Immunoenzymatic assay for total IgE quantification of blood spot on filter paper

RESEARCH

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ABSTRACT

This paper describes, the development of an immunoenzymatic method for the quantification of total IgE in dried blood collected on filter paper, by using UMELISA IgE kits for human serum. The optimal conditions for the assay were standardized and its analytical characteristics considering precision, accuracy, tracking record and specificity of the monoclonal antibody were determined. The standard curve 0 to 200 IU/mL showed a sigmoid behavior with a limit of detection of 0.7 IU/mL and a correlation coefficient of 0.9999 according to the International Standard. The variation coefficients intra-and inter-assays were lower than 10%. Accuracy was very good and the recovery test average was 97.86 ± 5.6. The results obtained with the UMELISA IgE test kit were compared with the commercial kit (Enzymun-Test IgE, Boehringer Mannheim) and the correlation index found was r=0.9583.

Key words: immunoenzymatic assay, quantification of total IgE, allergy, blood spot on filter paper

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RESUMEN

Ensayo inmunoenzimático para la cuantificación de inmunoglobulina E total en muestras de sangre seca sobre papel de filtro. Se describe el desarrollo de un ensayo inmunoenzimático para la cuantificación de la inmunoglobulina E (IgE) total en muestras de sangre secadas sobre papel de filtro, con el empleo de los reactivos del estuche UMELISA IgE para muestras de suero humano. Se estandarizaron las condiciones óptimas del ensayo y se determinó su precisión, exactitud, detectabilidad y especificidad. Se obtuvo una curva estándar con comportamiento sigmoidal en el rango de 0 a 200 U/mL, con un límite de detección de 0.7 U/mL y un coeficiente de correlación de 0.9999 con el estándar internacional. Los coeficientes de variación intra-ensayos e inter-ensayo son inferiores al 10%. El método desarrollado se considera exacto, con una desviación no mayor que 5.6% y el 97.86% del material recobrado. La correlación de los resultados del ensayo del UMELISA IgE para papel de filtro con los del *Enzymun-Test IgE* de la firma Boehringer Mannheim, mostró un índice de correlación igual a 0.9583.

Palabras claves: ensayo inmunoenzimático, IgE total, alergia, sangre seca sobre papel de filtro

*I*ntroduction

Allergic diseases have a significant impact on clinical practice due to their high prevalence. That prevalence was calculated to be 10% in the Cuban population [1], but in developed countries, there are even higher figures that tend to increase [2].

The clinical importance of IgE quantification is due to the need of reaching an objective discrimination within its hyperactivity, causing allergic symptoms, and the classic atopic diseases, since the correct and early diagnoses of those diseases, together with their early and appropriate treatment, would reduce the risk of chronic inflammation and histic destruction [3-5].

Besides atopic diseases, other health disorders can increase seric IgE levels. The most relevant ones are helminthic infestations [2], Hodgkin disease [6], multiple myeloma by the increase of the IgE levels [7], transplant-vs.-host disease, and immunodeficiency conditions caused by the Wiskott-Aldrich syndrome, and the acquired immunodeficiency syndrome (AIDS) [8], which have been used as forecasting markers [9]. There are several environmental factors that can cause a variation of IgE levels, though pollution is emphasized [10]. Sex and age also cause IgE variations. Its seric values are slightly higher in men than women; there is an increase of the values at 15 years of age and they decline after 30 [11, 12]. Therefore, IgE quantification must be carefully interpreted in the field of clinical signs and according to the results from other diagnostic tests [5, 12-15].

Several methods for IgE quantification have been developed throughout the years [16-21]. They must meet certain requirements, such as precision, accuracy, detection limit, specificity studies and others, all of which are regulated by the European Committee for Clinical Laboratory Standards, (ECCLS) that have made it possible to obtain high-quality diagnostic systems [22].

For collecting blood samples, filter paper is preferred due to its simplicity, easy transportation [23-25], stability and because it can be kept at 4 ºC or at room temperature [26-28]. These features make filter paper a very reliable alternative method [29] for any assay [30], especially in the neonatal screening systems [31-38].

The purpose of this paper is to develop and validate an immunoenzymatic assay for IgE quantification in dried blood samples on filter paper, by using the UMELISA IgE kit for human serum.

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Materials and methods

Samples

Serum and total blood samples were obtained by finger puncture from 58 adults and 42 children. The samples were spotted on filter paper. They came from the Vedado Blood Bank and the "Juan Manuel Marquez" Pediatric Hospital, respectively.

