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Dye-affinity purification of transthyretin from an unexploited by-product of human plasma chromatographic fractionation

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ABSTRACT

Dye-affinity chromatography of human plasma transthyretin on Remazol Yellow GGL–Sepharose from an unexploited by-product of chromatographic fractionation of plasma was optimized for large-scale preparation of a therapeutic product. With this system, transthyretin is only weakly bound to the gel. The residence time on gel and the transthyretin level in the by-product were observed to have no influence on the binding capacity of gel, and the optimum amount of transthyretin to be applied to the gel was found to be 1 g/l of gel. The adsorbent can be used more than ten times. The procedure resulted in the isolation, with a 30% yield with respect to plasma, of an 80% pure protein, which retained its thyroxine-binding capacity. Although the purity is acceptable for substitutive therapy, it can be improved further with a second chromatography on Cibacron Blue–Sepharose.

INTRODUCTION

Human plasma transthyretin (TTR), also called prealbumin, is a tetramer composed of four identical subunits of 14 000 relative molecular mass. This serum protein is involved in the transport of thyroxine (T4) and retinol-binding protein (RBP), which itself transports vitamin A [1]. TTR has been related to familial amyloidotic polyneuropathy (FAP) [2]. FAP is characterized by the presence of an abnormal TTR in amyloid fibrils and by low levels of total plasma TTR. Administration of exogenous purified TTR is postulated to have therapeutic substitutive value [3]. For this purpose, large amounts of purified TTR from plasma are required. Therefore, it appears desirable to develop a method suitable for large-scale purification without interfering with plasma fractionation. The first step of the chromatographic purification of human albumin by the Spherosil–Spherodex process [4] provides a by-product, unexploited until now, which contains high levels of TTR, trace amounts of albumin and no immunoglobulin G (IgG). TTR has already been isolated by dye-affinity chromatography using Remazol Yellow GGL [5,6], and the limiting factor of the purification yield is the presence of albumin and IgG in the final product. Development and optimization of a dye-affinity purification method using Remazol Yellow GGL-substituted gel and this by-product would allow the preparation, on an industrial scale, of TTR for therapeutic applications.

EXPERIMENTAL

Preparation of Remazol GGL gel

A 250-g amount of packed Sepharose CL-4B (Pharmacia, Uppsala, Sweden), 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

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ml of 0.25 *M* NaOH were added. After overnight agitation, the dye-substituted gel was washed with distilled water until absorbance at 400 nm reached zero. It was stored at 4°C in distilled water containing 0.2 g/l of NaN_3.

Transthyretin purification

Preparation of the TTR source. A by-product containing TTR was obtained from the first step of the chromatographic purification of human albumin by the Spherosil–Spherodex process [4]. Cohn supernatant II + III (SII + III) was dialysed against 0.01 *M* sodium phosphate buffer (pH 5.2) and applied to a DEAE-Spherodex (Sepracor-IBF, Villeneuve la Gazenne, France) column equilibrated in the same buffer. The column was then washed successively with equilibrating buffer and 0.02 *M* sodium acetate buffer (pH 4.5). A fraction containing TTR (DNaCl) was eluted with 1 *M* NaCl. The column was further regenerated with two successive washings with 0.1 *M* HCl and 0.5 *M* acetic acid–60% ethanol, respectively. The TTR-containing fraction was concentrated between four- and twenty-fold by ultrafiltration, dialysed against a 0.1 *M* sodium phosphate buffer (pH 7.4) (buffer A), sterile filtered and stored in this buffer at 4°C.

Affinity chromatography of TTR. TTR was purified from the DNaCl by-product according to the method described by Byfield [6]. The TTR-containing fraction was applied to a Remazol Yellow GGL–Sepharose CL-4B column, previously equilibrated with buffer A. The bound TTR was eluted with 10% ethanol in water, concentrated, dialysed against a 10 m*M* sodium phosphate, 50 m*M* NaCl buffer (pH 7.4) (buffer B) and applied to a column filled with Cibacron Blue–Sepharose CL-6B (Pharmacia) equilibrated with buffer B. The emerging breakthrough fraction containing TTR was finally concentrated by ultrafiltration.

