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Purification of biologically active human plasma transthyretin by dye-affinity chromatography: studies on dye leakage and possibility of heat treatment for virus inactivation

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ABSTRACT

The application of a purification procedure for the industrial preparation from human plasma of a therapeutic protein may be hindered by several safety concerns. The dye leaching from Remazol Yellow GGL-Sepharose used for the affinity chromatography of human plasma transthyretin was quantitatively studied by a sensitive competitive enzyme immunoassay. The possibility of including a heat treatment step for virus inactivation in the purification process while preserving the biochemical and functional characteristics of the protein is also reported.

INTRODUCTION

The value of Remazol Yellow GGL as a biomimetic ligand for the affinity chromatography of human plasma transthyretin (TTR) has been established [1–3]. Human plasma transthyretin is a protein with four identical subunits and a relative molecular mass of *ca.* 55 000. TTR is actively involved in the transport of thyroxine and retinol binding protein [4,5]. Variant transthyretins, characterized by single amino acid substitutions, have been described and are associated with different patterns of amyloid involvement, in particular familial amyloidotic polyneuropathy (FAP) [6]. Significantly reduced levels of TTR were found in patients with FAP [7]. Administration of exogenous TTR in order to normalize plasma levels in association or not with specific removal of abnormal TTR [8] could represent a potential treatment for this disease. Large amounts of purified TTR would therefore be required, and dye-affinity chromatography of TTR on Remazol Yellow GGL-Sepharose from a by-product of chromatographic fractionation of plasma has been optimized in order to allow large-scale preparations [3]. One of the main concerns for a ther-

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apeutic product is its safety [9]. Regarding the dye-affinity chromatography procedure, the storage of dye-sorbents in a bacteriostatic medium between uses was investigated, and the dye leakage was studied by means of a sensitive enzyme immunoassay [10]. Moreover, the risk of transmission of infectious viruses by a biological product derived from human plasma has prompted us to investigate the possibility of heat treatment for the purified TTR while preserving the biological activity of the therapeutic entity.

EXPERIMENTAL

Materials

The matrix used was Sepharose CL-4B from Pharmacia (Uppsala, Sweden). Remazol Yellow GGL was obtained from Vilmax (Buenos Aires, Argentina).

Antiserum to human TTR and standard serum were purchased from Behring (Marburg, Germany), [^{125}I]thyroxine (1.85 MBq/ml, 55.5 MBq/μg) from Amersham (UK) and gelatin from water fish skin from Sigma (St. Louis, MO, USA). A Remazol Yellow GGL–ovalbumin conjugate and biotinylated anti BSA-Remazol Yellow GGL rabbit immunoglobulins were prepared in our laboratory as previously described [10]. All other chemicals were of analytical-reagent grade.

Polyacrylamide gel electrophoresis (PAGE) was carried out using Phastgel gradient (8–25%) and a Phastsystem apparatus from Pharmacia (Uppsala, Sweden).

Costar microtiter plates were used for enzyme immunoassays.

Preparation of the dye-sorbent

A 250-g amount of settled Sepharose CL-4B, 25 g of NaCl and 2.5 g of Remazol Yellow GGL were mixed and made up to a volume of 450 ml with distilled water. The mixture was stirred for 30 min, then 50 ml of 0.25 *M* NaOH were added. After overnight agitation, the sorbent was washed with distilled water until the absorbance at 400 nm reached zero. It was stored at 4°C, either in distilled water containing 0.2 g/l NaN_3 or in a 20% ethanol aqueous solution.

Dye-affinity chromatography of TTR

An unused human plasma fraction (termed here DNaCl), obtained by elution with 1 *M* NaCl in the first chromatographic step of the purification of albumin using a so-called Spherodex–Spherosil process [11], was dialysed against 0.1 *M* sodium phosphate buffer (pH 7.4) (buffer A) and then applied to the dye-sorbent equilibrated with the same buffer. TTR was eluted with 10% ethanol in water, concentrated by ultrafiltration using a membrane with a 10 000 molecular mass cut-off, and dialysed against 9 g/l NaCl in water. The binding capacity of the sorbent was assessed by the amount of TTR eluted per ml of gel.

