediated ELISA (HELISA) technique in less than 3 h and gave similar ELISA values to those obtained by the conventional procedure carried out for 18 h on an untreated surface. A stable covalently bound solid phase is a prerequisite for the HELISA technique and was further verified when a solid phase prepared through adsorption onto an untreated surface showed less than half the ELISA absorbance values obtained with the activated surface at elevated temperatures. As surface activation can be achieved by application of a simple technique, the HELISA procedure could be a powerful alternative to conventional ELISA.

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1. Introduction

The sensitivity of an enzyme-linked immunosorbent assay (ELISA) procedure depends on the reagents and their concentration, the sensitivity of the assay molecule (Macy et al., 1988; Kemeny et al., 1989; Solling and Dinesen, 1983), the stability of the solid phase and

Abbreviations: FNAB, 1-fluoro-2-nitro-4-azidobenzene; PC, polycarbonate; PS, polystyrene.

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the incubation time. Purified antigen or antibody or the use of high affinity monoclonal antibodies increases the sensitivity of an ELISA procedure (Hoffman, 1981; Bruynzeel et al., 1979; Goldsmith, 1981). Enhancement of the sensitivity of an ELISA procedure can also be achieved by employing cofactor enzyme cycling (enzyme amplification method) (Seth et al., 1988) or using a sensitive tag such as fluorescent, chemiluminescent or radioactive moieties (Strouse et al., 1991; Pekary et al., 1986; Ehrlich and Moyle, 1983). Many workers have shown improved ELISA procedures using a stable solid phase prepared by covalently binding an antigen or an antibody to an activated matrix rather than a solid phase prepared through adsorption (Larsson et al., 1987, 1997; Roch et al., 1997; Yonezawa et al., 1993; Thomas et al., 1990; Hendry and Herrmann, 1980; Legastelois et al., 1994). Recently, we have demonstrated that the solid phase prepared on a photoactivated polystyrene surface gave around 1.5–2-fold higher readings than the untreated PS surface when the assay was carried out at 37 °C over 8 h (Bora et al., 2002). In most of these ELISA procedures, especially those used for determining IgE, the incubation period is 8–24 h at 4 or 37 °C. In this study, we have shown that an ELISA carried out at elevated temperature on a photoactivated plate requires an incubation time of less than 3 h and does not involve costly monoclonal antibodies or a sensitive tag.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), goat anti-human IgG, human IgG and goat anti-human IgG peroxidase conjugate were purchased from Sigma, USA. Goat anti-human IgE, IgE standard and goat anti-human IgE peroxidase conjugate were purchased from Bethyl Laboratories, USA. All other chemicals were of analytical grade and were purchased from Merck, India. UV irradiation at 365 nm wavelength was carried out in an UV Stratalinker (model-2400, Stratagene, USA). Polycarbonate (PC) PCR plate and polystyrene (PS) microtiter plates were purchased from Greiner labortechnik, Germany. Heat-mediated ELISA was carried out on PC plates in a thermocycler (Gene Amp PCR System 2400, Perkin Elmer).

Washings were carried out after each step of the ELISA using washing buffer (0.05% Tween 20 in 0.01 M PBS). For each experiment a corresponding control experiment was carried out using an untreated plate. Absorbance values were expressed as the mean of three readings $\pm$ S.D.

2.2. Introduction of active functional groups onto polycarbonate and polystyrene surfaces

1-Fluoro-2-nitro-4-azidobenzene (FNAB) was prepared according to Naqvi and Nahar (2004). Wells of a PC plate were loaded with the solution of FNAB (8 μ mol/50 μ l of methanol/well) and allowed to air dry in a fume hood. FNAB coated wells were then irradiated for 7.5 min by UV light and washed with methanol. The activated PC plates were dried and kept in a sealed polyethylene bag until required. PS plates were activated similarly using 10 μ mol of FNAB followed by 10 min UV irradiation as reported earlier (Nahar et al., 2001).