Analytical methods

Protein assays. The TTR level was determined by rocket electroimmunodiffusion [7] using antiserum and standard plasma from Behring (Marburg, Germany). The total protein content was measured using the method of Lowry *et al.* [8].

Purity control. The purity of the isolated TTR was ascertained by electrophoresis in polyacrylamide gel (Phastgel, Pharmacia) and FPLC-type analytical chromatography (Mono-Q HR 5/5 column and Superose 12 HR 10/30 column, Pharmacia).

Thyroxine binding. A 10-μl volume of purified TTR at 100 mg/l in buffer A was incubated with 10 μl of [^{125}I]thyroxine (1.85 MBq/ml, 55.5 MBq/μg, Amersham, UK) and 1 μl of bromophenol blue for 2 h at room temperature. A blank containing [^{125}I]thyroxine but no protein was treated in the same way. A 1-μl aliquot of each sample was subjected to native polyacrylamide gel electrophoresis (PAGE) using Phastgel gradient (8–25%) and a Phastsystem apparatus (Pharmacia). Electrophoresis was carried out according to manufacturer's instructions, except that it was stopped when bromophenol blue reached the anode buffer strip. The gel was sliced in various length sections, and each slice was assayed for radioactivity in a gamma counter and then stained with Coomassie Blue.

RESULTS

Purification on Remazol Yellow GGL-substituted gel

Yields. TTR was isolated on Remazol Yellow GGL–Sepharose from a by-product of the chromatographic purification of albumin with an average yield of 70%. The overall yield in relation to plasma was *ca.* 30%. As the first step of the chromatographic purification of albumin was achieved with a 80–100% yield in TTR, the greatest losses occur during pretreatment of plasma. Results (mean of five experiments) are summarized in Table I.

Quality of the purified TTR. Native PAGE analysis of the purified TTR showed a major band with anodal mobility and minor bands with lower mobilities (Fig. 1A). When analysed by sodium dodecyl sulphate (SDS) PAGE, two bands that are revealed with an anti-TTR antiserum by immunoblotting were observed, a major one and a minor one with relative molecular masses of

TABLE I

PURIFICATION OF HUMAN PLASMA TRANSTHYRETIN

The recovery was calculated from the TTR level in the previous purification step, and the overall recovery from the TTR plasma concentration. TTR 1 and TTR 2 are the values obtained after Remazol Yellow GGL–Sepharose chromatography and after Cibacron Blue–Sepharose chromatography, respectively.

Fraction	Recovery (%)	Overall recovery (%)	Amount of TTR per mg of protein (mg)	Apparent purification from plasma
Plasma	100	100	0.0039	1.0
SII + III	48	48	0.0044	1.1
DNaCl	89	43	0.0292	7.5
TTR 1	70	30	0.8333	214.0
TTR 2	95	28	0.9896	254.0

53 500 and 14 500, respectively. Small amounts of contaminants were present (Fig. 1B). Analysis using fast protein liquid chromatography (FPLC) revealed one important peak and small impurities (Fig. 2A). As can be seen in Fig. 3, when compared with plasma TTR and DNaCl TTR, purified TTR effectively bound [^{125}I]thyroxine since two separated radioactivity peaks were observed, one in the slice containing TTR and one in the anodal section. The elevated value of radioactivity obtained in the TTR slice with plasma is probably due to the presence of an excess of albumin (which also transports thyroxine) in this sample. No differences were observed among the other samples.

