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Liposome-based immunoaffinity chromatographic assay for the quantitation of immunoglobulin E in human serum☆

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ABSTRACT

Immunoglobulin E (IgE)-mediated type I allergies affect over 25% of the world's population; they are among the most common diseases in developed countries. Therefore, simple and rapid in vivo and in vitro methods for diagnosing allergies are becoming increasingly important. In this paper, we demonstrate the feasibility of using sulforhodamine B, a fluorescent dye, entrapped inside immunoliposomes, the outer surfaces of which were sensitized with IgE, as a signal amplifier for the development of a simple, rapid, and inexpensive colorimetric affinity chromatographic immunoassay for the detection of total IgE in serum. This assay operates based on competition between standards (or human serum samples) containing IgE and IgE-sensitized immunoliposomes for the limited number of antigen binding sites of immobilized anti-IgE antibodies at the antigen capture (AC) zone on the nitrocellulose membranes. The color density of the AC zone is indirectly proportional to the number of IgE units present in the test sample. The detection limit of this liposome-based immunoaffinity chromatographic assay was 0.37 ng in IgE-free serum solution (equivalent to 20 μL of a 18.5 ng mL^{-1} solution). A commercially available ELISA kit was used as a reference method to validate the proposed assay through the analysis of three human serum samples.

1. Introduction

Immunoglobulin E (IgE), discovered independently by Ishizaka, Ishizaka, and Hornbrook and by Johansson [1–3] in the middle of the 1960s, was the first recognized reaginic antibody [4]. IgE is an antibody subclass, found only in mammals, that is capable of triggering the most powerful immune reactions. IgE is usually found at low levels in human serum (ca. 0.3 μg mL^{-1}) [5], but it is present at much higher concentrations in patients afflicted with allergic asthma, atopic dermatitis, and other immune deficiency-related diseases (e.g., AIDS). Therefore, it is possible that IgE determination might allow the discrimination between atopic and non-atopic individuals [6] because the IgE concentrations in the circulation can reach over 10 times the non-atopic level in "atopic" individuals, who have increased risk of developing allergies [7].

An allergic reaction is commonly initiated through the cross-linking of an allergen to the IgE antibody, followed by binding to its high-affinity Fc receptor (FcεRI) on tissue mast cells or blood basophils [8]. The immediate reaction occurs within a few minutes of allergen provocation, resulting in the release of mediators that lead to symptoms characteristic of the target organ. A late-phase response associated with the influx of T cells, monocytes, and eosinophils may arise hours later. Hayfever, asthma, reactions to food, and eczema – the most common allergic responses – are normally caused by mast cell activation in mucosal tissues of the nose, lung, gut, and skin, respectively [7].

The prevalence of allergies has increased dramatically, resulting in high-cost diagnosis and treatment—a cumbersome burden on health care systems. Several in vivo and in vitro methods have been developed in the last few decades for diagnosis of allergies [9]. Because the IgE level in serum is very low (<1/40,000 of the IgG level), there is an urgent need for a highly sensitive method for the accurate diagnosis of allergies. Conventional in vivo testing methods, including skin prick tests with allergens, are sensitive and reliable, but it has the potential risk of causing adverse reactions, such as systemic reactions and anaphylactic shocks. The radioallergosorbent test (RAST), first described in 1967, was a clever means of allergy diagnosis; a similar assay concept, direct sandwich radioimmunoassay (PRIST), was described in 1972 [10,11]. Subsequently, the radioisotope label used in the original RAST or PRIST was replaced with a chromogenic enzyme immunoassay or fluorescent enzyme immunoassay for safety considerations [12]. The