2.3. Conventional ELISA

Goat anti-human IgG (90 μ l/well of a 250 ng/ml of 0.01 M carbonate buffer, pH 9.6) was immobilized onto a polycarbonate PCR or polystyrene microtiter plate by incubating at 4 °C for 11 h. Blocking (100 μ l/well of 2% BSA), human IgG binding (90 μ l/well of 125 ng/ml solution in 0.01 M PBS, pH 7.4) and goat anti-human IgG peroxidase conjugate incubations (90 μ l/well of a 1/5000, v/v dilution solution in 0.01 M PBS, pH 7.4) were carried out at 37 °C for 1, 3 and 3 h, respectively. Colour was developed by the addition of 90 μ l of substrate–dye solution (4 mg *o*-phenylenediamine dihydrochloride and 2 μ l H₂O₂ in 10 ml of 0.2 M citrate buffer, pH 5) for 5 min after which 10 μ l of stop solution (5% H₂SO₄) were added. The resulting solutions were transferred into the wells of a PS plate (when the ELISA was done on a PC plate) and absorbance was recorded at 490 nm.

2.4. Optimization of the concentration of goat anti-human IgG for immobilization onto the activated polycarbonate plate

A double dilution series (2000–0.061 ng/ml of 0.01 M carbonate buffer, pH 9.6) of goat anti-human IgG

was loaded into the wells (90 µl/well) of an activated PC plate and incubated at 50 °C for 1 h in a thermocycler. Remaining steps were carried out by conventional procedures as described.

2.5. Effect of incubation temperature and time on the immobilization of goat anti-human IgG to the activated polycarbonate plate

Wells of the activated PC plates were loaded with goat anti-human IgG (90 µl/well of a 250 ng/ml solution). To find the optimum temperature the plates were incubated for 60 min at 35, 40, 45, 50, 55, 60, 65 and 70 °C, respectively, in a thermocycler. In a separate set of experiments, the immobilization time was optimized by incubating goat anti-human IgG loaded plates at 50 °C for 10, 20, 30, 40, 50 and 60 min, respectively, in a thermocycler. The remaining steps of the ELISA in all the plates were then carried out conventionally.

2.6. Studies on incubation temperature and optimum time for blocking

Goat-anti human IgG immobilized (50 °C, 40 min) plates were loaded with 100 µl of BSA solution. One set of these plates were incubated for 1 h at 40, 45, 50, 55, 60 and 65 °C, respectively, to find the optimum temperature. Another set of plates was incubated at 40 °C for 10, 20, 30, 40, 50 and 60 min, respectively, to find the optimum blocking time. Absorbance was recorded after performing the remaining steps conventionally.

2.7. Determination of optimum concentration of human IgG for binding to the solid phase

The solid phase prepared (goat anti-human IgG, 50 °C in 40 min; BSA, 40 °C in 40 min) on activated PC wells was loaded with a 2-fold dilution series (1000–0.125 ng/ml) of human IgG solution and incubated for 3 h at 37 °C. The remaining steps of the ELISA were carried out as in the conventional method.

2.8. Effect of temperature and time on the binding of human IgG with the solid phase

The solid phase prepared on activated plates as in Section 2.7 were loaded with human IgG (90 µl/well

of a 125 ng/ml solution) and incubated for 60 min at 40, 45, 50, 55, 60 and 65 °C, respectively. The optimum time for human IgG binding to the solid phase was determined by incubating the human IgG loaded plates at 50 °C for 15, 30, 45, 60 and 90 min, respectively. The remaining ELISA steps were carried out conventionally.

2.9. Studies on the effect of temperature and time on the binding of goat anti-human IgG peroxidase conjugate with human IgG bound to the solid phase

Goat anti-human IgG binding, blocking and human IgG binding were carried out at 50 °C for 40 min, 40 °C for 40 min and 50 °C for 45 min, respectively. Six of these plates were incubated with goat anti-human IgG peroxidase conjugate for 60 min at 40, 45, 50, 55, 60 and 65 °C, respectively. Another six plates were incubated with goat anti-human IgG peroxidase conjugate at 50 °C for 10, 20, 30, 40, 50 and 60 min, respectively. Absorbance values were recorded after performing the remaining steps in all of the plates by conventional ELISA method.