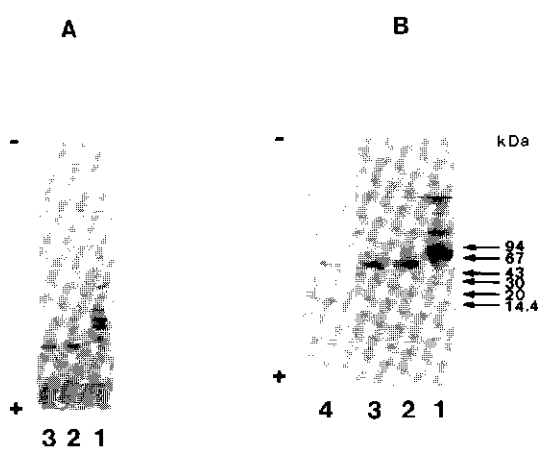

Fig. 1. Polyacrylamide gel electrophoresis analysis of isolated TTR. (A) Native PAGE on 8–25% gradient gel. (B) SDS-PAGE on 8–25% gradient gel. Gels were stained with Coomassie Blue. DNaCl (lane 1), the ethanol eluate from Remazol Yellow GGL–Sepharose chromatography (lane 2) and the emerging breakthrough fraction from Cibacron Blue–Sepharose chromatography (lane 3) were adjusted to a TTR level of 0.2 mg/ml. Immunoblotting with a specific anti-human TTR rabbit serum (lane 4) was performed after electrophoresis of the ethanol eluate.

Influence of buffer A washing time before ethanol elution. For first experiments, TTR was assayed at the outlet of the column in fractions recovered as follows: the flow-through protein peak, the end of washing with buffer A and the ethanol peak. As TTR was present at a higher level in the washing fraction in relation to the flow-through protein peak fraction, two experiments were performed in order to study the interaction between the dye and TTR. In the first, TTR desorption was achieved as soon as the absorbance had greatly decreased; in the second, it was achieved after the absorbance was maintained at a stable low value over a ten-column volume period. The flow-through was collected in 1/8 column volume fractions until desorption, and the TTR level was assayed both in flow-through fractions and in the ethanol peak. The total protein elution profile was noted to be different to the TTR one: the TTR level remained high even when the absorbance was greatly decreased and stable at a low value. Moreover, the amount of TTR desorbed by ethanol in the first experiment was four-fold higher than in the second. In all subsequent experiments, TTR desorption was carried out as soon as the absorbance had greatly decreased.

