A FAST AND SENSITIVE NON COMPETITIVE STREPTAVIDIN-BIOTIN ENZYME IMMUNOASSAY FOR HUMAN EPIDERMAL GROWTH FACTOR.

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SUMMARY

We have developed a sensitive enzyme immunoassay for the detection of recombinant human Epidermal Growth Factor (rhEGF). The assay is based on a previously developed **sandwich** ELISA which uses monoclonal antibodies (Mabs) CB-EGF.1 and CB-EGF.2, that has been modified to include streptavidin-biotin amplification.

General parameters influencing the performance of the assay as coating, blocking and biotinylation procedure were first optimized. We found that exposure of the coated Mab to a pH stress significantly reduced the amount of necessary antibody. Once these parameters had been adjusted, three different variants of the assay were compared: (1) a three-step procedure, (2) a two-step procedure with a preformed biotin-streptavidin complex, and (3) a two-step procedure with simultaneous incubation of the sample and the biotinylated second antibody.

We have found that the three methods result in the same sensitivity level. However, the third one was the fastest and most reproducible. The detection limit achieved was 30 pg/ml and the dynamic range between 50 and 1600 pg/ml. The intra and inter-assay coefficient of variation for the third method were 8.79 and 9.70%, respectively, while the mean analytical recovery was 99.87%. Finally, we have validated the usefulness of this assay in pharmacological studies with rhEGF in dogs.

RESUMEN

Se ha desarrollado un inmunoensayo enzimático de elevada sensibilidad para la cuantificación del factor de crecimiento epidérmico humano recombinante (rhEGF). Este inmunoensayo se basa en un ELISA tipo **sandwich** anteriormente reportado con los anticuerpos monoclonales (AcM) CB-EGF.1 y CB-EGF.2 y al cual hemos añadido un sistema de amplificación biotina-estreptavidina. Los diferentes parámetros que influyen en el comportamiento del ensayo, tales como recubrimiento, bloqueo y procedimiento de biotinilación, fueron optimizados. Se encontró que al someter al AcM de recubrimiento a un **stress** de pH, era posible reducir significativamente la cantidad necesaria de anticuerpos.

Una vez fijados estos parámetros se compararon tres variantes diferentes de ELISA: (1) un procedimiento de 3 pasos, (2) un procedimiento de 2 pasos, con el complejo preformado biotina-estreptavidina y (3) un procedimiento de 2 pasos con incubación simultánea de la muestra y el segundo anticuerpo biotinilado.

Se encontró que los 3 métodos logran el mismo nivel de sensibilidad, pero el tercero fue más rápido y reproducible. El límite de detección del sistema fue de 30 pg/ml, mientras que el rango dinámico del mismo estuvo entre 50 y 1600 pg/ml. Los coeficientes de variación intra e inter-análisis para el método (3) fueron 8.79 y 9.70%, respectivamente. El recobrado analítico promedio fue 99.87%. Se logró validar la utilidad de este ensayo durante estudios toxicológicos con rhEGF en perro.

INTRODUCTION

Human Epidermal Growth Factor (hEGF) is a 53-aminoacid polypeptide, weighting around 6000 Da, and with potent stimulatory activity on the proliferation of numerous cell types *in vitro* and *in vivo* (Moriyama *et al.*, 1991, Zapatasirvent *et al.*, 1993). The availability of large amounts of hEGF obtained by recombinant DNA techniques (Oka *et al.*, 1985) has promoted the clinical evaluation of this molecule as a wound healing drug (Martin *et al.*, 1992; Brown *et al.*, 1991).

While highly sensitive procedures, such as radioimmunassay and ELISA, are currently employed for the quantitative measurement of hEGF (Hayashi *et al.*, 1985a,b), these methods impose special demands for staff, laboratories and equipment. In order to avoid isotopic methods, to reduce the time of the assay, and to increase sensitivity, we have modified a previously reported **sandwich**-type ELISA, based on two specific monoclonal antibodies (Mabs) (Freyre *et al.*, 1989; Vázquez *et al.*, 1990) by including a streptavidin-biotin (SB) amplification step.