2.10. Comparative studies of ELISA by different conditions on polycarbonate and polystyrene surfaces for the detection of human IgG

ELISA procedures were performed by immobilizing goat anti-human IgG for 45 min at 37 °C, blocking for 1 h at 37 °C, human IgG binding for 3 h at 37 °C and conjugate binding for 3 h at 37 °C on the polycarbonate (PC1) and polystyrene (PS1) surfaces. In another experiment ELISA steps were carried out at 37 °C for 40, 40, 45 and 40 min, respectively (PC2 and PS2). In another plate (PC3) heat-mediated ELISA (HELISA) was performed by immobilizing goat anti-human IgG for 40 min at 50 °C, blocking for 40 min at 40 °C, binding human IgG for 45 min at 50 °C and conjugate binding for 40 min at 50 °C. HELISA on PS3 plates were carried out in an incubator similarly to the PC3 assay except that the temperature of the incubator at each step was 2 °C higher than in PC3 experiment carried out in a thermocycler. All these experiments were carried out with activated and untreated plates.

2.11. Quantitative determination of IgE in human blood sera by HELISA procedure

The presence of total IgE in sera from patients was determined and quantified by preparing standard curves by HELISA and conventional ELISA procedures. In the HELISA procedure, 75 µl/well goat anti-human IgE (1 µg/ml dilution) was immobilized in activated PC wells for 40 min at 50 °C followed by blocking with BSA at 40 °C for 30 min. The wells were then loaded with 75 µl of IgE standards as per the supplier's instructions in a double dilution series (1000, 500, 250, 125, 62.5, 31.25 and 15.34 ng/ml) and incubated for 45 min at 50 °C. To the solid phase in separate wells 1/4 (v/v) diluted human sera were incubated similarly. Goat anti-human IgE peroxidase (1/5000) solution was added to these wells and incubated for 40 min at 50 °C. After washing the plates were assayed for peroxidase activity. A conventional ELISA was then performed as described.

2.12. Inhibition studies to determine the specificity of antibodies

Goat anti-human IgE (90 µl of 1 µg/ml solution) was immobilized on the wells of activated polycarbonate plates for 40 min at 50 °C followed by blocking with BSA at 40 °C for 30 min. Separately, a double-dilution series of goat anti-human IgE (200–0 ng/90 µl of PBS) was taken in microcentrifuge tubes to which an equal amount of patient-derived sera (1/4, v/v dilution) was added before incubation for 1 h at 37 °C. 90 µl of this were added to the solid phase prepared as above and incubated for 45 min at 50 °C. Goat anti-human IgE peroxidase (1/5000) solution was added to these wells and incubated for 40 min at 50 °C. After washing the plates were assayed for peroxidase activity.

A conventional ELISA was then performed as described, except that in place of patient-derived sera, patient-derived sera co-incubated with goat anti-human IgE was used.

3. Results and discussion

Conventional ELISA procedures are normally carried out at 37 °C through adsorption on to

polystyrene microtiter plates. Customarily, ELISA has not been carried out at elevated temperatures, possibly anticipating the fact that the antigen or antibody would be desorbed from the solid surface at higher temperature resulting in lower ELISA values. In fact, when a solid phase was prepared on an untreated surface at higher temperature, ELISA values decreased slowly beyond 37 °C. In contrast, an activated surface showed increased immobilization of biomolecule beyond 37 °C, which prompted us to study ELISA performance at elevated temperatures. As we wished to study ELISA over a wide range of temperatures (30–70 °C), a thermo-stable PCR PC plate was found suitable. As a thermocycler can rapidly attain a particular temperature, it was used for HELISA experiments.