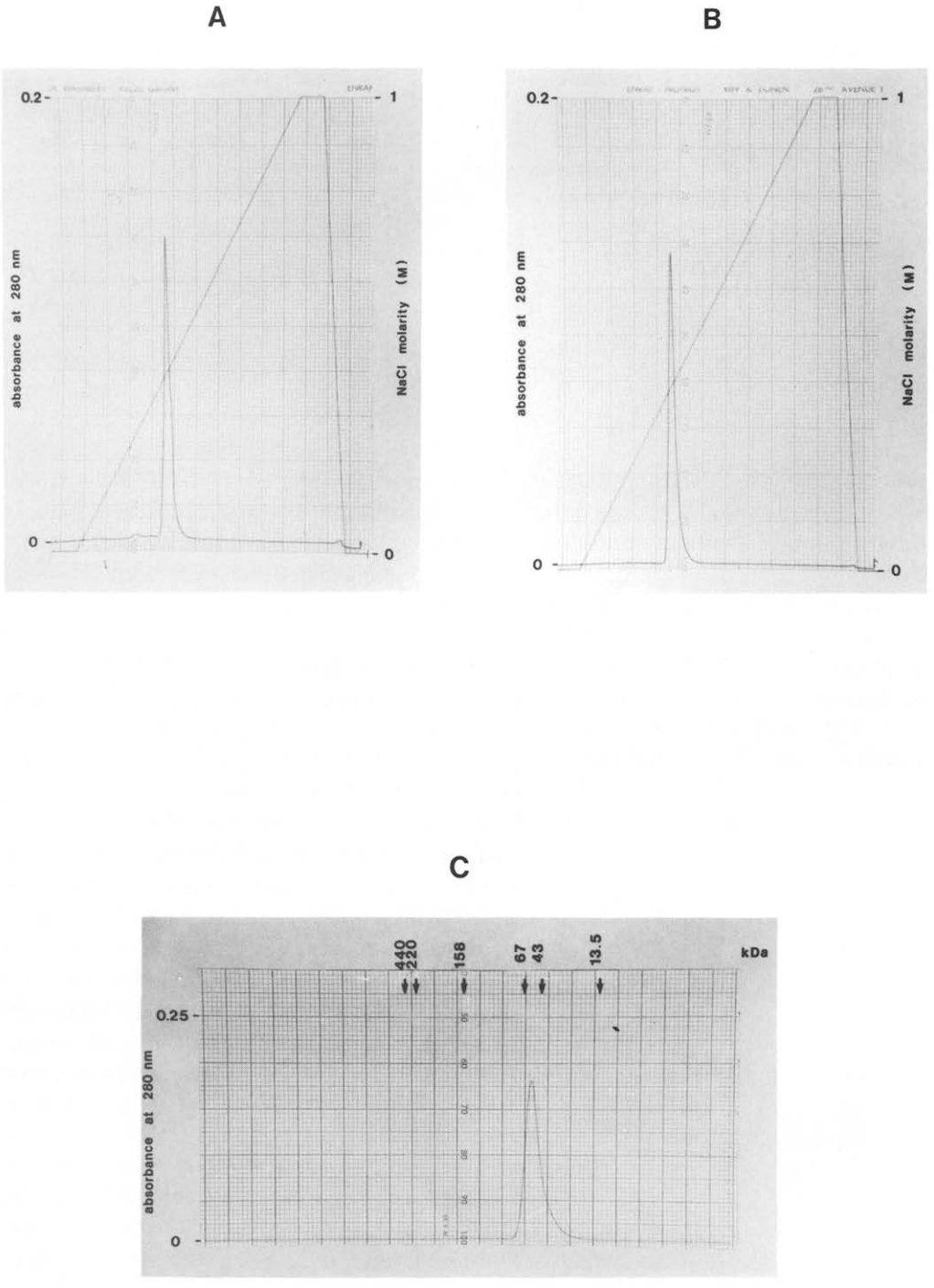

Fig. 2. FPLC ion-exchange and gel permeation chromatographic analysis of isolated TTR. FPLC ion-exchange chromatography: 40 μg of the ethanol eluate from Remazol Yellow GGL–Sepharose chromatography (A) and 160 μg of the emerging breakthrough fraction from Cibacron Blue–Sepharose chromatography (B) were applied to a Mono-Q HR 5/5 column equilibrated in 20 m*M* triethanolamine (pH 7.7) at a flow-rate of 1 ml/min. Proteins were eluted with a linear gradient of sodium chloride (0–1 *M*). (C) FPLC gel permeation chromatography: 67 μg of the emerging breakthrough fraction from Cibacron Blue chromatography were applied to a Superose 12 HR 10/30 column equilibrated in 0.05 *M* sodium phosphate, 0.15 *M* sodium chloride (pH 7.2) at a flow-rate of 24 ml/h.