The filter paper used was SS'2992, made by Schleicher & Schuell. The blood samples were collected according to the standard procedure by the US National Committee for Clinical Laboratory Standards (NCCLS) [23]. The samples were dried for 3 h at room temperature (20-25 ºC) in a device created for that purpose. Then, they were placed in paper envelopes, appropriately identified and kept at -20 ºC for later testing.

Diagnostic systems

The chemical and biological reagents used in the standardization of this assay were those of the UMELISA IgE kit, made by the Immunoassay Center (CIE), identified by the Sanitary Record 9401-06, for total IgE quantification in human serum. The kit is composed of:

- Three ultramicroELISA plates, coated with the anti-IgE monoclonal antibody (96 wells for each plate).
- Regulating solution TRIS 0.371 ml. /L, pH 7.8.
- Standard sera with concentrations of 0.5, 13, 32, 80 and 200 U/mL, calibrated against the 75/502 international standard of the World Health Organization (WHO).
- Control serum for a known IgE concentration (58.32 U/m)
- Anti-IgE/alkaline phosphatase polyclonal conjugate.
- 4-methylumbelliferyl-phosphate fluorogenic substrate.
- Regulatory solution for the substrate, diethanolamine 0.92 mol/L, pH 9.80.

The reference diagnostic kit used was Boehringer Mannheim's EnzymunTest IgE that is a colorimetric enzymatic immunoassay for determining seric IgE levels in human serum.

Equipment

The ultramicroanalytical system (SUMA) equipment and accessories were used for this assay. This technology, created and developed at the Immunoassay Center (CIE) only uses 10 µL the sample and reagents for the immunoenzymatic assays and it includes the PR-521 plate reader, a MAS 301 automatic washer, an ERIZO 101 multipipette and a p-52 driller. Moreover, we used a MicroELISA stirrer (Abbot, USA) and an incubator at 37 ºC (Retomed-Sakura, Cuba-Japan).

Standardization of the assay for blood spot on filter paper

The standardization of the assay for blood spot on filter paper is performed according to the following studies:

- Study of the elution conditions: to determine the elution conditions of the samples: sporadic manual stirring and steady shaking, in a microELISA plate shaker, with different elution periods: 1 h; 2 h; and 3 h at room temperature (between 20 and 25 ºC). The assays were repeated for several days in a humid chamber.

- Study of the incubation conditions of the eluate and the anti-IgE/alkaline phospatase conjugate: Different incubation temperatures were tested to determine incubation conditions: 37 ºC and room temperature (between 20 and 25 ºC), for different incubation periods: 2 h; 4 h and 18 h. The assays were repeated in a humid chamber for several days.

- Study of the incubation condition of the substrate. Different temperatures were tested to determine the incubation conditions of the substrate: 37 ºC and room temperature (between 20 and 25 ºC), for different incubation periods: 30 min; 1 h and 2 h. The assays were repeated in a humid chamber for several days.

Validation of the assay for dried total blood spots on filter paper

The analytical characteristics of the assay were determined according to the European Committee for Clinical Laboratories Standards (ECCLS) [22].

- Comparison of the IgE standard carried out at the Immunoassay Center (IAC) with the IgE international standard (IS) of the World Health Organization (WHO): For this, an IS ampoule, containing 5 000 U was reconstituted in 1 mL distilled water and it was diluted 25 times to 200 U; that gives the maximum point of the curve of the standard of the Immunoassay Center (CIE). It was then diluted 1: 2.5 times to obtain the 5 points of the curve: 0, 5, 13, 32, 80 and 200 U/mL. Each point of the curve of the IAC standard was reconstituted in 500 mL of distilled water. Then, both standards were compared, in duplicate, through UMELISA IgE. This procedure was performed for three days, by different technicians. The results were averaged and, the correlation coefficient between both curves was obtained.

- Within and between assay precision: The precision study was performed with 4 samples in different ranges of the curve. For each sample, 5 replicates were assessed on 2 plates and 2 different days.

- Linearity: A sample with a high IgE concentration (654 U/mL) was collected and serially diluted 1:2.5. At the same time, it was assessed by the IAC standard. Then, two assays were performed and the values were averaged. A regression study was used to compare the standard curve with the mean value of both assays and the linearity of the curve obtained from the sample was checked.

- Recovery: A definite amount of IgE was added to 5 known samples and the fluorescence values obtained in the IAC standard curve were interpolated. The recovery rate for each point was calculated for those values:

Recovery rate = true value (U/mL/expected value (U/mL) x 100%.