Goat anti-human IgG was immobilized onto an activated PC plate through a covalent linkage involving a fluoro group of the activated surface and an amino group of the protein. The mechanism for activation and immobilization was similar as that described for a PS surface (Bora et al., 2002). As an ELISA on a PC surface has not been previously reported, we have optimized the amount of goat anti-human IgG required for immobilization on the activated PC plate at 50 °C for 1 h in a thermocycler. The optimum concentration of goat anti-human IgG was found to be 250 ng/ml, which gave an absorbance value of 0.707 compared to 0.321 obtained on the untreated surface.

In HELISA experiments, each step of the assay was optimized by doing the subsequent steps in a conventional ELISA. The effect of temperature on the binding of goat anti-human IgG was studied by incubating the plates at different temperature in a thermocycler for 60 min. Maximum ELISA readings were obtained when the assay was carried out with the solid phase prepared on the activated PC surface at 50 °C. This is around 2-fold higher than that carried out at 37 °C. Any decrease in absorbance on both of the surfaces beyond 50 °C may be due to inactivation of protein. On the contrary, no increase in ELISA values was observed on the untreated PC surface beyond 35 °C (Fig. 1). When immobilization of goat anti-human IgG was carried out in a time dependent experiment at 50 °C in the thermocycler, 40 min was found to give maximum absorbance beyond which no further increase in absorbance was observed (Fig. 2).

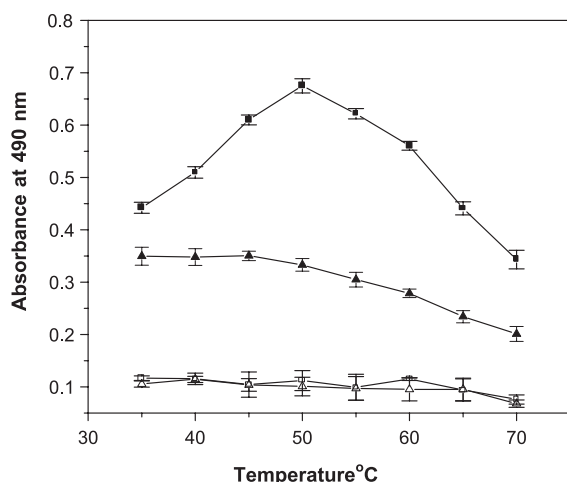


Fig. 1. Temperature dependent immobilization of anti-human IgG on photoactivated (■) and untreated (▲) PC plates over 60 min. The remaining ELISA steps were carried out as described in Section 2.3. The open symbols represent normal goat serum used as a negative control. The error bars indicate standard deviation.

The optimum temperature for blocking was found to be 40 °C. Nonspecific binding beyond 45 °C suggested the delicate nature of BSA binding. Being a weak binding, BSA may desorb from the solid phase at elevated temperatures giving rise to nonspecific binding. There was no non-specific binding after 40 min of incubation time at 40 °C for the blocking stage, which was taken as the optimum time.

Binding of human IgG to the solid phase was carried out by incubating an optimum dilution of human IgG (125 ng/ml) at different temperatures (40–65 °C) in the thermocycler for 60 min. Maximum binding was obtained at 50 °C, which is more than double that for the untreated surface at the same temperature. In contrast, binding on the untreated surface did not increase beyond 40 °C (Fig. 3). The optimum time for affinity binding of human IgG at 50 °C was 45 min beyond which the absorbance remain unchanged (Fig. 4).

Much higher absorbance values were obtained when second antibody-conjugate binding was carried out at 50 °C (0.622 ± 0.012) rather than at 40 °C (0.407 ± 0.010) for the same duration of incubation. However, absorbance values continuously declined on the untreated surface beyond 40 °C. A 40-min incubation time was found to be optimum for maximum binding of the second antibody-conjugate (Fig. 5).