MATERIAL AND METHODS

Epidermal Growth Factor

Recombinant hEGF (rhEGF) was kindly provided by the Production Division of the CIGB. The samples were titrated with hEGF from Boehringer Mannheim and used as a reference preparations.

Monoclonal Antibodies

Pure preparations of the Mabs CB-EGF.1 and CB-EGF.2 (Freyre *et al.*, 1989), specific for hEGF, and for both human and mouse EGF, respectively, were supplied by the Monoclonal Antibody Production Group of the CIGB.

Two washing buffers were used: 0.1% sodium citrate, containing 0.1% (w/v) bovine serum albumin (BSA), and PBS, containing 0.5% (v/v) Tween-20.

Biotin Labelling

Biotinylation of CB-EGF.2 was made as follows. The Mab was dialyzed against carbonate/bicarbonate buffer 50 mM, pH 8.5, at a protein concentration of 1.5 mg/ml. It was then mixed with 1.5 mg/ml, 1 mg/ml, 0.8 mg/ml and 0.4 mg/ml of biotinyl N-hydroxysuccinimide ester (BNHS, Pierce), and incubated for 2 h in ice. The mixture was then dialyzed overnight against phosphate buffered saline (PBS) pH 7.4, at 4°C.

Streptavidin-Peroxidase Conjugation

Streptavidin (Amersham) was coupled to peroxidase (Boehringer Mannheim) using sodium periodate as crosslinking reagent (Nakane and Kawaoi 1974).

Flat-bottom polystyrene microtiter plates purchased from Greiner, were always used, unless specified. In some experiments, Titertek (Flow), Maxisorp (NUNC) or Polysorp (NUNC) flat-bottom microtiter plates were used.

ELISA

Method 1 (three steps)

Polystyrene microtiter plates (Greiner) were coated overnight at 4°C with 5 μg of purified CB-EGF.1 per ml of coating buffer (0.06 M sodium carbonate/bicarbonate, pH 9.6), at 100 μl/well. The plates were washed three times with 0.1 M sodium citrate containing 0.1% (w/v) bovine serum albumin (BSA), vacuum-dried at room temperature. Samples (100 μl/well), prediluted with PBS containing 0.1% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20, were incubated for 1 hour at 37°C. The plates were washed four times with PBS-Tween 0.5%, and 100 μl of biotinylated CB-EGF.2 were added per well, and incubated for 1 h at 37°C. After washing as described above, the plates were incubated with 100 μl/well of the streptavidin-peroxidase conjugate for 30 minutes at 37°C, washed once, and the reaction developed with 100 μl of substrate (0.12 mg of 3,3':5,5' tetramethylbenzidine and 2 μl of 3% (v/v) H_2O_2/ml, diluted in acetate buffer pH 5.5), per well, for 15 min. at room temperature. The reaction was arrested by adding 50 μl 1M H_2SO_4 per well. The absorbance of each well was measured in a Titertek Multiscan MC340 spectrophotometer at a wavelength of 450 nm.

Method 2 (two steps)

The biotinylated CB-EGF.2 and the streptavidin-peroxidase conjugate were incubated for 1 h at 37°C under agitation (tridimensional agitator). The plates, already incubated with the samples and washed, were incubated with this mixture (100 μl/well). The rest of the procedure was carried out as described for method 1.

Method 3 (two steps).

Both sample and biotinylated CB-EGF.2 (50 μl/well, each), were added simultaneously to the pre-sensitized microplates, and incubated for 1 hour at 37°C. After washing, 100 μl of streptavidin-peroxidase conjugate was added per well, for 30 min at 37°C. The rest of the procedure was carried out as described for method 1.

