A NEW UMELISA FORMAT FOR THE QUANTIFICATION OF MATERNAL SERUM ALPHA-FETOPROTEIN

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ABSTRACT

In our country, the quantification of Alpha-fetoprotein is performed using a sandwich type heterogeneous enzyme immunoassay (UMELISA-AFP, 10 µL of samples and reagents)compatible with SUMA (Ultramicroanalytical system) technology designed mainly for screening purposes. This Kit has been modified, for improved manipulation and use. This paper shows standardization results of this new format (ready to use), its comparison with Enzymun-Test AFP of Boehringer Mannheim (Code 1361236) and screening results in our country. Stability is always higher than 80 % in the new presentation of the reagents that form the Kit (after 18 months). In the linear regression analysis performed between the new UMELISA format and the former one, a correlation of 0.9998, a slope of 1.07 and an angle of 44°, were obtained. In the comparison between the International Standard 72/225 of The World Health Organization (WHO) and our standard, we obtained a correlation of 0.9996, a slope of 0.9798 and an angle of 44°. Variation coefficients intra and inter-assay, obtained in the accuracy assay were less than 5 %. A correlation of 0.9380 was given by the correlation assay with 36 samples with the Enzymun-Test AFP of Boehringer Mannheim.

Key words: Alpha-fetoprotein, new UMELISA format, SUMA

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RESUMEN

En nuestro país la cuantificación de Alfa-fetoproteína se realiza a través de un ensayo inmunoenzimático heterogéneo de tipo sandwich, (UMELISA-AFP, 10 µL de muestras y reactivos), compatible con la tecnología SUMA (Sistema Ultramicroanalítico), designada fundamentalmente para propósitos de pesquisaje. Este estuche diagnóstico ha sido modificado con la finalidad de lograr una mejor utilización y manipulación del mismo. En el presente trabajo se reflejan los resultados de la estandarización de este nuevo formato (listo para el uso), su comparación con el Enzymun-Test AFP de la Boehringer Mannheim (Código 1361236) y los resultados en el pesquisaje realizado en nuestro país. La estabilidad obtenida en la nueva presentación de los reactivos que conforman el estuche siempre fue superior al 80 %, después de 18 meses. En el análisis de regresión linear realizado entre el nuevo formato y el de uso, se obtuvo una correlación de 0,9998, una pendiente de 1,07, y un ángulo de 44°. Al comparar nuestro estándar con el Estándar Internacional 72/225 de la Organización Mundial de la Salud (OMS) se obtuvo una correlación de 0,9996, una pendiente de 0,9798 y un ángulo de 44°. En el ensayo de precisión se obtuvieron coeficientes de variación intra e inter-ensayo menores del 5 %. La correlación con 36 muestras con el Enzymun-Test AFP de la Boehringer Mannheim dio como resultado una correlación de 0,9380.

Palabras claves: Alfafetoproteína, nuevo formato UMELISA, SUMA

Introduction

Alpha-fetoprotein (AFP) is a glycoprotein of approximately 70 000 Da, usually found in normal serum, in minimal concentrations which increase physiologically during pregnancy (1-3). Its quantification, in maternal serum, is relevant for the screening of neural tube defects (NTD), open defects in the anterior ventral wall, twin pregnancy, threats of abortion, fetal death and others (4-8). The low AFP maternal serum values are associated to chromosome abnormalities in the fetus, such as Down's Syndrome (9,10). It is a marker associated to hepatocellular carcinomas and tumors of the ovary and testicle germinal cells. Serum AFP also increases in the presence of other malignant tumors such as; gastric and pancreatic carcinomas, cholangiocarcinomas, esophageal carcinomas and non neoplastic hepatic diseases (hepatitis and cirrhosis).

The Ultramicroanalytical system (SUMA) was developed in our center in the last decade. It was designed mainly for screening purposes. This includes SUMA equipment and reagents used in the UltramicroELISA test (UMELISA), which combines high sensitivity of current ELISA tests with the use of ultramicrovolumes of samples and reagents (10 µL or less).

Immunoassays are highly related with quantification of Alpha -fetoprotein in maternal serum (11-14).