After optimizing temperature and time for all the steps of the assay, it was found that the optimum temperature for HELISA at any step was not more than 50 °C. This prompted us to use PS microtiter plates for HELISA since these can withstand such temperatures. A classical incubator was used for such plates but the temperature of the incubator was kept 2 °C higher than that used in the thermocycler. We then compared HELISA and ELISA procedures on both untreated and activated PS and PC surfaces using different conditions. Thus, ELISA procedures were carried out on these surfaces (PC1 and PS1) at 37 °C in an incubator for 7 h 45 min according to an improved ELISA procedure (Bora et al., 2002). When the ELISA was carried out for the same time (2 h 45 min) as that used for the HELISA but at 37 °C (PC2 and PS2), the absorbance values were much less than in the former experiment. Finally, the HELISA was carried out on both untreated and activated polycarbonate plates (PC3) in a thermocycler at 50 °C (blocking 40 °C) and on polystyrene microtiter plates (PS3) in an incubator at 52 °C (blocking 42 °C) for 2 h 45 min. The absorbance values were found to be a little higher than those obtained in the ELISA performed for 7 h 45 min at 37 °C. Similar results with both of the activated surfaces suggest that the

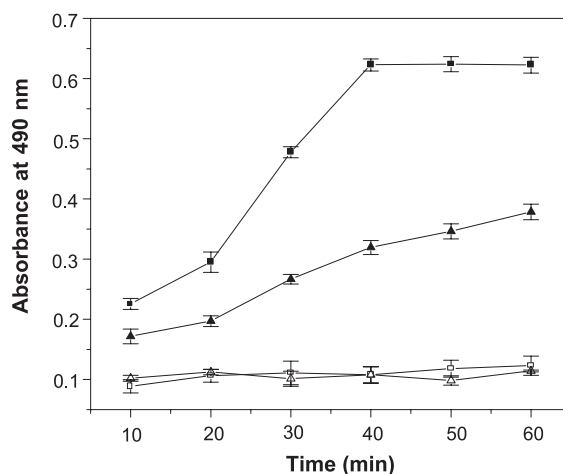


Fig. 2. Optimization of incubation time for immobilization of anti-human IgG at 50 °C in activated (■) and untreated (▲) PC plates. Absorbance was recorded after performing the subsequent ELISA steps by conventional methods. The open symbols represent negative controls. The error bars indicate standard deviation.

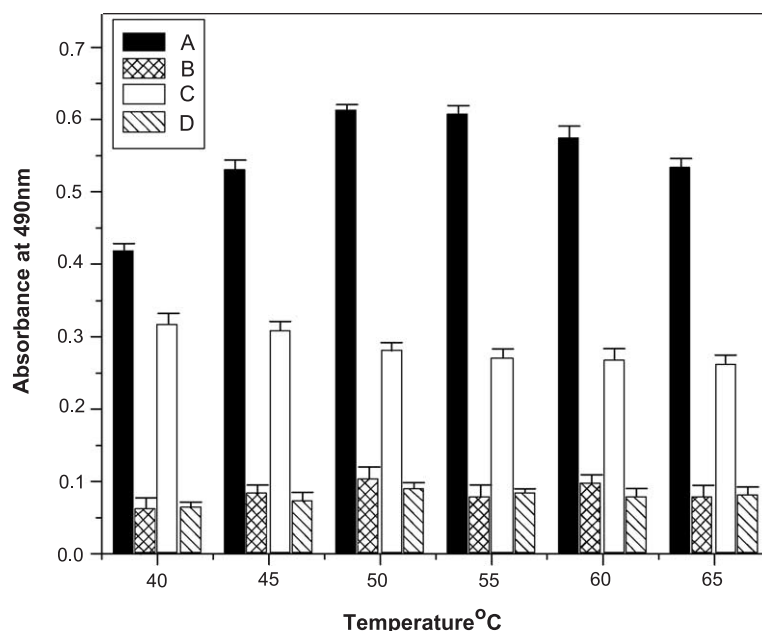


Fig. 3. Binding of human IgG at different temperatures (40, 45, 50, 55, 60 and 65 °C) over 60 min to anti-human IgG immobilized on activated (A and B) and untreated (C and D) wells of PC plates. Absorbance values were recorded after performing subsequent ELISA steps conventionally. (A) and (C) represent positive test sera, whereas (B) and (D) represent negative control sera. The error bars represent standard deviation.