Heat treatment

The purified TTR, adjusted at a concentration of 50 g/l in a 9 g/l NaCl solution, was heated in a water-bath at 60°C for 10 h.

TTR assay

The TTR concentration was determined by rocket immunoelectrophoresis [12] using a specific antiserum and a serum as standard.

Thyroxine binding capacity

A 10-μl volume of sample containing TTR at a concentration of 100 mg/l (plasma, plasma depleted in TTR by yellow dye-affinity chromatography, or purified TTR) was incubated with 10 μl of [^{125}I]thyroxine and 1 μl of bromophenol blue for 2 h at room temperature. A 1-μl aliquot of the mixture was subjected to native PAGE. The gel was sliced in lanes and each lane in various length sections. Each slice was assayed for radioactivity in a gamma counter.

Remazol Yellow GGL dye assay

Microtiter plates were coated with 1 μg/ml Remazol Yellow GGL–ovalbumin conjugate in 50 m*M* sodium carbonate (pH 9.6) (100 μl/well) at 4°C overnight. After washing with 130 m*M* NaCl, 5 m*M* Na_2HPO_4 and 1 m*M* KH_2PO_4 (pH 7.2) containing 0.05% Tween 20 (PBS–Tween), the plates were blocked with 125 μl of 0.5% gelatin in PBS for 3 h at 37°C. During this time, four volumes of samples or standard were added to

one volume of biotinylated anti BSA-Remazol Yellow GGL rabbit immunoglobulin G (IgG) and incubated for 2 h at 37°C. The plates were washed with PBS–Tween, and 100-μl aliquots of each mixture were added to the wells and incubated for 2 h at 37°C. The plates were washed with PBS–Tween, then 100 μl of streptavidin–peroxidase were added and incubated for 15 min at 37°C. After washing with PBS–Tween and 140 m*M* sodium acetate–citrate (pH 6.0) (buffer B), 100 μl of 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine and 0.01% H_2O_2 in buffer B were placed in each well. The enzyme reaction was stopped by addition of 25 μl of 2 *M* H_2SO_4. The coloration was measured at 450 nm in an automatic micro ELISA reader.

RESULTS

The influence of several parameters on the fixation and the elution of TTR has already been studied [3]. The efficiency of the purification has been shown to be unchanged whatever the residence time and the TTR level in the DNaCl fraction. On the other hand, the capacity of the dye-sorbent is influenced by the washing volume with equilibrating buffer to elute non-bound proteins and by the amount of TTR applied to the sorbent. No loss of binding capacity of the dye-sorbent was observed over twenty successive purification procedures. In optimum working conditions, the dye-affinity chromatography resulted in the isolation of a 80% pure TTR with a 70% yield.

Storage of dye-sorbents and measurement of binding capacity

In order to prevent the dye-sorbent from bacterial contamination, the possibility of storage between runs in a 20% ethanol aqueous solution known as a bacteriostatic agent was investigated. Five identical runs were performed on a dye-sorbent. Between the first three runs the sorbent was stored in buffer A, then it was stored in ethanol overnight before run 4 and for two days before run 5. As shown in Fig. 1, the binding capacity remained unchanged after storage in 20% ethanol.

