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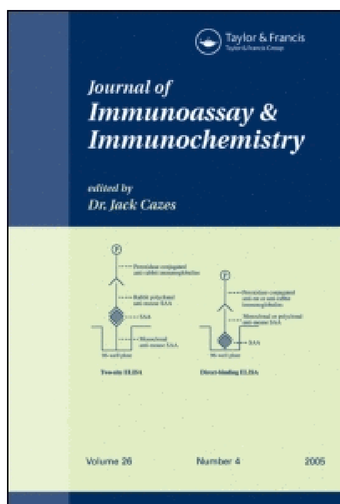

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A SENSITIVE ENZYME IMMUNOASSAY FOR THE DETECTION OF A SYNTHETIC AFFINITY LIGAND, THE REACTIVE YELLOW 13 DYE

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ABSTRACT

Dye-affinity chromatography is widely and increasingly used for the isolation of various proteins. In particular, the purification of transthyretin can be efficiently achieved by affinity chromatography on immobilized Reactive Yellow 13. Measurement of trace-amounts of dye leaching from affinity columns is important because of possible toxicity or side effects linked with the presence of dye in therapeutic transthyretin preparations. A competitive enzyme immunoassay was developed to monitor yellow-dye column leaching. Biotinylated rabbit anti-Reactive Yellow 13 antibodies (immunoglobulin G fraction) were used as principal reagent. The assay is specific, sensitive to 1 ng/ml of Reactive Yellow 13, has a good reproducibility and allows the accurate detection of the dye in the presence of transthyretin. (KEY WORDS : Reactive Yellow 13, enzyme immunoassay, dye-affinity chromatography, anti-dye antibodies).

INTRODUCTION

Dye-affinity chromatography on Reactive Yellow 13 is an attractive method for isolating plasma transthyretin (TTR) due to its simplicity, selectivity and rapidity (1,2,3). However, if the purified TTR is intended for therapeutic purpose, a major safety concern is the unavoidable release of dye. Indeed,

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affinity chromatography has been associated with ligand leakage whatever the ligand, the support or the eluting buffers used (4). Although several publications deal with toxicity of affinity ligands such as Protein A (5,6,7) or adverse reactions to nanogram amounts of immunogenic proteins leaching from affinity columns (8,9,10,11), little information is available about dyes. Cibacron Blue leached from agarose-based sorbents has already been studied and linked with toxicity in *in vitro* models (12). In order to evaluate the contaminating Reactive Yellow 13 in TTR preparations, a highly sensitive Reactive Yellow 13 dye ELISA was developed which allows detection at nanogram levels in the presence of high TTR concentrations.

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MATERIALS AND METHODS

Dye

Reactive Yellow 13 (also called Remazol Yellow GGL, CAS n° 12769-09-4) was synthetized by VILMAX (Buenos Aires, Argentine). Reactive Yellow 13 was coupled to carrier proteins. 1 g of bovine serum albumin (BSA) or ovalbumin was mixed with respectively 161 or 252 mg of dye and 1.6 g of sodium chloride in 29 ml of distilled water. After a 30 min incubation at room temperature, 3.2 ml of NaOH, 0.25 mol/L, was added and the mixture was stirred overnight at room temperature. The complex dye-protein was separated from the native dye by gel filtration on G-25 (Pharmacia).

Polyclonal Antibodies Preparation

Rabbits were immunized by five intramuscular injections at intervals of 1 week with 5 mg of Reactive Yellow 13-BSA complex in 1 ml phosphate-buffered saline suspended in 1 ml Freund's adjuvant. Complete Freund's

adjuvant was used for the first injection and incomplete Freund's adjuvant for the following injections.

Whole rabbit immunoglobulins G (IgG) were separated from immune serum using protein A-Sepharose CL-4B (Pharmacia). The gel was equilibrated in 1.5 mol/L glycine, 3 mol/L NaCl, pH 8.9. The immune serum was applied to the column (1.0 x 6.3 cm) at a flow-rate of 10 ml/h. After washing the column with equilibration buffer, IgG was eluted with 0.1 mol/L sodium citrate, pH 6 and then dialyzed against a bicarbonate buffer, 50 mmol/L, pH 8.5. IgG purity was ascertained by polyacrylamide gel electrophoresis. The electrophoresis was carried out using Phastgel gradient 4-15 (Pharmacia) in presence of SDS. IgG concentration was determined by absorbance at 280 nm using an extinction coefficient of 14.0 for a 1 % solution.