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commercialized CAP system routinely used nowadays in clinical practices for determining total and allergen-specific IgE is manufactured by Pharmacia (Uppsala, Sweden). In spite of the availability of in vitro assays for diagnosing allergies, most of them are expensive, labor-intensive, and time-consuming (each assay takes ca. 3–4 h) [12], requiring fairly large amounts of reagent and serum [13]. To overcome these limitations, in 2000, Wiltshire et al. developed the detection of multiple allergen-specific IgEs on microarrays through an immunoassay using rolling circle amplification [14]. Soon after, a microarray biosensor based on chemiluminescence for the detection of IgE was presented—it had been developed previously for the highly sensitive detection of pesticides and other environmental contaminants in drinking water [15]; an improved system is soon to be commercialized by ATTO-TEC (Siegen, Germany). In 2002, Hiller et al. [16] reported an allergen microarray based on fluorescence detection; a year later, the Weller and co-workers applied parallel affinity sensor array (PASA) technology to automatically perform allergy diagnosis [12], where purified recombinant and natural allergens, as well as allergen extracts, were immobilized onto glass slides for the detection of IgE, followed by detection using enhanced luminol chemiluminescence with the aid of label peroxidase. In addition, a self-assembled monolayer-based piezoelectric crystal immunosensor has also been developed for the quantification of total human IgE [17]. Furthermore, disposable screen-printed carbon electrode technology and amperometric detection have been employed in the development of an immunosensor for determining IgE in blood samples [18]. A label-free protein biosensor based on aptamer-modified carbon nanotube field effect transistors was demonstrated by Maehashi et al. in recent years [19].

The study presented herein is an application of a liposome immunoassay (LIA), using fluorescent sulforhodamine B (SRB) dye-loaded liposomes, the surfaces of which were sensitized with IgE, for the detection of allergen-specific IgE in human serum, performed in a test strip format. The proposed immunoaffinity chromatographic assay has potential to serve as a rapid and inexpensive point-of-care diagnostic assay.

2. Experimental

2.1. Reagents and materials

All inorganic chemicals and organic solvents were of analytical grade or the highest purity commercially available and used as received. IgE from human myeloma plasma was purchased from Athens Research & Technology (Athens, GA, USA). Anti-human IgE antibody was purchased from Abcam (Cambridge, MA, USA). A human IgE ELISA quantitation kit was purchased from Bethyl (Montgomery, TX, USA). Dipalmitoylphosphatidylamine-*N*-[4-(*p*-maleimidomethyl)cyclohexane-carboxamide] (PE MCC), dipalmitoylphosphatidyl choline (DPPC), and dipalmitoylphosphatidyl glycerol (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-Succinimidyl-*S*-acetylthioacetate (SATA) and SRB were purchased from Molecular Probes (Eugene, OR, USA). Cholesterol, Sephadex G-50, *N*-ethylmaleimide (NEM), hydroxylamine hydrochloride, ethylenediaminetetraacetic acid (EDTA), casein, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), polyvinylpyrrolidone, 3,3′,5,5′-tetramethylbenzidine (TMB), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). The IgG fraction of anti-biotin (goat) was purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). Dialysis tubing [molecular weight cutoff (MWCO): 12,000–14,000] was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Nitrocellulose membranes were purchased from Millipore (Bedford, MA, USA). All solutions were prepared with deionized water having a resistivity not less than 18 MΩ cm (Milli-Q, Bedford, MA, USA).

2.2. Methods

2.2.1. Preparation of SATA-modified IgE

Thiolated immunoglobulin molecules were prepared using a modification of a procedure described elsewhere [20]. In short, human IgE (0.2 mg) was dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.4). A sample of SATA (0.37 mg) in DMSO was added to the IgE solution. The reaction mixture was then mixed on a shaker for 90 min at room temperature. The acetylthioacetyl-IgE was deprotected by adding hydroxylamine hydrochloride (10 μmol) to obtain free sulfhydryl groups. Subsequently, the thiolated IgE was purified using a Microcon® Centrifugal Filter Device (Millipore, Billerica, MA, USA) having a 50-kDa MWCO.

2.2.2. Preparation of maleimide-derivatized, SRB-encapsulated liposomes

Liposomes were prepared using a reverse-phase evaporation method [21–23]. A lipid mixture, consisting of DPPC, cholesterol, DPPG, and PE MCC (molar ratio = 5:5:0.4:0.14), was first dissolved in a mixture of chloroform, isopropyl ether, and methanol (6:6:1, v/v/v; 4 mL). A 1.0-mL aliquot of a warmed solution of 90 mM SRB was then added to the lipid mixture. After sonication of the solution for an additional 3 min with occasional swirling, the organic solvent was evaporated at 45 °C, leaving a dark-purple gel-like suspension of liposomes. An additional 1.0 mL of SRB was added, followed by another 3 min of sonication at 45 °C. The liposome preparation was incubated in a water bath at 45 °C for 30 min and then it was passed through a 0.4-μm polycarbonate filter to produce a homogeneous suspension of uniform size. Any unencapsulated dye or traces of organic solvent were removed from the liposome preparation through gel filtering on a 1.5-cm × 25-cm Sephadex G-50-150 column at room temperature.

