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Note

ISOLATION OF HUMAN SERUM PROTEIN BINDING CARDENOLIDES BY AFFINITY CHROMATOGRAPHY

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INTRODUCTION

Drug action, metabolism, and excretion are dependent on the binding of drugs to serum proteins. Several authors have studied the binding of digoxin and other related cardenolides to serum albumin, but little is known about ouabain transport. The methods used for these experiments were based on conventional chromatographic procedures or membrane equilibrium dialysis (1–3). Spector et al. (4), using ultrafiltration experiments, supported the hypothesis that the binding of cardiac glycosides to serum proteins is directly related to molecule polarity. Therefore ouabain, being very polar, is only slightly bound. Affinity chromatography is more rapid and specific than conventional techniques used in the study of this important problem. Thus drugs can be attached to a solid support in order to obtain a specific resin capable of retaining only the serum proteins which transport the given drug in the blood.

In this communication we report experimental data showing that several human serum proteins can be isolated by affinity chromatography on strophantidin–Sepharose and ouabain–Sepharose.

MATERIALS

Sepharose was purchased from Farmacia, CNBr from Fluka. Strophantidin-3-bromoacetate was synthesized essentially according to Hokin et al. (5). Alkylation was lengthened from 1.5 to 16 h.

SYNTHESIS OF THE SPECIFIC ADSORBENTS

The synthesis of the specific adsorbent which contains strophantidin bound to Sepharose was performed as follows. Synthesis of

diaminodipropylamino–Sepharose was carried out according to Cuatrecasas (6): 0.8 g of strophantidin-3-bromoacetate in 20 ml of 50% (vol/vol) *N,N*-dimethylformamide at pH 7 was added to 20 ml of diaminodipropylamino–Sepharose; pH was kept constant with a pH-stat. The reaction course was followed measuring the resin amino residues by the method of Favilla and Santi (7). No variation in the amount of amino residues was observed after 15–16 h. The residual amino groups of the resin were blocked by treatment with 10 mM acetic anhydride for 10–20 min in 0.2 M sodium phosphate buffer pH 6.0. Then the adsorbent was thoroughly washed with 50% (vol/vol) dimethylformamide to remove the physically adsorbed bromoacetylstrophantidin. The adsorbent containing ouabain was synthesized as follows: adipic acid dihydrazide–Sepharose was prepared as described by Wilchek and Lamed (8).

Ouabain was oxidized as follows: 0.022 g of sodium periodate was added to a water solution containing 0.073 g of ouabain. The reaction mixture was kept in the dark at 0°C for 1 h. Then the oxidized ouabain was added to 30 ml of adipic acid hydrazide–Sepharose suspended in 25 ml of 0.1 M acetate buffer pH 5.5, and stirred for 17 h at 5°C. The resin was then thoroughly washed with a 1 M solution of NaCl and then with water. Solid sodium borohydride 0.1 g in 50 ml of 0.5 M tris-HCl at pH 8 was added to the suspension three times at hourly intervals. The degree of coupling of strophantidin or ouabain to Sepharose was determined colorimetrically according to a modification of the method of Ulubelen (9).

AFFINITY CHROMATOGRAPHY PROCEDURE

The specific adsorbents were tested for their ability to bind human serum proteins: 25 ml of human serum, diluted 1:1 with 20 mM tris-HCl buffer pH 7.4, was added to 20 ml of strophantidin–Sepharose. The mixture was incubated and gently stirred for 5 h at 4°C. The resin was then poured into a 2.5 × 7 cm column. The column was washed with the incubation buffer until absorbance at 280 nm approached zero. The column was washed again with the incubation buffer containing 0.5 M NaCl, until the absorbance at 280 nm was again negligible, in order to remove the proteins adsorbed by nonspecific ionic interactions. The gel was removed from the column and was stirred overnight at 4°C with an equal volume of 4 mM ouabain in 10 mM tris-HCl buffer pH 7.4 (specific buffer). Strophantidin could not be used as eluent, since it is practically insoluble in aqueous solvents.

The mixture was poured back into the column and the elution was performed at 20°C with 4 mM ouabain in the same tris-HCl buffer. In Fig. 1 is shown a typical elution pattern of human serum for strophantidin–Sepharose adsorbent.