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Rabbit anti-BSA-dye IgG were biotinylated according to the supplier's instructions. The procedure consisted of addition of 0.4 mg of NHS-LC-biotin (Pierce) to a 20 mg/ml antibody solution in bicarbonate buffer, 50 mmol/L, pH 8.5 followed by incubation for 2 h in an ice bath. Excess NHS-LC-biotin was then removed by diafiltration against a sodium phosphate buffer, 100 mmol/L, pH 7 containing 1 g/l NaN_3.

Assay Procedure

96-well flat bottom ELISA plates (Costar) were coated with 100 µl of 1 µg/ml Reactive Yellow 13-ovalbumin conjugate in carbonate buffer, 50 mmol/L, pH 9.6 and incubated overnight at 4°C. The plates were washed with NaCl, 130 mmol/L ; Na_2HPO_4, 5 mmol/L ; KH_2PO_4, 1 mmol/L, containing 0,05 % of Tween 20 (PBS-Tween), blocked with 100 µl of 0,5 % gelatin (cold water fish skin, Sigma) in PBS for 3 h at 37°C. During this time, 160 µl of

samples or the standard were added to 40 µl of 6,5 µg/ml biotinylated anti-BSA-dye IgG in a glass tube. All solutions were diluted in PBS-Tween and the mixture was incubated for 2 h at 37°C. The plates were washed with PBS-Tween. The mixtures were added in 100 µl aliquots to the wells and incubated for 2 h at 37°C. Wells were set aside that contained only 100 µl of PBS-Tween to serve as blanks. The plates were washed with PBS-Tween, then 100 µl/well of streptavidin-peroxidase was added and incubated for 10 min at 37°C. After washing with PBS-Tween and sodium acetate-citrate buffer, 140 mmol/L, pH 6, 100 µl/well of 3,3'-5,5'-tetramethylbenzidine (TMB) peroxidase substrate was added and incubated approximately 5 min at room temperature. 25 µl of H_2SO_4, 2 mol/L, was added to stop the enzyme reaction. The intensity of yellow color generated was determined by measuring the absorbance at 450 nm on a Titertek Multiskan plus micro ELISA reader (Flow Laboratories). The average value of the blanks was subtracted to eliminate background.

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RESULTS

Antibody Specificity

Rabbits, immunized with a BSA-dye conjugate, developed antibodies against the dye and against BSA. The dye was complexed with ovalbumin to coat the plates, and the rabbit sera were checked for reactivity against ovalbumin. As shown in Fig. 1, the immune sera reacted identically to non-immune serum against ovalbumin-coated plates, whereas specific binding occured with both BSA and dye-coated plates.

Optimal Assay Conditions

The influence of the amount of ovalbumin-dye complex, dilution of anti-Reactive Yellow 13 antibody, antibody/dye ratio and incubation times were