2.2.3. Conjugation of maleimide-derivatized liposomes with SH-containing antibody

The coupling of the thiolated IgE to maleimide-derivatized liposomes was achieved by incubating overnight at 4 °C. Unreacted sulfhydryl groups on the antibody were subsequently capped with *N*-ethylmaleimide. After dialysis (MWCO = 12–14 kDa), the antibody-tagged liposomes were collected and stored in the dark at 4 °C.

2.2.4. Preparation of test strips and assay procedures

Immobilization of anti-human IgE antibodies on plastic-backed nitrocellulose membranes was performed through manual pipetting, leading to a final concentration of 0.5 μg of antibody/strip. The antibody-coated bands were air-dried in the hood for 30 min and then further dried under vacuum (10 psi) at room temperature for 60 min. The coated nitrocellulose sheet was then immersed in a blocking solution comprising 0.5% polyvinylpyrrolidone and 0.03% casein in Tris-buffered saline (TBS) at pH 7.0 for 60 min on a rotating shaker, followed by drying under vacuum (10 psi) at room temperature for 1.0 h. The prepared sheets were then cut into 0.5 cm × 9.0 cm strips using a paper cutter, producing strips with the antigen capture (AC) zone positioned 1.5 cm above the bottom of the strip. The prepared strips were packed with desiccants and stored at 4 °C until required for use. The format for the newly developed assay consists of an immunoliposome solution of optimal volume and concentration, an appropriate amount of the test sample in a glass tube (12 mm × 75 mm), and a nitrocellulose test strip featuring the immobilized anti-IgE antibodies. Five minutes after inserting the test strip into the glass tube containing only the sample solution, a solution of the IgE-tagged immunoliposomes was added to the same glass tube and then the solution front was allowed to reach the upper end of the test strip. After removing the strip and air-drying, the signal intensity of the test line

was estimated qualitatively (visually) or quantitatively [after conversion into grayscale readings using an HP scanjet 4370 scanner (Palo Alto, CA, USA) and Biosoft Quantiscan software (Cambridge, UK)]. The collected data was transformed into a binding ratio using the equation

$$\text{binding ratio} = \frac{I}{I_0} \times 100\%$$

where I is the grayscale reading generated at a given concentration of IgE and I_0 is the reading obtained at zero IgE.

2.2.5. Human IgE ELISA quantitation kit

A commercial human IgE ELISA quantitation kit was used in this study for the quantitative analysis of IgE in standard solutions or human serum samples and the validation of the proposed new assay as well. The procedure was performed using the manufacturer's method. Briefly, each tested samples were transferred to an anti-IgE antibody-coated well, wherein sample IgE molecules were bound to the anti-IgE antibody binding sites. Unbound sample IgE and other soluble substances were then rinsed away; the HRP-conjugated detection antibody was then added. Development of color occurred as a result of the presence of bound conjugate, which reacted with the TMB substrate solution. Stop solution (0.18 M H_2SO_4) was added to stop the enzymatic reaction; the absorbance (OD_{450}) of the resulting solution was measured. The values of absorbance of the samples were compared with those of controls; using a standard curve, IgE concentrations of samples were then calculated.

3. Results and discussion

3.1. Characteristics of liposomes

Size uniformity (homogeneous liposomal biolabels of a similar size) and the surface charge of liposomes are very important key elements in the development of a reliable diagnostic liposome immunoassays; these characteristics can be determined using a Brookhaven 90 plus particle size analyzer and a ZetaPlus Zeta Potential Analyzer (Holtsville, NY, USA), following the manufacturer's method. The integrity of the liposomes correlates with the shelf-life of the fluorescent liposomal biolabels; it can be investigated by measuring the fluorescence generated from SRB before and after lysis. According to our previous studies, almost instantaneous total lysis of liposomes occurs at room temperature when a solution of 50 mM *n*-octyl β-D-glucopyranoside (OG) is added. For these fluorescence tests, the SRB dye was excited at a wavelength of 544 nm and the fluorescent emission intensity was measured at 596 nm.