Dye leakage

The stability of the dye-affinity support itself has been already studied by quantification of the amount of dye leached in several buffers with pH values ranging from 1 to 14 [13]. In the pH range 4–10, as well as in the solutions used during the chromatographic purification procedure, the

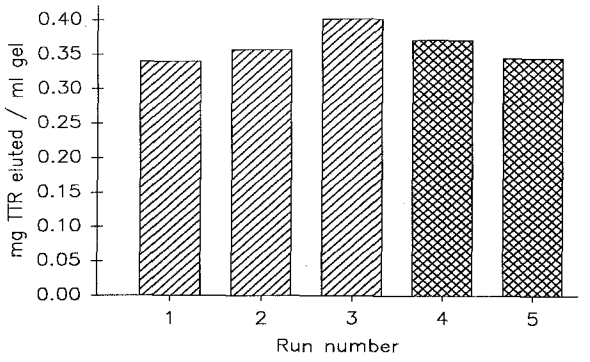

Fig. 1. Binding capacity of dye-sorbents stored in 20% ethanol. Each run was achieved by applying 14 ml of a DNaCl fraction (TTR level of 500 μg/ml) to the dye-sorbent (1.6 × 7 cm) at a flow-rate of 50 ml/h. Between the first three runs the sorbent was stored in buffer A, and before the two last runs in a 20% ethanol aqueous solution.

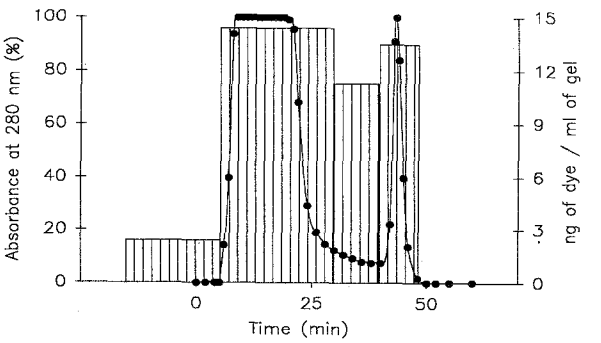

Fig. 2. Dye leakage during the chromatographic procedure. Dye was assayed in four fractions recovered as indicated on the typical elution pattern of DNaCl chromatographed on Remazol Yellow GGL-Sepharose. The dye-sorbent was washed with buffer A, and the DNaCl fraction was applied to the sorbent at time 0.

leakage of the dye remained at a minimal value (*ca.* 5 μg of dye per g of sorbent for a 24-h incubation time).

Dye leaching during the purification procedure. As the competitive enzyme immunoassay used to quantitate the dye has been shown to allow the accurate measurement of both free dye and dye complexed to TTR [10], the dye leakage was quantified during the purification procedure. Dye was assayed at the outlet of the column in fractions recovered as follows: the equilibration of the sorbent with buffer A, the flow-through protein peak, the end of washing with buffer A to elute non-bound proteins and the ethanol peak dialysed against NaCl. As can be seen in Fig. 2, dye leakage is increased in protein-containing fractions. Although the TTR concentration is significantly higher in the ethanol fraction in relation to the flow-through protein fraction, similar amounts of leached dye are measured in these solutions. The dye leakage is thus not only dependent on the TTR concentration in the fraction.

Influence of the concentration of the eluted TTR on dye leakage. In order to elucidate the influence of the amount of adsorbed and eluted TTR on dye leakage, various volumes of a DNaCl fraction were applied to identical dye-sorbent columns stored in buffer A, and the dye was assayed in eluted TTR. The dye concentration in the ethanol fraction remained relatively unchanged, whereas the TTR recovery increased as the amount of TTR loaded increased. For increasing amounts of TTR loaded less than 1–1.5 g of TTR per litre of sorbent, the TTR concentration in the eluate increased whereas the ethanol eluate volume was constant. For increasing amounts of TTR loaded greater than 1–1.5 g of TTR per litre of sorbent, the TTR concentration in the eluate remained at a same value but the ethanol eluate volume increased. Consequently, the ratio μg of dye per g of TTR decreases and levels out for amounts of TTR loaded greater than 1–1.5 g of TTR per litre of sorbent (Fig. 3).