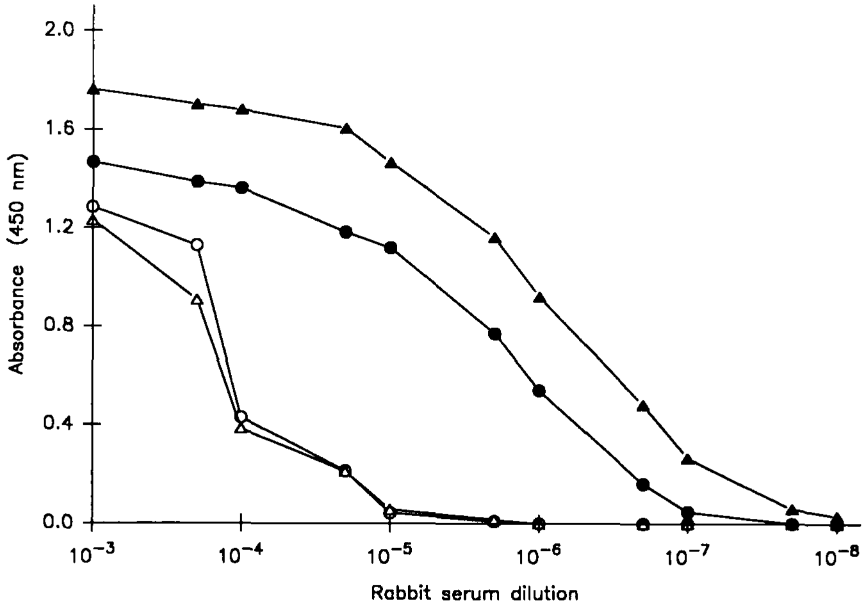

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Figure 1 : Reactivity of non-immune and immune rabbit serum with ovalbumin, BSA or Reactive Yellow 13 coated plates. Plates were coated with 100 ng of each protein or dye and a biotinylated donkey anti-rabbit IgG was used as second antibody. Similar results were obtained for non-immune serum with all the 3 sets of plates and only the results obtained with dye-coated wells are shown (○). Reactivity of immune serum with dye coated wells (●) and BSA coated wells (▲) is compared with reactivity with ovalbumin coated wells (△).

determined. Optimal binding of the antibody occurred in the wells containing 100 ng of ovalbumin-dye complex. Reproducible results were obtained with 2 h incubation times for the antibody with free dye and the antibody with dye adsorbed to the wells. Both dilution of anti-Reactive Yellow 13 antibody and antibody/dye ratio were found to influence the assay sensitivity and variability. Dilutions of the 6.5 mg/ml biotinylated anti-dye antibody stock solution were prepared and maximal differences in absorbance values between dye concentrations were achieved with an antibody dilution of 1/1000 (Fig. 2).

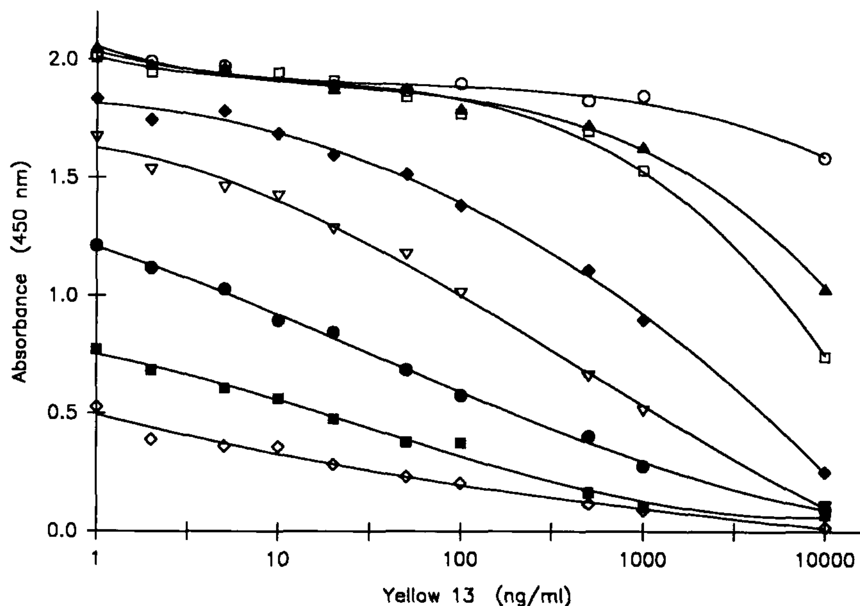

Figure 2 : The effect of conjugate antibody dilution on the standard curve for Reactive Yellow 13 ELISA. The antibody/dye ratio (1:4) was constant for all curves. 8 dilutions of the 6.5 mg/ml biotinylated antibody stock solution, 1/10 (○), 1/50 (▲), 1/100 (□), 1/500 (◆), 1/1000 (▽), 1/2500 (●), 1/5000 (■) and 1/10000 (◇) were assayed on the same plate.

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Various ratios of free dye and biotinylated antibody at a 1/1000 working dilution were assayed and the detection limit was best with a 1:4 antibody/antigen ratio (Fig. 3).

Assay Sensitivity

In order to determine the working range of the assay under optimal conditions as above mentioned, concentrations of dye ranging from 0.1 ng/ml to 100 µg/ml were tested in quadruplicate. The results (Fig. 4) show a linear standard curve over the range 1-1000 ng/ml. Replacement of the biotinylated