· True value. IgE concentration value obtained by interpolating the fluorescence of the IgE-enriched sample, in the IAC standard curve.

· Expected value. The expected IgE concentration of the sample after the addition of a theoretically calculated definite amount of IgE.

- Parallelism: Four samples of different IgE concentrations were collected and diluted serially 1:2 four times. The fluorescence values obtained were interpolated in the curve and the results of the IgE concentration for each dilution were multiplied by

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the corresponding dilution factor. If there was a real accuracy, all dilutions should give a similar concentration for each sample, with a variation coefficient lower than 10%.

- Specificity of the assay antibodies: Two techniques were used for this study:

· Ouchterlony's Double Immunodiffusion [39] to the conjugate anti IgE polyclonal antibody to check if there was any reactivity between it and the analyzed plasmatic proteins (G. M. A immunoglobulins and human seralbumin), supplied by the Protein Purification Laboratory of IAC and assessed at the normal seric concentration range.

· To assess the coating anti-IgE monoclonal antibody specificity, an Indirect ELISA was used and plasmatic proteins were added, the same ones used for the former assay, having the same origin and concentration.

- Correlation between Enzymun-Test IgE (serum) and UMELISA IgE (filter paper) with the purpose of correlating the new UMELISA IgE assay for filter paper with the Enzymun-Test IgE from Boehringer Mannheim.

Statistical Analysis

The data were processed in an Excel worksheet, a program from Microsoft Corporation, 2000 Version. The mean, standard deviations (SD) and of coefficients variation (%) were estimated for the variables requiring them in each assay.

Results and discussion

Standardization of the assay for total blood spot on filter paper

- Study of the elution conditions: The average values of the results from the elution kinetics of the samples are shown in table 1. fluorescence values similar to those of the serum were obtained with an elution of 2 h with sporadic manual stirring, at 20-25 ºC or with an elution of 1 h with a steady shaking. Therefore, both ways can be used. Even though the elution periods are longer, no substantial changes are observed to justify an extension of the time period.

- Study of the incubation conditions of the eluate and the anti-IgE/AP conjugate: The results are shown in table 2. The best results were obtained at room temperature (RT) and with an 18 h incubation. The reaction was not properly completed at shorter periods. Similar results were obtained at 37 ºC with a 4 h incubation, but they had higher variation coefficients (even over 10%, as recommended in the consulted literature) [22]. This could be due to the evaporation occurring in the mixture in this period, because there were hardly 20 µL for each well. Therefore, it is recommended to incubate them for 18 h at room temperature in a humid chamber to reduce evaporation.

- Study of the incubation conditions of the substrate: The results of that study are shown in table 3. Similar results were obtained with a 1 h incubation at room temperature or for 30 min at 37 ºC, but it is important to take into account that incubation at that temperature increases the value of the assay targets, hence, the accuracy of the samples with low values of IgE concentration could be affected. It is recommended to incubate the substrate for 1 h at room temperature in a humid chamber.

Table 1. Results of the fluorescence of the samples for different conditions.

Concentration IgE (IU/mL)	Samples of serum (F)	Dried blood sample spots on filter paper (F)					
		Sporadic manual stirring			Steady shaking		
		1 h	2 h	3 h	1 h	2 h	3 h
0	4	3	6	5	5	5	6
5	14	10	15	17	14	13	16
13	29	21	30	32	32	30	32
32	58	43	61	70	57	60	63
80	98	79	102	99	105	106	103
200	145	113	150	140	147	139	151

*F: fluorescente

Table 2. Results of fluorescence of the samples for different conditions.

Concentration IgE (IU/mL)	Samples of serum (F)	Dried blood sample spots on filter paper (F)						
		At room temperature				At 37 ° C		
		2 h	4 h	18 h		2 h	4 h	
				F	CV (%)		F	CV (%)
0	4	3	3	5	9.1	4	6	15.3
5	13	6	9	14	7.2	8	12	23.2
13	32	11	14	29	5.3	14	26	16.5
32	65	24	30	58	6.1	32	52	11.1
80	100	49	55	97	4.4	62	96	7.9
200	135	69	81	142	3.9	96	140	12.5

* F: fluorescente
* CV: coefficient of variation

Validation of the assay for total blood spot on filter paper

- Comparison of the IgE standard made at IAC with the WHO IgE International standard (IS): correlation coefficient of 0.9999 was obtained when correlating the 0; 5; 13; 32; 80 and 200 U/mL standard sera with the WHO international standard 75/502. The slope was 1.0115 and the intercept was -0.825. The standard curve was a sigmoid in the 0 - 200 U/mL range. The linearity obtained at low concentrations showed a detection limit of 0.7 U/mL, obtained from the analysis of the first point of the curve: 0 U/mL+2 SD (Standard Deviation) figure 1.