The key characteristics of the liposomes were briefly described as follows: the average diameter of the liposomes used in this study was 238 ± 31 nm, suggesting that the average volume of a single liposome was 7.1×10^{-12} μL, with an entrapped volume (assuming a bilayer thickness of 4.0 nm) of 6.4×10^{-12} μL. Assuming that the SRB concentration inside the liposomes was equal to that in the original solution, and comparing the fluorescence of the lysed liposomes with that of standard SRB solutions, we estimated that there were 4.0×10^{12} liposomes per milliliter and that each liposome contained 3.4×10^5 molecules of SRB. Because the average surface areas of DPPC and cholesterol molecules are 71 and 19 Å^2 [24], respectively, we estimated that an average of 77 molecules of IgE were located on the outer surface of each liposome—assuming that all the IgE molecules were fully thiolated with excess SATA and that they were subsequently successfully coupled with 0.70 mol% PE MCC presented on the outer surface of liposome. The ζ potential of the liposomal biolabels under study was −15.3 ± 1.4 mV, indicating that these liposomes were dispersed uniformly in the solution. The IgE-sensitized, SRB-containing liposomes were stable at 4 °C in PBS for at least two months without any significant leakage of the encapsulated SRB moieties or appreciable loss of activity for the IgE tags sensitized on the outer surfaces of the liposomes.

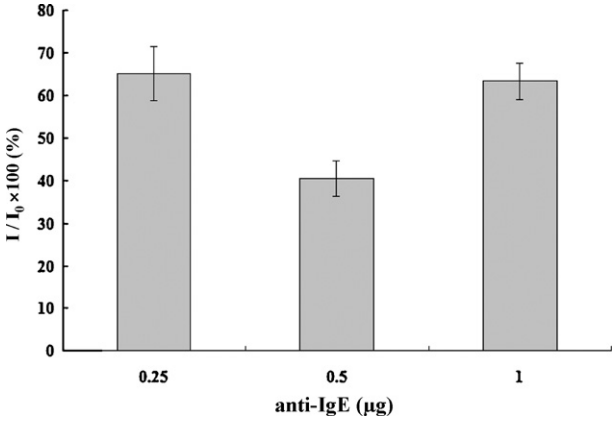

Fig. 1. Effect that the amount of anti-IgE antibody immobilized on the nitrocellulose membrane has on the generation of the signal intensity at IgE levels of 0 and 10 μg mL^{-1}. A mass of 0.5 μg of the anti-IgE antibody immobilized on a single strip was chosen as the optimum because it provided a more-noticeable signal difference between the signals obtained from the 0 and 10 μg mL^{-1} IgE (analyte) solutions.

3.2. Optimization of parameters

The amount of antibody immobilized on the test strip is a central consideration to the performance of this assay. Therefore, we investigated the optimal concentration of the capture antibody immobilized on the strip. Fig. 1 reveals the effect of the amount of immobilized capture antibody on the performance of the immunoaffinity chromatographic assay for IgE; we manufactured test strips dot-blotted with 0.25, 0.5, and 1 μg of anti-IgE antibody. Fig. 1 reveals a much more-noticeable signal difference between 0 and 10 μg mL^{-1} IgE (analyte) solutions when using the immunoaffinity chromatographic assay featuring 0.5 μg of the anti-IgE antibody immobilized on a single strip. This level was, therefore, chosen as the optimum for use in subsequent experiments.

Fig. 2 depicts the effect of the amount of liposome on the performance of the immunoaffinity chromatographic assay. Here, we found that the optimal amount of added liposome was 3 μL; this sample, which contained ca. 1.2×10^{10} liposomes and encapsulated ca. 6.9×10^{-9} mol of SRB dye, produced the highest signal difference between 0 and 10 μg mL^{-1} IgE solutions.

Fig. 3 displays the effect of the volume of the liposome solution used as signal biolabels on the performance of the immunoaffinity chromatographic assay for measuring IgE. We diluted samples of the liposome solution (3 μL, containing ca. 1.2×10^{10} liposomes) by

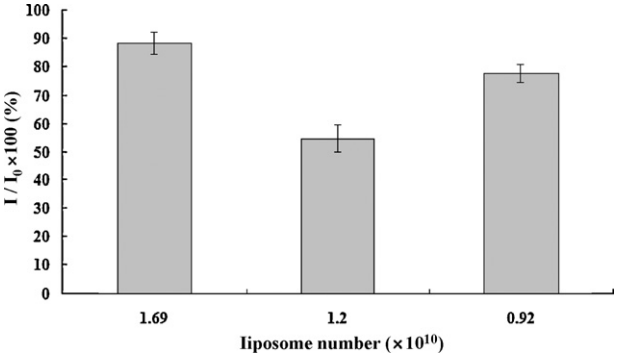

Fig. 2. Effect of the amount of added liposome on the generation of the signal intensity. Volumes of 2.2, 3, and 4.2 μL of the liposome preparation were investigated (containing 0.92×10^{10}, 1.2×10^{10}, and 1.69×10^{10} liposomes, respectively).