Dye leakage over runs. The amount of dye leaching from the affinity support was then quantified over runs in TTR preparations. Ten identical runs were performed by applying 0.5 g of TTR per litre of sorbent. Between uses, the dye-sorbent was stored at room temperature overnight or at 4°C for a few days in 20% ethanol. Both the amount of TTR eluted and the amount of leached dye remained constant over runs (Fig. 4). The dye leakage was found to be two-fold higher when the dye-sorbent was stored in ethanol rather than in buffer A (Fig. 3). Although the dye-sorbent was washed with ten column volumes of buffer A before use, such more elevated values were observed throughout the purification procedure.

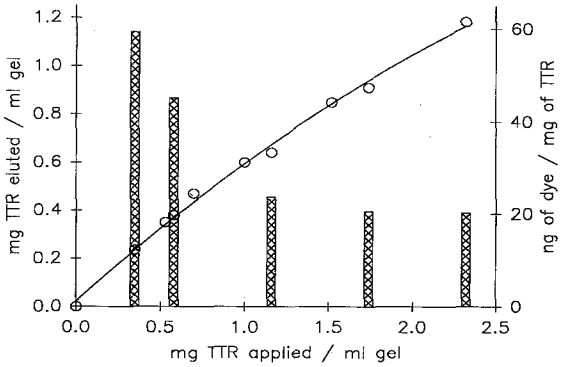

Fig. 3. Influence of the amount of TTR applied to the sorbent on binding capacity and dye leakage. Volumes of DNaCl (TTR concentration of 1.16 mg/ml) ranging from 3 to 20 ml were applied to the dye-sorbent (1.6 × 5 cm) at a flow-rate of 50 ml/h. Dye was assayed in eluted TTR (bars).

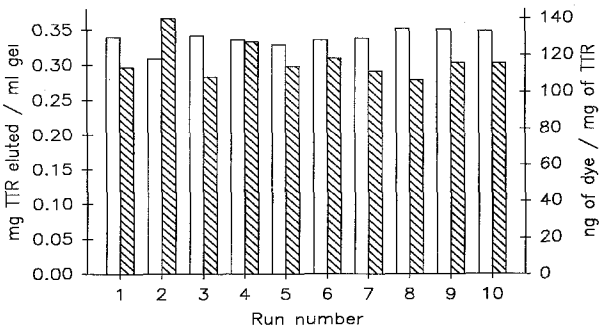

Fig. 4. Multiple use of dye-sorbents and dye leakage. For each run, 10 ml of a DNaCl fraction (TTR level adjusted to 500 μg/ml) were applied to the dye-sorbent (1.6 × 5 cm) at a flow-rate of 50 ml/h. Between runs, the sorbent was stored in 20% ethanol. Before each run, the sorbent was washed with ten column volumes of phosphate buffer. For each run, the binding capacity of the dye-sorbent (□) was expressed as the amount of TTR eluted per ml of sorbent.

TTR pasteurization

In order to achieve virus inactivation in the purified protein, TTR was concentrated to *ca.* 50 g/l and heat-treated at 60°C for 10 h (these conditions have been successfully used for human albumin [15]). The biochemical and functional characteristics of the heated TTR were compared with those of the unheated protein. Heating had no significant effect on the measured contents of TTR, Remazol Yellow GGL dye or total protein. Native PAGE analysis of the two samples showed identical results, only one band with anodal mobility (Fig. 5).

The stability of the biological activity of the protein was assessed by means of its thyroxine binding capacity. As shown in Fig. 6, the thyroxine binding capacity of TTR was altered neither during the dye-affinity chromatography step nor during the pasteurization step. When calculated as a molar ratio, one molecule of thyroxine was bound per molecule of TTR. In order to investigate the capacity of the purified protein to restore functional TTR activity in TTR-deficient plasma, experiments were performed with a normal plasma, the same plasma depleted in TTR, purified TTR, and purified TTR in the depleted plasma. An elevated value of radioactivity in the TTR slice was observed for plasma (*ca.* 2.6-fold higher than for purified TTR). It could be expected that other proteins with an affinity for thyroxine are present in this slice. The TTR depletion of the plasma was achieved by yellow dye-affinity chromatography and led also to a 20% loss of total protein content. The radioactivity is greatly decreased for the depleted plasma and represented only 4% of the radioactivity obtained for the starting plasma. When purified TTR was added to the depleted plasma to restore the initial level, the radioactivity represented 80% of the value obtained for the starting plasma.