Samples

Serum samples, collected from dogs under preclinical studies with rhEGF, were supplied by the Division of Clinical and Pre-clinical Studies (CIGB). The samples were stored at -20°C for periods ranging from one month up to 6 months before use.

pH Stress

The effect of pH stress on coating antibody was tested using method 1. Different concentrations of coating antibodies were incubated during 5 min with 0.1 M glycine/HCl, pH 2.6. This solution was diluted 1:10 in 1 M potassium phosphate buffer, pH 8.3, and 100 μl of treated antibody were added per well. Controls of CB-EGF.1 diluted in both 0.1 M carbonate buffer, pH 9.6, and 1 M potassium phosphate buffer, pH 8.3 were included. Maxisorp and Polisorp (NUNC) plates were also used in these experiments.

Statistical procedures

The detections limits of the rhEGF assay were established taking into account two standard deviations above the mean absorbance of the negative controls. Estimation of the antigen content in the samples was based on the use of a reference standard curve.

RESULTS

Biotinylation of CB-EGF.2

Biotin labelling of CB-EGF.2 was done at BNHS/protein ratios (w/w) of 1, 0.66, 0.53, and 0.27 (the molar ratios of BNHS/protein were 269:1, 178:1, 142:1 and 73:1). Different dilutions of biotinylated CB-EGF.2 were tested in the 3-step method. The best results obtained for each BNHS/protein ratio are present in figure 1 as the relationship between the lower (50 pg/ml) and higher (2000 pg/ml) points of the standard curve, with the background values. A BNHS to anti-hEGF ratio of 0.53 gave the best sample/background proportion values (1.54 and 14.0 for 50 and 2000 pg/ml, respectively), and was used for subsequent

studies. Using higher biotinylation ratios the assay was less sensitive because of the higher background, while for lower ratios a diminution in the absorbance values was found.

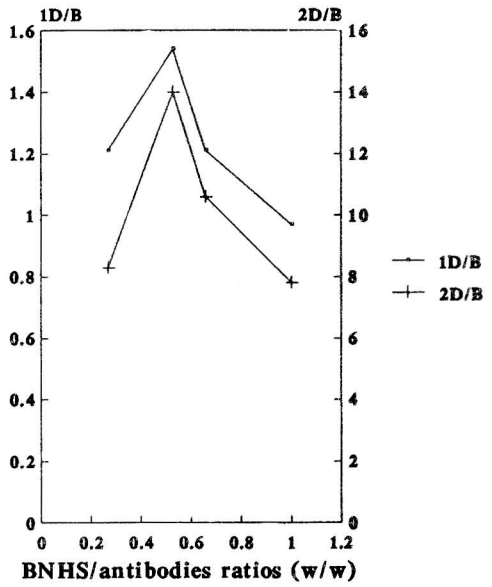

Fig. 1. Proportion between the lower (50 pg/ml) and higher (2000 pg/ml) points of the standard curve with the background values. (1D/B) Optical density to 50 pg/ml of hEGF and background ratios, (2D/B) Optical density to 2000 pg/ml of hEGF and background ratios.

Blocking and Coating Conditions

Method 1 (three steps) was used as the basis for the establishment of the blocking and coating conditions, as follows:

(a) Blocking

The background values were lowered using PBS, 0.1% BSA 0.1% and 0.05% Tween-20 as sample buffer. Higher concentrations of BSA and Tween slightly decreased the optical density values (data not shown). A previous washing step with BSA diluted in sodium citrate buffer prevented non specific binding in washing the same form as when it was incubated postcoating.

(b) Coating

In order to determine the optimal concentration of the coating antibody, the plates were incubated with 1.25, 2.5 and 5 μg/ml of CB-EGF.1 (100 μl per well). We found that concentrations above 2.5 μg/ml did not increase the absorbance values.

pH Stress

The pH stress study showed that the antigen binding capacity was higher in the treated Mab-coated plates than in the control ones. The practical benefit of this phenomenoum is that the amount of Mab needed for coating can be reduced four-fold (0.625 μg/ml), with the same final values as for 2.5 μg/ml. These observations were true for Maxisorp (figure 2a) and Greiner (data nor shown) microplates, but a similar effect was not observed when Polysorp (figure 2b) or Titertek microplates were used.