- Within and between-assay precision: The results of the precision study are shown in table 4. The values for the within and between-assay variation coefficients were below 10%, a parameter used for similar systems [30-33, 36].

- Linearity: The results of the linearity study for a sample with a high IgE concentration (654 U/mL) are shown in figure 2. The regression study indicated

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Table 3. Results of fluorescence of the samples for different conditions.

Concentration IgE (IU/mL)	Samples of serum (F)	Dried blood sample spots on filter paper (F)					
		At room temperature			At 37 ° C		
		30 min	1 h	2 h	30 min	1 h	2 h
0	5	3	4	8	7	10	12
5	12	7	12	16	13	15	21
13	24	13	26	31	27	32	44
32	50	28	49	64	52	65	87
80	90	54	92	119	95	115	134
200	138	72	139	164	141	156	177

*F: fluorescencia

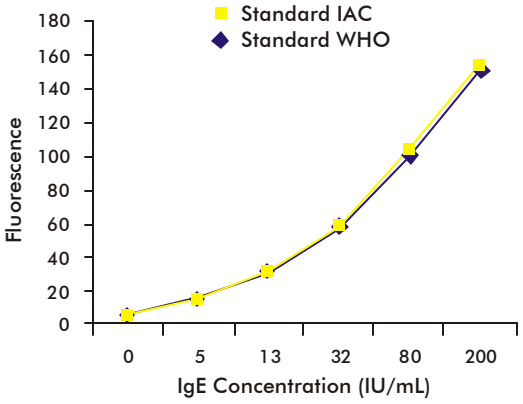

Figure 1. Correlation between the WHO Standard and the IAC Secondary Standard.

Table 4. Precision of the UMELISA IgE with dried blood spots on filter paper.

Concentration IgE (UI/mL)	Within-assay (n = 10)		Between-assay (n = 40)	
	SD*	CV (%)	SD*	CV (%)
22.3	1.6	7.8	2.2	8.1
46.3	2.2	4.1	2.6	5.2
95.8	4.8	6.7	5.7	6.5
142.6	6.1	7.4	7.9	7.3

SD*: Standard deviation
n: number of samples

that the assay had a high efficacy, with r = 0.9951. The linearity of the curve was verified and the accuracy of the assay was confirmed, as found in other assays reported [14, 16, 32].

- Recovery: In figure 3, the recovery rates are between 80 and 120%, of the recommended range; mean recovery was 97.86 ± 5.6%.

- Parallelism: The serial dilution of 4 samples in the assay range had no notable effect in determining the IgE concentration when it was multiplied by the dilution factor, and the coefficient of variation was 2.8-9.9% (Figure 4).

- Specificity of the assay antibodies:

· The results of the Ouchterlony double immunodiffusion technique (39) applied to the conjugate anti-

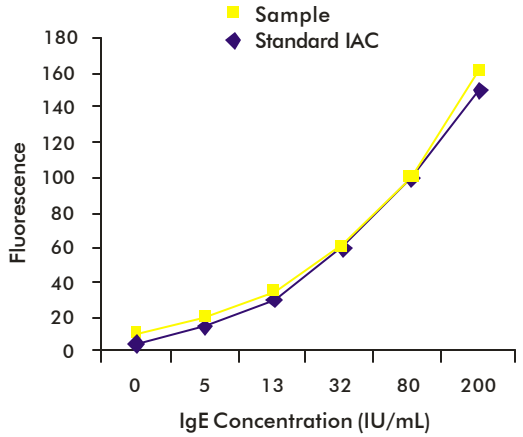

Figure 2. IgE UMELISA linearity with blood spots on filter paper.