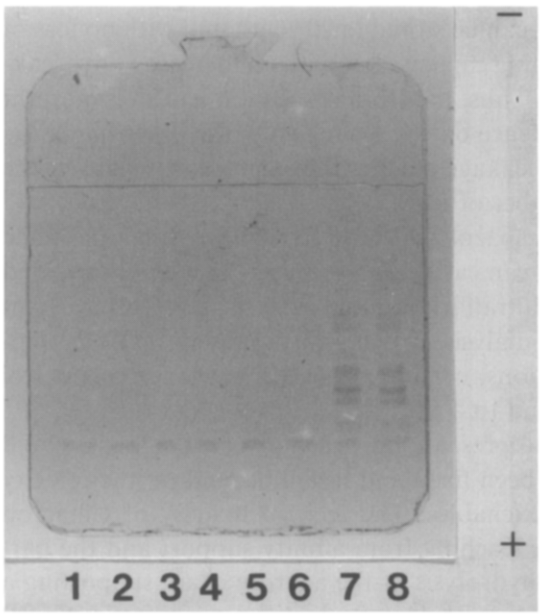

Fig. 5. PAGE analysis of the purified TTR. Native PAGE was carried out in a continuous 8–25% gradient gel zone with 2% cross-linking. The gel was stained with Coomassie Blue. The DNaCl fraction (lanes 7 and 8), the unheated purified TTR (lanes 1–3) and the heated purified TTR (lanes 4–6) were adjusted to a TTR concentration of 0.5 mg/ml.

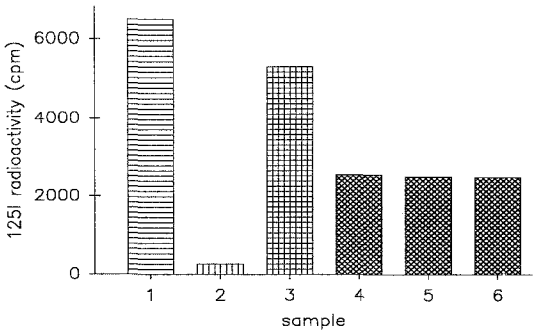

Fig. 6. Biological activity of the purified TTR. The binding of thyroxine to TTR (see Experimental) was compared in various samples: plasma (sample 1), the same plasma depleted in TTR (sample 2), DNaCl fraction (sample 4), purified TTR (sample 5), heat-treated purified TTR (sample 6) and heat-treated purified TTR in the TTR-depleted plasma (sample 3). Samples 1, 4, 5 and 6 were adjusted to a TTR level of 100 mg/l. The TTR-depleted plasma was diluted as the native plasma, and purified TTR was added at a final concentration of 100 mg/l in sample 3.

DISCUSSION

The development of a purification procedure for plasma proteins destined for therapeutic uses is associated with several requirements: (1) the isolation procedure must be suitable for large-scale preparation of the protein with a conve-

nient level of purity; (2) the safety of the purified product; (3) the preservation of the biological activity of the purified protein.

Dye-affinity chromatography of TTR on Remazol Yellow GGL from a by-product of the chromatographic purification of human albumin by a classical process [11] is an attractive method. This by-product was chosen because it is unexploited until now, and because it contains high levels of TTR; other by-products of plasma fractionation that also contain significant levels of TTR are also usable. The application of affinity techniques for large-scale preparations may be hindered by the cost of production of specific ligands or by interference with classical plasma fractionation. The use of a synthetic dye and of a by-product of the plasma fractionation could circumvent the problems of the high cost of production of affinity supports involving highly specific ligands, such as anti-TTR antibodies, retinol binding protein or thyroxine, and of interference with plasma valorization, respectively. As a first step in assessing the application of the procedure to the preparation of a therapeutic TTR, the method has been previously optimized in order to be easily scaled up, and has been shown to result in the isolation of a TTR with a purity acceptable for substitutive therapy because the main contaminant is albumin.