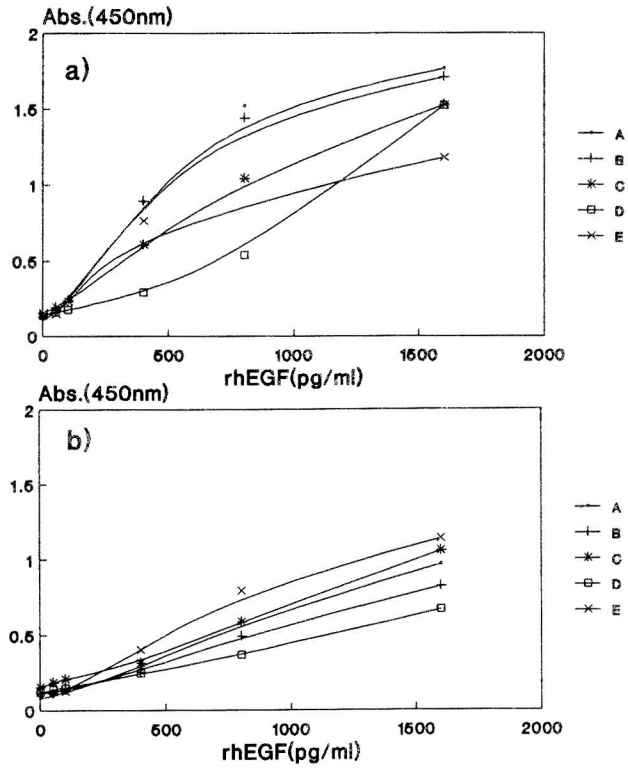

Fig. 2. The pH stress studies in (a) Maxisorp and (b) Polysorp microplates. Determinations were made in duplicate. 0.625 vg/ml (A), 1.25 vg/ml (B), and 2.5 vg/ml (C) of coating antibody with pH stress, 2.5 vg/ml (D) of coating antibody in potasium phosphate buffer, 2.5 g/ml (E) of coating antibody in carbonate-bicarbonate buffer.

Comparison of the Three ELISA Methods

(a) Optimal dilution of reagents

A 1:1000 dilution was optimal for both the biotinylated antibody and the streptavidin-peroxidase conjugate, in method 1. In method 2, the CB-EGF.2-biotin conjugate was tested at dilutions ranging from 1:250 to 1:1000, with streptavidin-peroxidase at dilutions ranging from 1:250 to 1:1000. Maximal activity was attained using a 1:1000 dilution of biotinylated CB-EGF.2, with a 1:250

dilution of streptavidin-peroxidase. Figure 3 shows a comparison of methods 1 and 2. A similar comparison was done between methods 1 and 3. As shown in figure 4, with a 1:500 dilution of the biotinylated antibody, and 1:1000 for the streptavidin-peroxidase conjugate, analogous values for the standard curve were obtained.