HELISA can be carried out on either of the surfaces provided it is capable of making solid phases through stable covalent bonding (Fig. 6). The procedure does

not require any additional care or instrument except that the temperature of the reagent has to be adjusted to the HELISA temperature before placing the plate

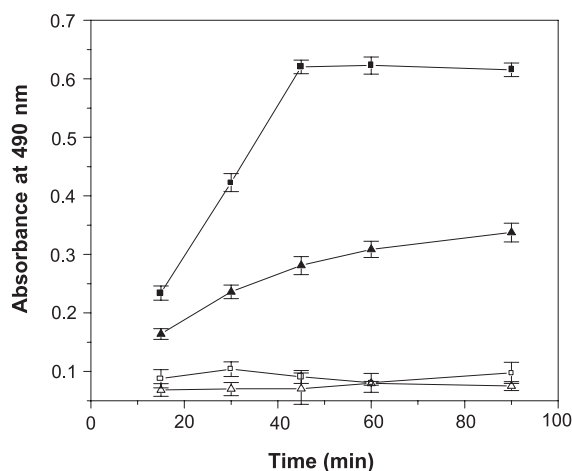


Fig. 4. Binding of human IgG at 50 °C for 15, 30, 45, 60 and 90 min, respectively, to anti-human IgG immobilized on activated (■) and untreated (▲) PC plates. Absorbance values were recorded after performing the subsequent ELISA steps. The open symbols represent normal goat serum used as a negative control. The error bars represent standard deviation.

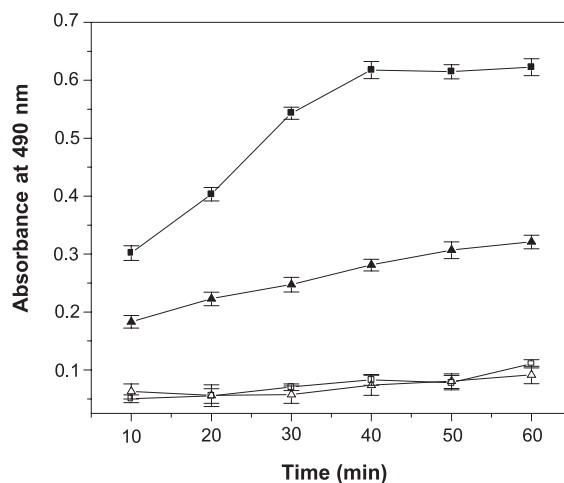


Fig. 5. Optimization of time (10, 20, 30, 40, 50 and 60 min) for the incubation of antibody–enzyme conjugate to human IgG bound to a solid phase at 50 °C. (■) and (▲) represent absorbance values recorded in assays carried out in the activated and untreated wells, respectively. The open symbols represent a negative control serum. The error bars represent standard deviation.