A sandwich type heterogeneous enzyme immunoassay for the quantification of AFP in pregnant women between 15 and 19 weeks of pregnancy for pre-natal diagnostic (15) was introduced in our

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country in 1982. It was compatible with the SUMA technology, making the assay specific, accurate, rapid, of low cost and applicable to a large number of samples at the same time and very useful in screening protocols (16-19).

The useful life time of the Kit format was affected by the low stability of the reagents, mainly the fluorogenic substrate (4-methylumbelliferyl phosphate) and that is the reason why a new one has been developed. Another limitation was the solid phase used (irradiated polystyrene plates with a capacity of 39 duplicated determinations). If the amount of samples to process were less than this number, the rest of the plate positions could be wasted or results would be delayed on awaiting to complete this number.

Materials and Methods

Solid Phase
Irradiated polystyrene plates (8 X 12 strips) and with titanium dioxide (Greinerlabortechnik, Germany) were used as a solid phase (20).

Antibodies
Polyclonal antibodies to AFP were obtained from the Immunoassay Center. Amniotic fluid with raised level of AFP (in a concentration higher than 200 µg/mL, quantified by electroimmunodiffusion) was used as an antigen. Antigen was purified by ionic exchange chromatography in DEAE Sephadex matrix and a K26/40 ion-exchange column in order to attain a high purity level. Afterwards, antigen was purified by affinity chromatography (AH-Sepharosa 4B-anti AFP) and it was dialized in Sephadex G-25 Coarse. Then, sheep were immunized using this antigen.

The antibodies obtained, were purified by affinity chromatography AH-Sepharose 4B glutaraldehyde-AFP and eluted with a 0.2 M glycine buffer, pH 2.8. They were desalted versus phosphate-buffered saline, pH 7.4 (PBS), and stored in the same buffer at -20 °C until their use.

Antibody-coated Solid Phase
The solid phase used was coated with 15 µL of polyclonal AFP antibodies at a concentration of 8 µg/mL in 0.1 M carbonate-bicarbonate buffer, pH 9.6; the plates were incubated during 4 hours in a humidity chamber at 45 °C, and then they were blocked with PBS containing 0.1 % bovine serum albumin (BSA) and sealed.

Washing buffer
Tris (hydroximethyl)methylamine-HCl, 0.015 M pH 7.8, (BDH Chemicals Ltd. Poole, England).

Preparation of standard sera, control serum and samples
The standard sera and control serum were prepared in CIE from normal human serum with amniotic fluid (Maternal Hospitals and from Blood Banks, in Havana City), and they were negative to anti-HIV 1+2, HBsAg and Anti-HCV. The standard serum was presented in a lyophilized form at a concentration of 100 IU/mL; the new standard serum is presented in the form of independent concentrations which are: 96 IU/mL, 48 IU/mL, 24 IU/mL, 12 IU/mL, 6 IU/mL and 0 IU/mL (this has only Human Serum).

In the reconstitution of the Standard Sera and the Control Serum and in the dilution of samples, a sheep serum was used in two different forms: at 20 % and without dilution; both were reconstituted with Tris, obtaining a final concentration of 5 %. The dilution of samples was 1:2 in both solutions.

Antibody-enzyme Conjugate
A polyclonal anti-AFP antibody was conjugated with alkaline phosphatase EIA-grade I (Boehringer Mannheim GmbH, Germany), using the one-step glutaraldehyde method developed by Avrameas (21). This conjugate was used in a concentrated and ready to use solution (diluted in Tris pH 8.0 and BSA at 1 %).

Substrate preparation
The 4-methylumbelliferyl phosphate substrate (4-MUP) (Koch Light Ltd. Haverhill, Suffolk, England), was prepared in two different forms: a lyophilized form and a 0.5 mM concentrated solution. Diethanolamine buffer 0.92 M pH 9.8 (Merck, Schuchardt, for synthesis) was used for its dilution. As a reference solution, 0.057 mM 4-methylumbelliferone was used.

Equipment-SUMA fluorimeter 421
To measure the intensity of the emitted fluorescence due to the enzymatic activity and the non-enzymatic hydrolysis a computerized fluorimeter (SUMA) was used. The SUMA has a high sensitivity and low operation cost; it reads fluorescence in less than 1 minute. The reading is performed between the 420 and 540 nm wavelengths.

ERIZO Multipipette 201
It is used to manipulate liquids and to prepare samples in immunotesting techniques that require great precision, in a volume interval between 2 and 200 µL.