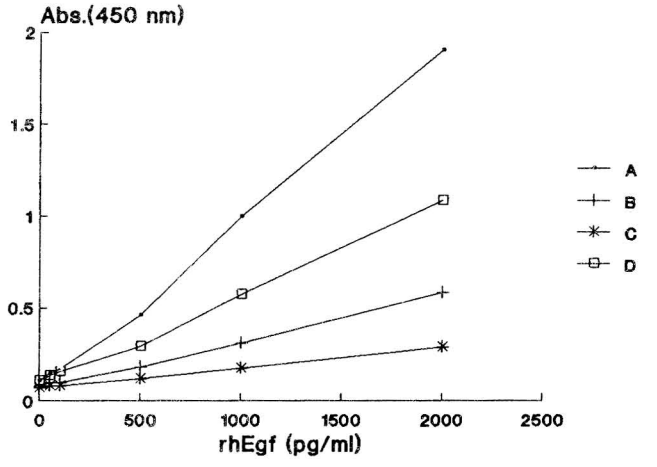

Fig. 3. Comparison of Methods 1 and 2, with different dilutions of the streptavidin-peroxidase conjugate in the preformed complex. Method 2 with 1:250 (A), 1:500 (B), and 1:1000 (C) dilutions of the streptavidin-peroxidase; Method 1 (D). Determinations were made in duplicate.

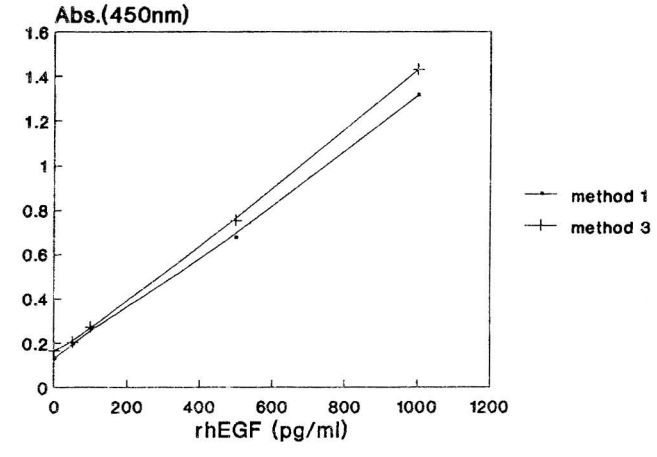

Fig. 4. Comparison of Methods 1 and 3. Determinations were made in duplicate.

(b) Dynamic Range and Sensitivity

The dynamic range for the calibration curve in methods 1 and 2 lied between 50 and 2000 pg/ml, while for the method 3 the range was from 50 to 1600 pg/ml, with a steeper slope.

The three methods presented equivalent sensitivity with a detection limit of 50 pg/ml. However, method 3 was the fastest, most economical, and reproducible. method 1 takes 165 minutes, versus 135 minutes for method 2 and 105 minutes for method 3. The consumption of reagents in method 3 is lower, due to the higher dilution of the CB-EGF.2-biotin conjugate.

Low pH treatment did not improve the sensitivity of the assay. Figure 5 shows that the minimum detection level (mean of background for n=4, plus 2 times the standard deviation) was 30 pg/ml for method 3, with or without pH stress. Lower rhEGF concentrations gave absorbance values below these limits. Figure 2a shows that the measurable concentration range was from 50 to 1600 pg/ml.

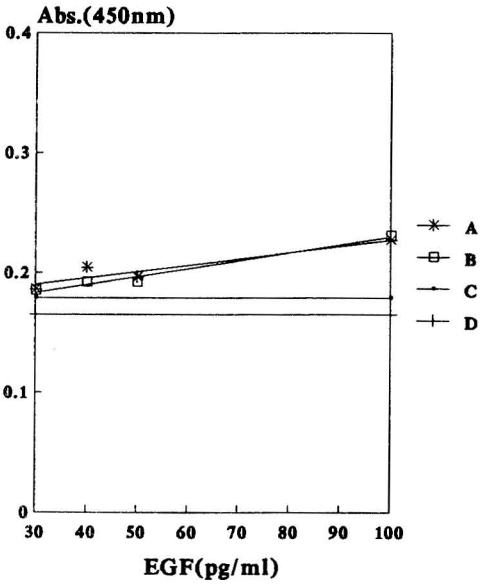

Fig. 5. The minimun detection level of the method 3 with non-treated coating antibody (A) and treated coating antibody (B).The detection limit was 2 standard deviations plus the mean of backgrounds of the system with non-treated coating antibody (C) and treated coating antibody (D). Determinations were made in duplicate.