A major safety concern for affinity chromatography is the unavoidable leakage of ligand. The results obtained and reported here emphasize the need to study the stability of affinity supports in equilibrating, washing and eluting solutions, and in protein solutions. The dye leakage is strongly influenced by the presence of proteins, since the amount of dye present in the flow-through DNaCl fraction is six-fold higher than in the phosphate buffer alone. As the amount of dye leached in TTR ethanol eluates was shown to be *ca.* ten times more important than in ethanol solution [13], the dye leakage was thought to be dependent on the amount of TTR adsorbed and eluted. However, when the amount of TTR eluted was increased by increasing the amount loaded, the dye level in the eluate (calculated in ng/ml) remained unchanged. Dye leakage is therefore a complex phenomenon involving several factors. The dye increase in protein-containing solutions is not entirely explained by the amount of TTR adsorbed and eluted.

For a therapeutic protein, the dye leakage is expressed as the ratio of the amount of dye to the protein level. This ratio reaches a minimal value for amounts of TTR loaded greater than 1 g of TTR per litre of sorbent. This result is in good agreement with the previously defined optimum working conditions for which optimum effectiveness of the dye-sorbent is obtained with 1 g of TTR loaded per litre of sorbent.

Regeneration and multiple use of dye-affinity supports contribute to lowering the cost of the purification method. It therefore appears desirable to store the dye-sorbent in safe conditions between runs in order to avoid bacterial contamination and facilitate the production of a sterile, apyrogenic and atoxic protein. Storage in 20% ethanol has been chosen because ethanol is bacteriostatic and non-toxic. It ensures the preservation of sterility for dye-sorbents prepared and used in sterile conditions. The dye-affinity support can be stored in 20% ethanol with no loss of binding capacity but with an increase in dye leakage. Thus, the storage solution has an important influence on dye leakage. On the other hand, the dye leakage reaches the same value whatever the number of uses.

Remazol Yellow GGL dye is a small molecule that can easily pass through the membranes used for ultrafiltration and dialysis. Even after extensive dialysis, dye is always present in TTR preparations, meaning that the leached dye is partly bound to TTR.

At present, no acceptable leakage threshold has been fixed and it will depend on the toxicity of leachables. The *in vitro* toxicity of Cibacron Blue leaching from affinity support and the partial hydrolysis of the agarose chain supporting a molecule of dye covalently immobilized have already been reported [14]. Studies have to be performed with native Remazol Yellow GGL and dye leached from the dye-sorbent in order to elucidate the toxicity of free dye, dye bound to TTR, or dye associated with a small part of the matrix.

For therapeutic proteins derived from human plasma, virus inactivation strategies are generally dictated by the lability of the virus and the stability of the biological activity of the protein in question. It has been established that human serum albumin in the presence of suitable stabilizers can be heated in solution at 60°C for 10 h and the viral safety of such preparations has been demonstrated [15]. The functional activity of TTR was studied by means of its thyroxine binding, which occurs in an internal channel of the TTR tetramer [16]. The possibility of heating TTR in solution at 60°C for 10 h without stabilizers, while preserving its thyroxine binding capacity, has been proved. Our finding of a single thyroxine binding site for the TTR tetramer is consistent with the strong negative cooperativity effect of thyroxine on the second binding site of the TTR tetramer [17]. Moreover, the thyroxine binding capacity of plasma selectively depleted in TTR is totally restored by the addition of heated purified TTR. Model virus studies are now required to provide the confidence needed and satisfy the legal directives regarding the viral safety of the heated TTR before starting clinical trials in humans.

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