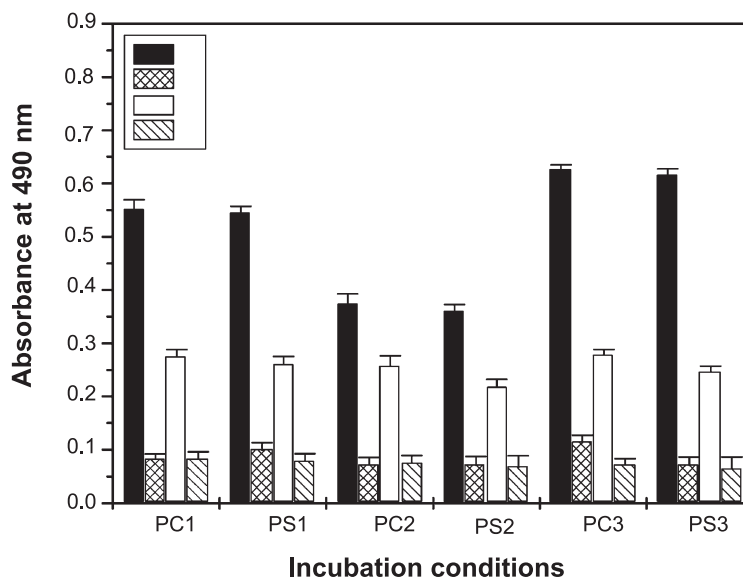


Fig. 6. Comparative studies of ELISA procedure carried out on photoactivated and untreated polycarbonate (PC) and polystyrene (PS) surfaces at different conditions: (a) time and temperature of the ELISA steps are 45 min, 1 h, 3 h and 3 h at 37 °C (PC1 and PS1), (b) ELISA conditions: 40, 40, 45 and 40 min at 37 °C (PC2 and PS2), (c) ELISA conditions: 40 min, 50 °C; 40 min, 40 °C; 45 min, 50 °C and 40 min, 50 °C (PC3 and PS3). (A) and (B) represent an activated surface and (C) and (D) represent an untreated surface. (A) and (C) represents positive test sera, whereas (B) and (D) represent negative control sera. The error bars represent standard deviation.

inside the preheated incubator. Stringent monitoring of the temperature during incubation is usually not required, as there is not much variation in the ELISA value, even if a slight deviation of temperature or time from optimum occurs (Figs. 1–5).

Finally, to generalize our method, we have carried out the HELISA procedure using the optimized condition as above (40 min 50 °C, 40 min 40 °C, 45 min 50 °C and 40 min 50 °C for 1st to 4th step, respectively) for the detection of total IgE in human sera on a photoactivated PC surface. IgE detection through a conventional ELISA procedure in human blood sera requires extended times of incubation. Standard curves prepared by performing the ELISA by the reported and conventional procedures were found to increase linearly with increased concentrations of human IgE (Fig. 7). Total IgE detected in the sera of patients by the reported method (ELISA value 0.369 ± 0.012 , 262.3 IU) was similar to that obtained by the conventional ELISA procedure (ELISA value 0.376 ± 0.015 , 265.5 IU). Similar results obtained from both procedures suggest that the HELISA procedure did not influence the specificity of the assay, which was further confirmed by doing an inhibition study. In two

separate experiments goat antihuman IgE was immobilized under conventional (11 h) and HELISA (40 min 50 °C) conditions. Inhibition patterns in both the plates

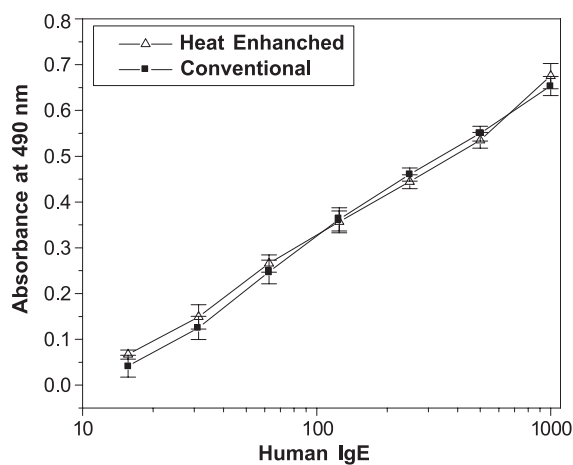


Fig. 7. Comparison between standard curves for determining IgE in human sera obtained by carrying out conventional ELISA (■) and HELISA (△) procedures. Incubation time and temperature for different steps of (a) HELISA were 40 min, 50 °C; 40 min, 40 °C; 45 min, 50 °C and 40 min, 50 °C; and (b) conventional ELISA were 11, 1, 3 and 3 h at 37 °C.

(results not shown) showed similar results confirming that higher incubation temperatures do not lead to any loss of the specificity of the assay.

The heat-mediated ELISA procedure was found to enhance assay speed without affecting specificity and sensitivity. This procedure also permits the detection of minute quantities of human IgE in less than 3 h with comparable results to those obtained in about 18 h by conventional methods. Although a photoactivated plate was used for the HELISA, any surface capable of covalently producing a solid phase can be used for such a purpose. The HELISA procedure is a potential alternative to the conventional ELISA carried out at 37 °C. The method may also find potential applications in PCR-ELISA and other solid phase based assays.

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