Accuracy and Precision of Method 3

The most reproducible and quick method 3 was evaluated in more detail. Three rhEGF samples were assayed in 1:150,000 or 1:300,000 dilutions. Table 1 shows that the average coefficient for intra-assay

precision was 8.79 % (2.83-14.75%). Table 2 illustrates an average coefficient of 9.70% for inter-assay precision (6.83-12.56%).

Table 1.-
Intra-assay precision of the streptavidin-biotin assay (Method 3) for rhEGF.

Range	Samples	n	Conc. mean (ng/l)	S.D.	C.V. (%)	C.V. (%) for range
low	1	4	5.6	0.7	13.5	15.4
	2	3	29.4	5.6	19.0	
	3	3	25.4	0.9	3.7	
medi	4	3	82.8	1.8	2.2	7.2
um	5	13	68.7	6.9	10.0	
high	6	2	123.6	1.6	1.2	3.8
	7	4	179.6	6.3	6.3	

Note: S.D.= standard desviation; C.V.= coefficient of variation; n = number of replicas tested in the assay; conc. = concentration.

Table 2.-
Inter-assay precision of the streptavidin-biotin assay (method 3) for rhEGF

Range	Samples	n	conc. mean (ng/ml)	S.D.	C.V. (%)	C.V. (%) for range
low	1	3	11.3	1.7	15.6	13.6
	2	3	13.9	1.4	10.5	
medium	3	3	42.9	8.2	19.1	8.1
	4	3	38.0	0.8	2.1	
	5	3	77.5	2.4	3.1	
high	6	3	176.0	13.8	7.8	8.0
	7	3	215.4	17.6	8.2	

Note: S.D.= standard desviation; C.V.= coefficient of variation; n = number of replicas tested in the assay; conc. = concentration.

The accuracy of this method was tested by adding known amounts of rhEGF in serum samples from dogs. These samples were diluted 1:500. The hEGF concentrations were measured and compared with the expected values (table 3). The mean analytical recovery was 99.87 (88.27-111.47%).

DISCUSSION

The very high affinity of avidin for biotin ($Kd = 10^{-15}$ M) has been exploited in a number of biochemical applications, including cell labelling, affinity chromatography, and immunoassays (Wilchek *et al.*, 1984). Many of the problems associated with avidin, most significantly its nonspecific binding, have been circumvented by using streptavidin, a protein extracted from the bacterium *Streptomyces avidinii*, which has a neutral isoelectric point and lacks the carbohydrate moieties of the avidin molecule.

Table 3.-
Accuracy of the streptavidin-biotin assay (method 3) for hEGF

Range	Sample	Conc. (ng/ml)	added conc. (ng/ml)	expected conc. (ng/ml)	obtained conc. (ng/ml)	rec. (%)
low	1	60.0	68.0	128.0	153.1	119.0
			53.4	113.4	92.2	81.4
	2	48.9	68.0	116.9	124.9	106.8
			53.4	102.3	93.4	91.3
medium	3	378.1	66.1	444.2	449.8	101.0
			172.8	550.9	470.9	85.5
	4	210.8	66.1	276.9	303.6	109.6
			172.8	383.6	426.6	111.0
	5	210.1	66.1	276.2	305.7	110.0
			172.8	382.9	345.8	90.3
high	6	669.8	66.1	735.9	692.3	94.0
			172.8	842.6	830.0	98.5

Note: S.D.= standard desviation; C.V.= coefficient of variation; rec.= recovery; conc = concentration.

Guesdon *et al,* (1979) first applied the biotin-avidin system to non-competitive solid phase immunoassay. In this procedure, a solid phase immobilized antibody capture the antigen, which is then recognized by the biotinylated second antibody. The biotin is then detected directly by avidin, which is covalently conjugated to a label (three-step assay) (Guesdon *et al.*, 1979, Yolken *et al.*, 1983, Vilja *et al.*, 1988).