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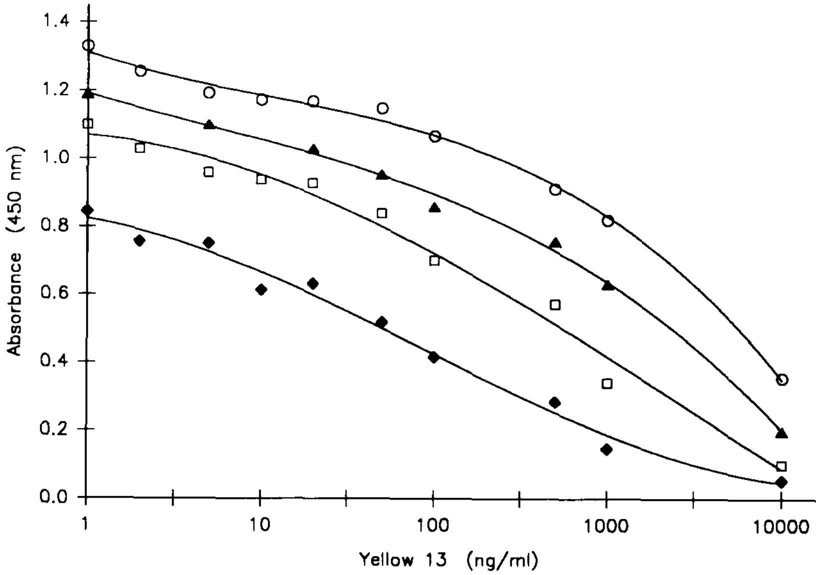

Figure 3 : The effect of antibody/dye ratio on the standard curve for the Reactive Yellow 13 ELISA. The dilution of conjugate antibody (1:1000) was constant for all curves. Results obtained with 4 antibody/dye ratios, 1:4 (◆), 1:1.5 (□), 1.5:1 (▲) and 4:1 (○) were compared on the same plate.

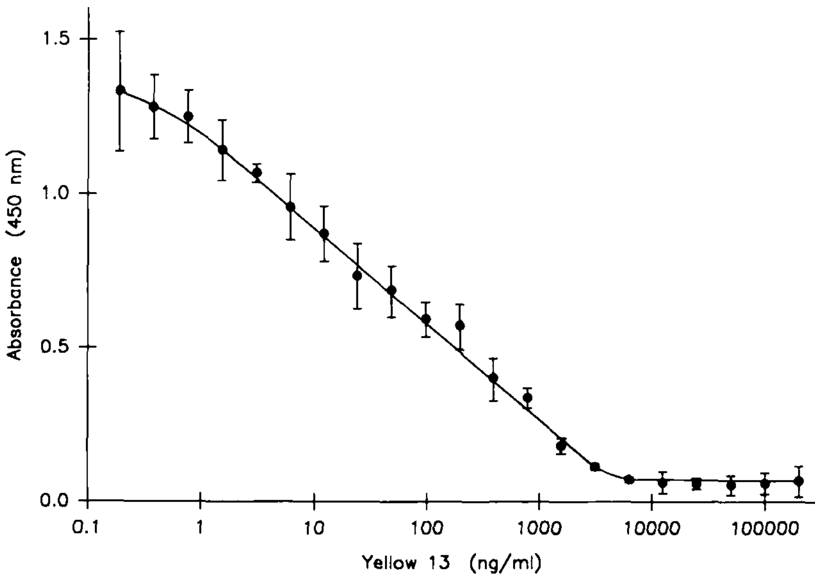

Figure 4 : Standard curve for the Reactive Yellow 13 ELISA. Assay was perfomed as described in Materials and Methods. Each sample was assayed in quadruplicate and vertical lines indicate the value for a 99 % confidence interval.

rabbit anti-dye IgG with rabbit anti-dye IgG followed by a donkey anti-rabbit IgG-biotin failed to improve the assay sensitivity.

The sensitivity of the assay was studied by assaying 32 times a blank containing no dye. The sensitivity, derived from the mean absorbance of the blank minus three standard deviations, was 1 ng/ml.

The intraassay precision was calculated for seven samples with different dye concentrations. Each sample was assayed 24 times and the results showed a mean value of 3.5 % for the intraassay coefficient of variation.

<u>Assay Validation</u>

The Reactive Yellow 13 ELISA was developed to quantitate Reactive Yellow 13 in the presence of TTR. To determine whether the presence of TTR would interfere with the assay, two solutions of TTR at a concentration likely to be present in the test material (0.43 and 0.15 mg/ml respectively) purified to homogeneity by immunoaffinity chromatography on immobilized monoclonal anti-TTR antibodies were spiked with 0.5 μg/ml of Reactive Yellow 13. As control, the ELISA dilution buffer was also spiked with 0.5 μg/ml dye. The mixtures were incubated for 1 h at room temperature and then diluted for assay. The results are shown in Fig. 5. The shapes of the two sets of curves (spiked and unspiked samples) are similar. The slight differences observed between values that are independent of TTR concentration over the range tested can be attributed to within assay variability.