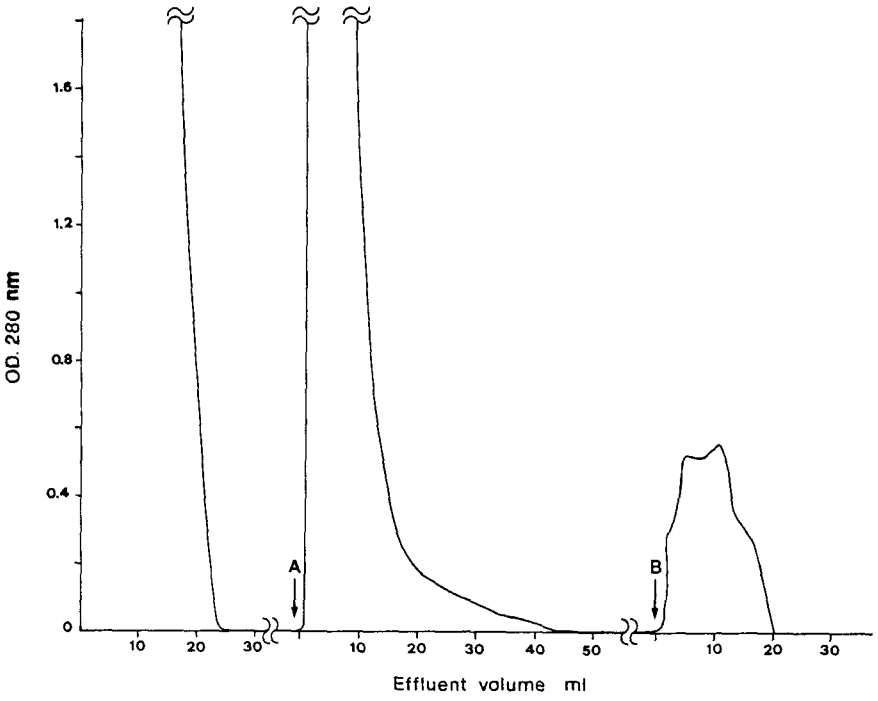

FIG. 1. Elution pattern for the purification of serum proteins binding ouabain: 25 ml of human serum diluted 1 : 1 in 20 mM tris-HCl pH 7.4 was incubated for 5 h at 4°C and poured in a 2.5 × 7 cm column. Washing was performed with 10 mM tris-HCl pH 7.4, and then (arrow A) 0.5 M NaCl was added to the washing buffer. Gel was removed and incubated overnight at 4°C with 17.5 ml of 10 mM tris-HCl buffer containing 4 mM ouabain. The mixture was then repoured into the column (arrow B) and the elution was performed at 25°C.

Ouabain–Sepharose resin was eluted in a manner similar to the resin just described. The elution pattern was similar but the proteins recovered tended to precipitate below 4°C. A control experiment was carried out to demonstrate the specificity of the column. The serum was chromatographed on a column of acetylated diaminodipropylamino–Sepharose and the complete elution procedure repeated. No proteins were eluted by the specific buffer containing ouabain. In order to exclude the possibility of hydrophobic interactions of the serum proteins with the specific adsorbents, the columns were washed as in the preceding experiments, followed by 3.3% ethylene glycol in 10 mM tris-HCl buffer pH 7.4. By this procedure no proteins were recovered. The column was then treated with the specific buffer, and the same proteins as those obtained by the standard procedure were recovered. An experiment to determine the binding capacity for serum proteins of the two different adsorbents was designed.

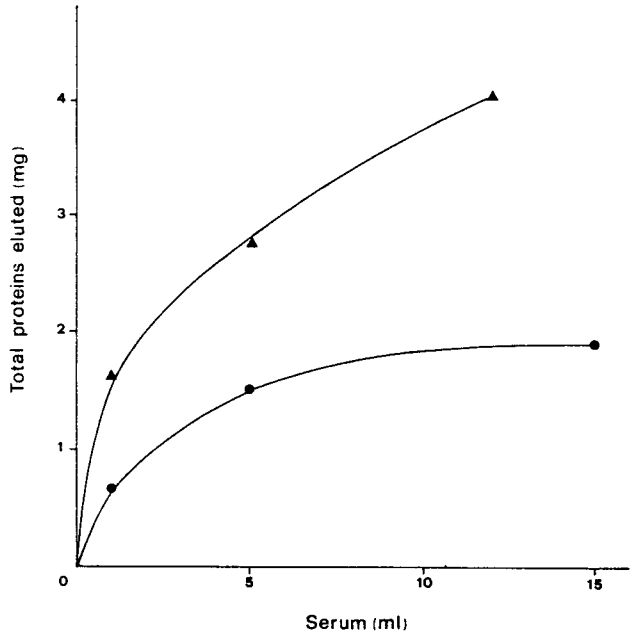

FIG. 2. Two columns (2 × 3 cm) of strophantidin–Sepharose (▲) and ouabain–Sepharose (●) were incubated with three different volumes of serum and eluted as described in Fig. 1 and in the text.

In Fig. 2 are reported the quantities of proteins recovered after incubating the columns with increasing volumes of serum. The amount of proteins recovered in three different elutions are in agreement with a typical saturation curve. The lower yields of proteins recovered from ouabain–Sepharose are due to the smaller amount of ouabain attached to the resin. Samples eluted from the specific adsorbents were analyzed with polyacrylamide disk gel electrophoresis using 7.5% acrylamide, according to the method described by Davis (10), with a discontinuous buffer system at pH 9.5. The electrophoretic pattern of the proteins eluted by the specific buffer is shown in Fig. 3. The pattern of the proteins eluted from strophantidin–Sepharose showed three bands: the first one migrated about 1.4 mm from the origin in the S-α-2-glubulin zone. This band was also stained by periodic acid–Schiff reagent (10), indicating the presence of carbohydrates in the protein. The second band was positioned in the zone of haptoglobins; the third comigrated with serum albumin. Slight differences were observed for the proteins eluted from ouabain–Sepharose. The first two bands are not so intense, while albumin seems to bind to ouabain–Sepharose with greater efficiency.

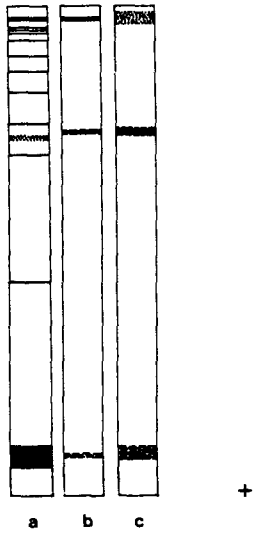

FIG. 3. Schemes of the electrophoretic patterns of the proteins eluted from the adsorbents strophantidin–Sepharose (b) and ouabain–Sepharose (c). The pattern of some serum proteins, taken as reference, is shown in scheme (a): from the bottom one may observe albumin, transferrin, haptoglobins, and finally at the top the S-α-2-glycoprotein band.

Our data show that serum proteins other than albumin may act as carrier for cardenolides. Further studies using the same technique are being carried out in order to detect other plasma proteins capable of binding different types of cardiac glycosides.

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