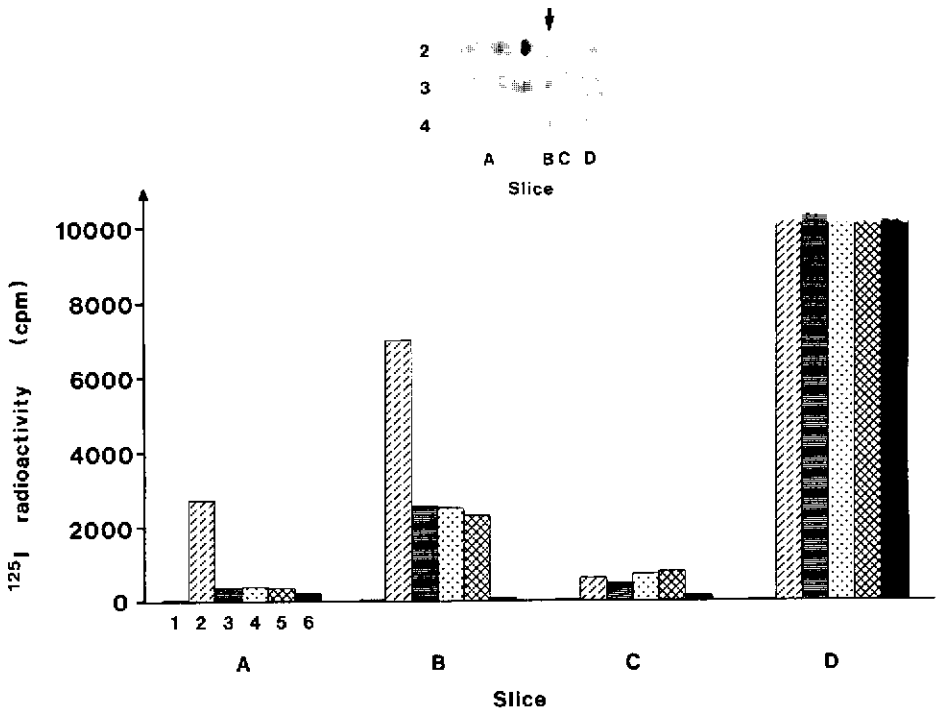

Fig. 3. Thyroxine binding: several samples were subjected to electrophoresis, TTR without thyroxine (1), thyroxine without TTR (6), human serum (2), the DNaCl by-product (3), the ethanol eluate from Remazol Yellow GGL–Sepharose chromatography (4), and the emerging breakthrough fraction from Cibacron Blue–Sepharose chromatography (5). For each of the samples 2, 3 and 4 the reconstituted gels after slicing, assay for radioactivity and staining are shown. The arrow indicates the position of TTR.

Influence of the amount of TTR applied to the gel. Various volumes of a DNaCl pool were applied to a same adsorbent at a low flow-rate in order to ensure maximum adsorption. Then, the same volume of DNaCl with various concentrations of TTR was applied to a same adsorbent. As can be shown in Fig. 4, the amount of TTR eluted increased as the amount of TTR loaded increased. The increase was not linear for amounts of TTR greater than 1 g of TTR per litre of gel. Thus, optimum effectiveness is obtained with 1 g of TTR per litre of gel. Moreover, the results indicate that the amount of TTR eluted was not dependent on the TTR level in DNaCl.

Influence of the residence time. A similar volume of DNaCl was applied to an adsorbent at residence times (ratio of the adsorbent volume to the flow-rate) ranging from 12 to 105 min. No differences in the amount of TTR eluted were observed.

Multiple uses of adsorbents. Ten runs were performed on a 300-ml adsorbent column without

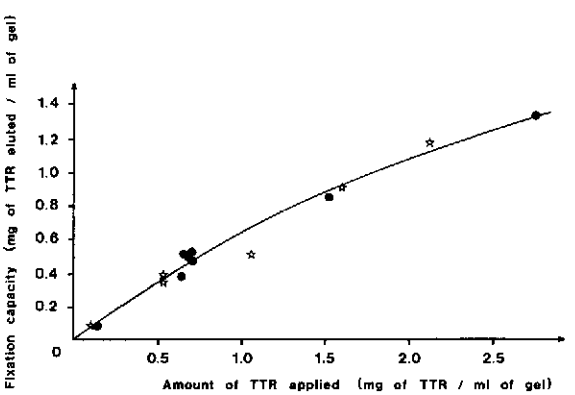

Fig. 4. Influence of the amount of TTR applied to the gel. Volumes of DNaCl (TTR concentration of 0.4 mg/ml) ranging from 120 to 1990 ml were applied to an adsorbent (7 × 7.8 cm) at a linear flow-rate of 5 cm/h (●). The same volumes of DNaCl with TTR concentrations ranging from 0.1 to 2 mg/ml were applied to an adsorbent (1.1 × 10 cm) at a linear flow-rate of 52 cm/h (☆).

TABLE II

MULTIPLE USE OF ADSORBENTS

The different runs were achieved by applying various amounts of DNaCl to an adsorbent (7 × 7.8 cm) at a linear flow-rate of 5 cm/h. The recovery was calculated from the TTR level in the DNaCl fraction, and is given only for experiments carried out using the same volume of DNaCl.