This report describes the optimization of a solid phase enzyme immunoassay for quantitative determination of hEGF. The use of the biotin-streptavidin system has improved the sensitivity from 400 pg/ml (Vázquez *et al.*, 1990) to 50 pg/ml, using the same monoclonal antibodies CB-EGF.1 and CB-EGF.2. The presence of four high affinity binding sites in streptavidin for biotin leads to the amplification of the antigen-antibody reaction, which in turn results in improved sensitivity.

BNHS can be covalently linked to free amino groups of proteins. Our results confirm that though biotinylation is a mild procedure, a too high BNHS/protein ratio may decrease the immunological reactivity of antibody or antigen (Guesdon *et al.*, 1979). On the other hand, too low

amount of bound biotin limits the binding of streptavidin, and decreases sensitivity. We have found that the optimal degree of biotinylation for CB-EGF.2 was obtained with a 0.53 BNHS/protein ratio (w/w), or a 142:1 BNHS/protein molar ratio.

The pH stress had been reported before to cause a selective denaturation of the Fc fragment of the antibodies, leading to a preferential binding of this region to the polystyrene surface (Vandenbranden, 1981, Van Erp *et al.,* 1992). Therefore, the antigen binding domains of the coating antibodies can be better oriented towards the solvent, thus increasing the antigen binding capacity.

We have only found a positive effect of ph stress for Mab CB-EGF.1 in Maxisorp NUNC microplates, that have polar groups on the surface (Esser, Nunc laboratories, 1988). In plates with predominantly hydrophobic surface, the ph stress had no major impact in the test. These results suggest that Mab CB-EGF.1 tend to react with the water molecules by hydrogen bonds rather than bind to the Polysorp surface by the weaker hydrophobic bonds, even when it has a more hydrophobic Fc region after ph stress. Additionally, we have demonstrated that the pH stress did not increased the sensitivity of our assay, although it resulted in a significant reduction in the amount of coating Mab.

The standard curve of the method 2 was occasionally obtained, some difficulties in the repeatability emerged. It seems that small changes in the proportion of the components in the preformed complex leads to a significant change in the absorbance values. Because the streptavidin molecule of the streptavidin-peroxidase conjugate has at least two free biotin-binding sites (according to information from Vector Laboratories), it may bind two molecules of biotinylated antibody (Tijssen, 1985). This may result in the formation of an extended and complex molecular lattice that may affects by steric hindrance the antibody/antigen interaction or the specific activity of the enzyme. This may explain why a high excess of streptavidin peroxidase- conjugate is needed, because in that way all available biotin molecules will be compromised or blocked and no aggregates will be formed.

Method 3 was the fastest, and the most economic and reproducible. This procedure has been described before by Vilja *et al.* (1991) for the measurement of a monomeric antigen (lactoferrin, MW 80 000 Da) using polyclonal antibodies, and the detection of a heterodimeric antigen (lutropin) using Mabs. In our case, rhEGF (MW ca. 6000 Da) as a very little molecule could be more sensitive to some steric hindrance by CB-EGF.1. Our results corroborate those of Vázquez *et al.,* (1990) in the sense that this coincubation does not affect the sensitivity of the assay.

The intra-assay (8.79%) and inter-assay (9.70%) coefficients of variation and the mean analytical recovery (99.87%) for hEGF demonstrate the precision and accuracy of the method 3. This high recovery also indicates that components of the dog serum does not block the binding of Mabs with hEGF, neither by binding to the hEGF nor through its interaction with the Mabs. These features make this system attractive for pharmacological studies in this animal model.

Two different two steps NSBA (utilizing preformed complexes of biotinylated antibodies and streptavidin peroxidase conjugate, or incubating antigen and biotinylated-antibody together) have been developed for the detection of hEGF using a sandwich ELISA system.

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