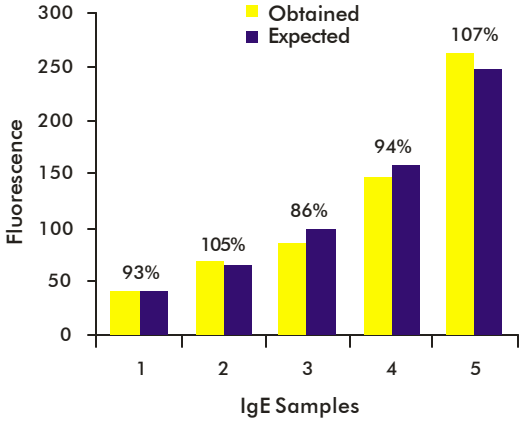

Figure 3. IgE UMELISA Recovery with blood spots on filter paper.

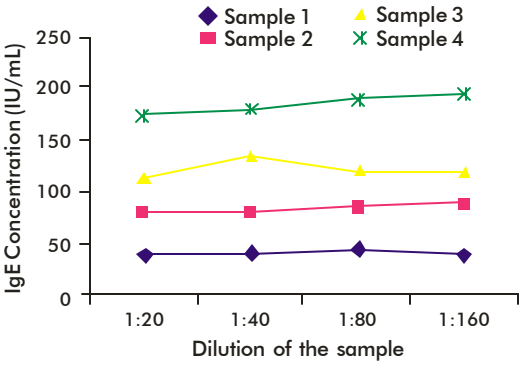

Figure 4. UMELISA IgE parallelism with blood spot samples on filter paper.

body showed that there was no reactiveness between it and the analyzed plasmatic proteins (G, M, A immunoglobulins and human seroalbumin). A precipitation band appeared only in IgE, and that confirmed the specific recognition of this immunoglobulin by the polyclonal antibody used in the assay.

· In figure 5, the results of the indirect assay for assessing the specificity of the coating monoclonal

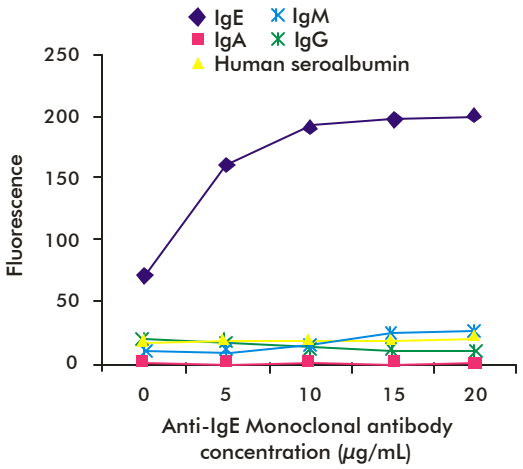

Figure 5. Cross reactivity of the human coating Anti-IgE monoclonal antibody versus other seric proteins.

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antibody are shown. There were no fluorescence signs evidencing recognition by the monoclonal antibody, of albumin and other seric immunoglobulins showing a certain structural similarity with IgE, which could have produced unspecific reactions by cross reactivity.

- Correlation between the Enzymun-Test IgE (serum) and the UMELISA IgE (filter paper): The results of the correlation study between the Enzymun-Test IgE (serum), of Boehringer Mannheim, and the UMELISA IgE (filter paper) of IAC, with blood samples of children and adults are shown in figure 6. The correlation coefficient was 0.9583, which is even higher than others reported for those systems in the reviewed literature [30].

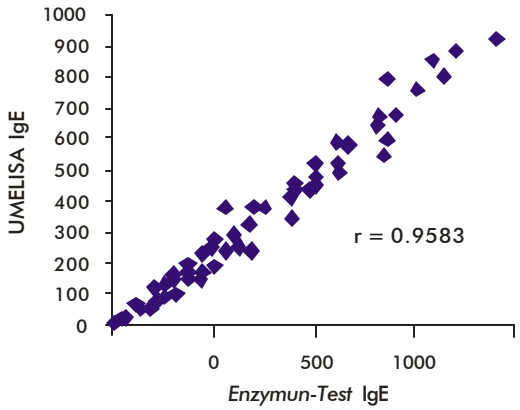

Figure 6. Correlation between Enzymun-Test IgE and IgE UMELISA in blood spots on filter paper. 100 samples from children and adults.

Conclusions

An immunoenzymatic method for determining total IgE in dried blood sample spots on filter paper was developed. The optimal conditions for the assay were as follows:

- Elution of the sample for 1 h with steady shaking or for 2 h with sporadic manual stirring at 20-25 ºC.

- Incubation of the eluate with the IgE/AP conjugate: for 18 h at 20-25 ºC.

The standardized UMELISA IgE for samples spots on filter paper meets the quality parameters of precision, accuracy and clinical assessment, accepted for enzymatic immunoassays [22].

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