Run	Chromatographic recovery of TTR (%)	Amount of TTR per mg of protein in the purified fraction (mg)
1	72.0	0.89
2		0.74
3		0.73
4	75.5	0.79
5	75.4	0.89
6		0.86
7	67.7	0.86
8		0.84
9		0.81
10	71.8	0.90

significant changes in the performance of the gel or in the quality of the purified TTR (Table II). Various amounts of DNaCl were applied to the adsorbent, and the chromatographic recovery of TTR is listed only for identical runs.

Purification on Cibacron Blue-substituted gel

In order to improve the TTR purity, a Cibacron Blue–Sepharose chromatography was performed after the Remazol Yellow GGL–Sepharose chromatography. The chromatographic recovery of TTR was 95% (Table I), and this step allowed the preparation of pure TTR. Analysis of the recovered material by native PAGE revealed only one band with anodal mobility (Fig. 1A). When analysed by SDS-PAGE and immunoblotting, the purified TTR migrated as two bands that reacted with an anti-TTR antiserum (Fig. 1B). Just one symmetrical peak was obtained using FPLC analysis (Fig. 2B). The relative molecular mass of the final TTR was estimated to be 54 500 from FPLC gel permeation chromatography (Fig. 2C). Finally, the purified TTR effectively bound [^{125}I]thyroxine (Fig. 3), and its binding capacity was not decreased during this purification step.

DISCUSSION

Human plasma TTR must be available in large amounts and with sufficient purity for studies of its potential therapeutic value. Purification of TTR by dye-affinity chromatography, which takes advantage of the interaction with the dye Remazol Yellow GGL, is faster and simpler than the affinity chromatographic methods previously described [9,10]. In studies reported by other authors [5,6], TTR was isolated from serum. However, for large-scale purification, use of serum as the starting material will interfere with the fractionation of plasma. Moreover, serum contains albumin and IgG, which interact with Remazol Yellow GGL and contaminate the final product. The isolation of TTR from a by-product of the chromatographic purification of albumin, as described here, offers the following advantages.

(1) This by-product, which has not been exploited previously, is available in large amounts, and contains TTR with a 40% recovery in relation to plasma.

(2) This by-product contains no IgG and only a trace amount of albumin which allows the preparation of purer TTR (73–90%) than that prepared from plasma (40–50%) [6].

(3) The thyroxine binding capacity of TTR was investigated in plasma, in the by-product used as starting material, and in TTR samples obtained after Remazol Yellow GGL chromatography and after Cibacron Blue chromatography. The functional activity of TTR, as represented by this capacity, was not altered throughout the purification procedure.

The isolation procedure described here can purify TTR 28-fold in a single chromatographic step on Remazol Yellow GGL–Sepharose, with an average 70% yield. Analysis of results gives the following conclusions.

(1) TTR is weakly bound to the dye. In fact, TTR is partially eluted with the equilibrating buffer. This phenomenon has already been reported by other authors [5]. They observed that TTR in presence of plasma proteins was rather strongly bound to the dye, in contrast to the isolated protein. Their results indicated that, appar-

ently, a certain affinity for albumin is necessary to enhance the TTR–dye interaction. This finding is in good agreement with our results, since the by-product used as source of TTR contains only trace amounts of albumin. In order to obtain a good yield, it is preferable to elute the bound TTR quickly after washing with equilibrating buffer.

(2) Elution of TTR from Remazol Yellow GGL gel did not require drastic conditions, which left the gel and the protein unchanged and increased the safety of an injectable product.

(3) The efficiency of the dye-affinity purification procedure is unchanged whatever the residence time and the TTR level in the by-product. Moreover, the TTR binding capacity of the gel is relatively important (in optimum working conditions, 0.65–0.7 g of TTR are eluted per litre of gel for a load of 1 g of TTR per litre of gel). Consequently, the purification method can be easily scaled up.

(4) The gel can be used ten times with no loss of binding capacity.

CONCLUSION

Dye-affinity isolation of TTR on Remazol Yellow GGL-substituted gel from a by-product of the chromatographic purification of albumin is a method suitable for large-scale preparation. The quality of the TTR obtained may be acceptable for substitutive therapy. Remaining impurities (essentially albumin) can be removed by Cibacron Blue chromatography with no significant loss in recovery.

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