MAS Washer 201
It is a fully-automatic device used to guarantee uniformity in the washing procedures of the UltramicroELISA plates.

The accessories of SUMA laboratory and the software package UMELISA-AFP were also used.

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Determination of residual humidity using Karl Fisher's method
Determination is performed as a dead-stop endpoint (DSEP) titration using a double platinum electrode, with the Radiometer TTT 83KF-TITRATOR (Copenhagen, Denmark).

The potentiometric titration is based on the reaction between the iodine methanolic solution and water in the presence of sulphur dioxide and pyridine. Chemical reactions abruptly change the resistance and these sudden changes, detected by the introduction of a double platinum electrode in the solution, indicate that the equivalence point has been reached.

Test Procedures
Samples are incubated in strip wells during 1 hour at 37 °C. Unbound elements are removed after three washings with Tris buffer and only the antibody/AFP complex remains in the wells. After washing them, 10 µL of alkaline phosphatase (A.P.)-conjugate anti AFP were added and incubated 1 hour at 37 °C. Strips are washed again to eliminate the excess of the conjugate. Then the wells were incubated for 30 minutes at room temperature with 10 µL of 4-MUP that is hydrolyzed by the enzyme of the conjugate. The fluorescence intensity will be proportional to the AFP concentration in the sample.

Statistical analysis
The statistical parameters were obtained using the appropriate tests in the StatGraphic software on an IBM-compatible computer.

Precision
Analyte concentration was measured repeatedly in three human sera that contained AFP in three ranges of values: high, medium and low.

Sensitivity
The lowest limit of detection (mean + 2 SD of results for zero standard) was calculated from the results of assaying twenty replicates of the zero standard.

Comparison between the International Standard of AFP and standard serum of the new UMELISA format
A linear regression analysis between the standard serum by independent concentrations and WHO International Standard 72/225 was performed.

Correlation between the commercial assay and the new UMELISA format
A correlation of 36 samples of low, medium and high concentrations was made, using the new UMELISA format and the Enzymun-Test AFP of Boehringer Mannheim (Code 1361236).

Results and Discussion

Standardization
In this paper reagents of the new format and the former one, using UMELISA AFP are compared.

Solid phase irradiated polystyrene plates (8 X 12 strips) and with titanium dioxide
The stability of the reaction strips without their protective cover and stored with desiccant in the given bags was higher than 95 % after 6 months.

Table 1. *Stability of the new reagents.*

Points of the curve	Solid- phase (6 months)			Tris 25x (2 months)		
	Control	Strips	% recovery	Control	R.T.U.	% recovery
1	5.50	3.57		6.34	6.80	
2	26.00	22.00	84.61	31.84	30.18	94.78
3	42.12	35.26	83.72	52.33	45.21	86.39
4	62.01	55.01	88.71	79.40	72.18	90.90
5	94.59	86.42	91.36	112.97	101.98	90.27
6	128.43	120.51	93.89	140.97	131.02	92.94
Points of the curve	**Sheep serum (4 months)**			**Conjugate (12 months)**		
	Control	R.T.U.	% recovery	Control	R.T.U.	% recovery
1	5.36	6.55		6.09	6.70	
2	31.85	31.55	99.05	21.54	20.90	97.04
3	50.79	50.51	99.44	33.51	36.94	110.20
4	78.98	76.94	97.41	56.57	52.72	93.20
5	111.92	112.93	100.90	88.09	79.12	89.80
6	149.85	146.70	97.891	31.28	115.10	87.75

R.T.U. = Ready to use

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Table 2. *Non-enzymatic hydrolysis of 4-MUP.*

Time (months)	Relative fluorescence	
	Concentrated solution	Lyophilized substrate
0	0.90	1.21
18	1.80	32.15

Tris Buffer 25X (liquid)
It was diluted in distilled water and stored at 4 °C during 2 months; stability was higher than 95 %.

Sheep serum 20X (liquid)
It was diluted 1:4 with ready to use Tris buffer and was stored at 4 °C during 4 months, then stability was above 95 %.

Alkaline phosphatase-conjugate Anti-AFP (ready to use)
The stability of the conjugate in the ready to use form was above 85 % in 12 months. This presentation decreases laboratory mistakes. Table 1 presents the results obtained.