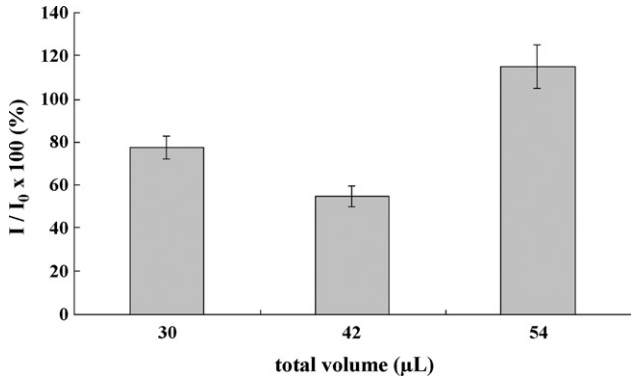

Fig. 3. Effect of the dilution factor of the liposome solution on the generation of the signal intensity. Dilutions of the 3-μL liposome solution by 10-, 14-, and 18-fold were investigated, with 14-fold dilution providing the optimal sensitivity.

10-, 14-, and 18-fold. A 14-fold dilution (3 μL of liposome diluted to 42 μL in the appropriate buffer) appeared to provide the optimal concentration—i.e., it gave the lowest value of I/I_0 and, hence, the most evident signal difference.

3.3. Assay performance

We performed an analytical calibration of IgE to measure the sensitivity of the immunoaffinity chromatographic assay toward IgE, testing using various concentrations of the target analyte (18.5–500 ng/mL). The calibration curve for the detection of IgE was obtained by recording, at each concentration, the grayscale reading of a test strip under the optimal experimental conditions. Each point of the calibration graph corresponds to the mean value obtained from at least three replicate determinations. Fig. 4 displays the dose–response curve obtained for immunoaffinity chromatographic strips prepared with 0.5 μg of anti-IgE antibody and using IgE standards. It plots 100 I/I_0 (%) as a function of the logarithm of the amount of IgE. The inset reveals that the correlation coefficient (r^2) of the data for the linear part of the curve, between 18.5 and 500 ng/mL for IgE, was 0.988. Set by the International Union of Pure and Applied Chemistry (IUPAC), the operational detection limit is defined here as the concentration corresponding to a signal three standard deviations below the mean for a calibrator that is free of IgE. Accordingly, the limit of detection (LOD) for IgE measured by the immunoliposome strip coated with 0.5 μg of anti-IgE antibody was 0.37 ng of IgE (equivalent to 20 μL of an 18.5 ng mL^{-1} solution) at a 99.7% level of confidence. This immunosensor function with acceptable reproducibility; the responses at various concentrations of IgE yielded an RSD of no greater than 15%.

Table 1
Detection limits, linear dynamic ranges, and sensitivities of the liposomal.

	Liposome immunoassay	Commercially available ELISA
LOD	0.37 ng	1.56 ng
Linear dynamic range	0.37–10 ng (18.5–500 ng mL^{-1})	1.56–100 ng (15.6–1000 ng mL^{-1})
Sensitivity|slope|	26.7	1.11

Table 2
Concentrations (ng/mL) of IgE in serum samples, detected using both the ELISA kit and the immunoliposome strip.