DISCUSSION

The development of affinity chromatography methods for the preparation of proteins destined for therapeutic uses is associated with safety requirements, in particular development of highly sensitive assays to quantitate ligand release.

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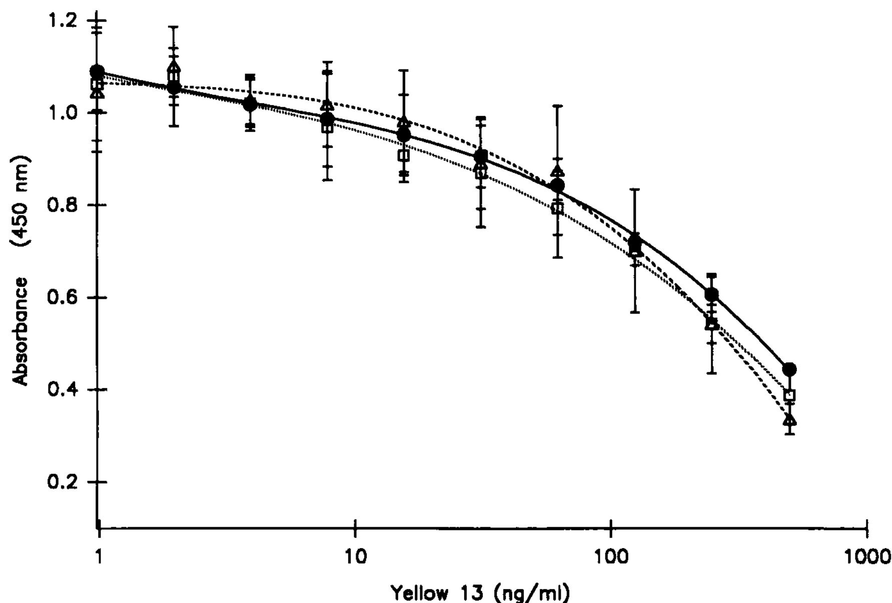

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Figure 5 : The effect of TTR on the Reactive Yellow 13 ELISA. 3 sets of standards, free dye (●), dye in solutions containing respectively 0.15 mg/ml of TTR (□) and 0.43 mg/ml of TTR (△) were diluted and assayed. Assay was performed as described in Materials and Methods. Each sample was assayed in quadruplicate and vertical lines indicate the value for a 99 % confidence interval.

Release of immobilized Protein A or IgG has already been studied using such assays (13,14,15). The assay described here is a competitive enzyme immunoassay for the detection of Reactive Yellow 13. It was specially designed to monitor leaching of this dye from affinity columns.

Although there are currently no guidelines for the maximum levels of dye that may contaminate proteins purified on immobilized dyes, the sensitivity of spectrophotometry (about 1 µg/ml) is inappropriate for the measurement of trace-amounts of dye. ELISA represents a convenient alternative since it has the

potential for measurement in the nanogram range. The sensitivity of the assay developed (about 1 ng/ml) is one thousand fold greater than spectrophotometric assay. Attempts to lower the detection limit by using a noncompetitive two-site immunoassay technique, in which dye is trapped onto antibody-coated solid phase and is estimated by enzyme-labelled antibody were unsuccessful.

Assay specificity is potentially a problem, given the need for haptens as dye molecules of carrier proteins both for antibody preparation and coating of plates. The choice of the two carrier proteins used, bovine serum albumin and ovalbumin was suitable for these purposes since the cross-reactivity studies failed to reveal interference.

As our requirements involved assaying Reactive Yellow 13 in the presence of TTR, the assay developed needed to detect both free dye and dye complexed to TTR. Thus the antibodies used in the ELISA must be specific for epitopes on the dye molecule. The reactivity of the polyclonal anti-Reactive Yellow 13 antibodies with two other yellow dyes having the same chromophore group as Reactive Yellow 13 was studied and found to be identical for all dyes. Interference studies showed that TTR at a concentration range likely to be present in the test material had no significant effect.

With regard to reproducibility, the intraassay coefficient of variation is less than 5 %.

In conclusion, the competitive enzyme immunoassay described here is sensitive, accurate and reproducible for the measurement of Reactive Yellow 13 dye. It provides a powerful tool for the investigation of Reactive Yellow 13-affinity support stability and toxicity studies of leachables.

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