Standard and control serum (lyophilized)
Stability was higher than 90 % after 12 months of reconstitution and storage at 4 °C. Residual humidity in the lyophilized material was between 3 and 3.5 %. The standard for independent concentrations prevents errors in the preparation of the calibration curve.

4-MUP substrate (concentrated solution)
The non-enzymatic hydrolysis of 4-MUP was checked by measuring relative fluorescence (Table 2). The recovery of the enzymatic activity in the assay was over 90 %.

Standard curve
Table 3 and Figure 1 show the standard curve obtained after 12 months of preparation.

Precision
The intra-assay (n = 30) and inter-assay (n = 90) reproduction was evaluated with 3 known specimens. Coefficients of variation obtained were below 5 % (Table 4). The precision of the assay is adequate.

Table 3. *Standard curve of the new format (12 months).*

Points of the curve	Control	New format	% recovery
1	5.33	5.59	
2	23.23	21.90	94.27
3	34.26	32.10	93.69
4	56.60	54.42	96.14
5	87.34	81.97	93.85
6	123.54	118.14	95.62
Control	69.87	67.54	96.66

STATISTICAL ANALYSIS
Intercept = -0.69
Correlation = 0.9998
Slope = 1.0701
Angle = 44°

Sensitivity
Sensitivity of the new format is approximately 1.0 IU/mL. This was calculated as the concentration which was distinguishable from the zero standard, that is, two standard deviations above the fluorescence of the 0.00 IU/mL standard serum.

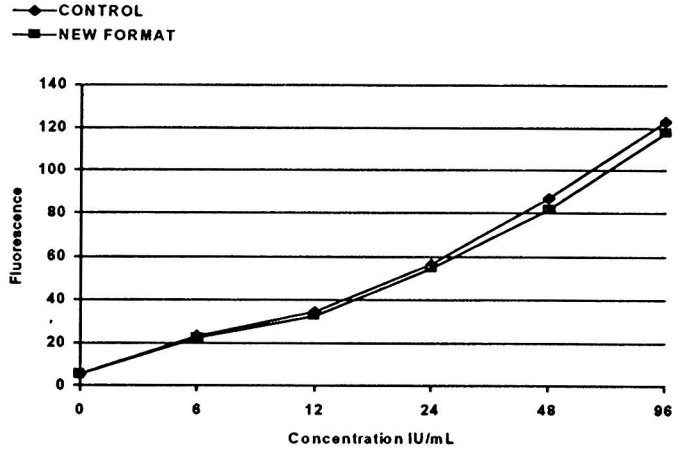

Table 4. *Precision.*

Sample	Intra-assay (n = 30)			Inter-assay (n = 90)		
	X	S.D.	C.V.	X	S.D.	C.V.
Low	26.03	1.14	4.40	26.62	1.25	4.72
Medium	67.10	2.68	3.99	67.94	3.10	4.65
High	133.34	3.90	2.92	133.60	4.30	3.25

S.D. = standard deviation
C.V. = coefficient of variation

Linear regression analysis
In the comparison between the WHO International Standard 72/225 and the present standard, a correlation of 0.9996, a slope of 0.9798 and an angle of 44° was obtained.

Correlation assay
The correlation coefficients for UMELISA versus Enzymun-Test AFP of Boehringer Mannheim was 0.9380.

Screening in our country
Table 5 shows a screening study of pregnant women started in Cuba in 1982 ; since then the data have remained the same. Table 6 shows the results for the introduction of this new UMELISA format in our country.

Conclusions

It can be concluded that with the introduction of these changes time was saved, special laboratory abilities are not required and there is a better use of the Kit. The new format has a higher precision and works better than the former one.

Quantification is similar for new UMELISA AFP and Boehringer Mannheim format since the correlation coefficient obtained is 0.9380.

Table 5. *Screening in Cuba since 1982.*

	Number of pregnant women
Study of pregnant women	1 605 409
NTD diagnosis	2 024
Other malformations	1 288
Other diagnoses	11 007
Incidence X 1 000	2.06

Table 6. *Screening with the introduction of the new format.*

	Number of pregnant women
Study of pregnant women	112 206
NTD diagnosis	91
Other malformations	47
Other diagnoses	284
Incidence X 1 000	1.20

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