	Sample 1 (average)	Sample 2 (average)	Sample 3 (average)
ELISA kit	34.4	296.3	491.4
Immunoliposome strip	39.5	298.0	509.4

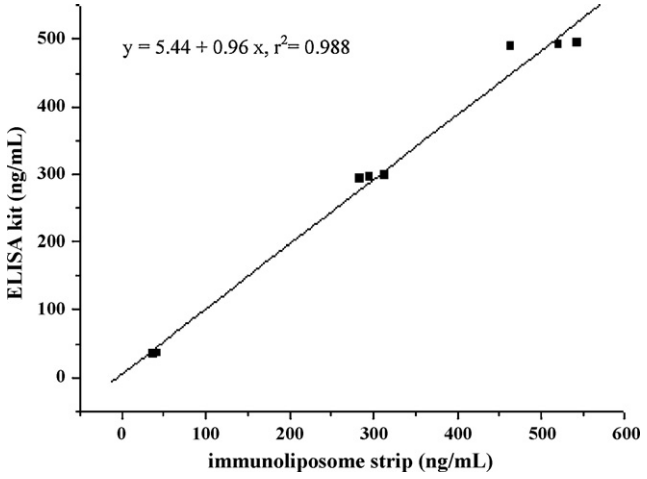

Fig. 5. Correlation between the results obtained using the ELISA and liposomal immunoaffinity chromatographic strip assay for the detection of IgE.

3.4. Validation of the sensor and serum sample analysis

One of the essential elements in sensor development is an adequate validation system; therefore, we employed a commercial ELISA system to validate the accuracy of our proposed technique by examining human serum samples obtained from laboratory personnel [25]. Table 1 lists the detection limit, linear dynamic range, and sensitivity obtained using both our liposomal immunoaffinity chromatographic assay and the ELISA kit. Table 2 presents mean quantitative data based on triplicate analyses for each sample. The best-fit regression line of the average of the liposomal

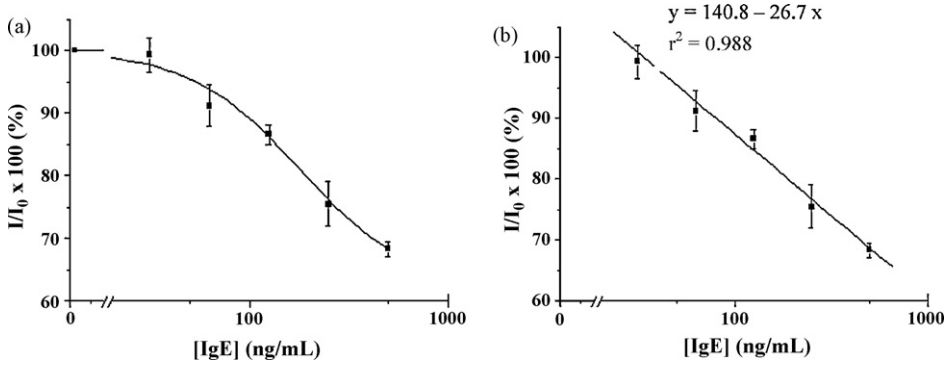

Fig. 4. (A) Dose–response curves in the IgE-free serum system obtained from the immunoliposome strip coated with anti-IgE antibody; error bars: ± 1 standard deviation. (B) Linear part of the main curve. Table 1 summarizes the detection limit, linear dynamic range, and sensitivity.

immunoaffinity chromatographic strip's triplicate analyses versus those determined using the commercial ELISA kit indicated a strong correlation between the two data sets ($r^2 = 0.988$; Fig. 5), suggesting that the liposomal immunoaffinity chromatographic strip assay is comparable to commercial ELISA method for the detection of IgE in serum samples. As a result, our proposed liposomal immunoaffinity chromatographic strip assay might be an alternative method for the detection of human IgE in clinical diagnostics.

4. Conclusions

We have demonstrated the possibility of measuring IgE at elevated levels in human serum samples using a liposomal immunoaffinity chromatographic strip assay. A good correlation ($r^2 = 0.988$) existed between the levels obtained using ELISA (commercial available kits) and the proposed assay (response (y) = 5.44 + 0.96[IgE] (x)). The final assay was optimized to measure IgE in serum in a competitive immunoassay format within 30 min. This strip assay format obviates the necessity for washing and incubation, commonly required in ELISA procedures, thereby making this assay less prone to errors and further reducing the cost. This strip assay provided a detection limit of 0.37 ng of IgE (equivalent to 20 μL of an 18.5 ng mL^{-1} solution) in addition to its advantages of disposability and cost-effectiveness. The immunoliposomes prepared in this study are feasible for use in competitive immunoassays that allow the simple, rapid, and inexpensive densitometry-based quantitation of IgE in serum samples. Future studies will be focused on the detection of allergen-specific IgEs in such samples.

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