PURIFICATION OF SEX HORMONE-BINDING GLOBULIN USING AN AFFINITY MATRIX IN AN ELECTROPHORETIC CELL SYSTEM

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1. Introduction

Several attempts to purify specific steroid-binding proteins such as sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) using affinity chromatography have been reported [1–5]. In these, elution of the immobilised protein has been effected using relatively large volumes of either a buffer containing a competing steroid or a buffer of extreme pH or salt concentration. Such procedures necessitate subsequent removal of the ligand by exhaustive dialysis, and concentration of the eluate.

Recently the use of affinity matrices as electrophoretic media has been suggested [6]. This report describes a small scale preparative method in which SHBG specifically bound to an androstanediol–agarose matrix is electrophoretically desorbed from the affinity matrix, obviating the need for a competing ligand or concentration of eluate volumes. This is a specific application of a technique with wide-ranging potential.

2. Materials and methods

The affinity matrix was prepared as follows: Aminohexyl-Sepharose 4B was prepared as in [7] and 5α-androstane-3β, 17β-diol-3-hemisuccinate was coupled to this using dicyclohexylcarbodiimide. Androstanediol-aminohexyl–Sepharose, 25 g, were washed on a No. 1 sintered Buchner funnel with 500 ml 0.05 M Tris, pH 7.4, containing 0.005 M $CaCl_2$ and 1.0 M NaCl followed by 500 ml 0.05 M Tris, pH 7.4, containing 0.005 M $CaCl_2$.

SHBG was immobilised by stirring 10 g washed gel at 4°C with 5 ml Tris buffer and 5 ml post-partum plasma for 1 h. Following the incubation, the gel was washed again as above (until no protein could be detected in the washes) and the 'loaded' affinity gel transferred to the electrophoresis cell.

The electrophoretic desorption of the SHBG from the affinity gel was carried out in a cell (fig.1), prepared from a 20 ml polypropylene syringe barrel and incorporating an hermetically-sealed elution chamber cut off from the anode buffer compartment by a dialysis membrane (Visking membranes, Scientific Supplies Ltd, England). The apparatus was maintained below 10°C by placing it in an ice bath and electrophoresis carried out using 0.05 M Tris, pH 7.4, buffer in both anode and cathode compartments at 10 mA and 110 V for 5 h after which the elution chamber fluid was collected for analysis.

Binding capacity and association constants were assessed by Scatchard analysis for 1:5 dilutions of both the elution chamber fluid and original plasma as in [8]. This method involves two-step chromatography incorporating Cibacron blue F3G-A–Sepharose 4B, to remove plasma albumin and the steroid bound to it, above Sephadex LH-20 to remove the free steroid; thus the emergent fluid contains the specifically bound steroid. Radioactivity in the Cibacron blue F3G-A–Sepharose 4B was determined as in [8].

Analytical disc PAGE electrophoresis was carried out as in [9] on duplicate samples of the neat elution chamber fluid, the latter 2-times concentrated (minicon cell, Amicon, England) and the original plasma, after pre-equilibration with tritiated 5α-DHT (spec. act. 100 Ci/mmol, Radiochemical Centre,

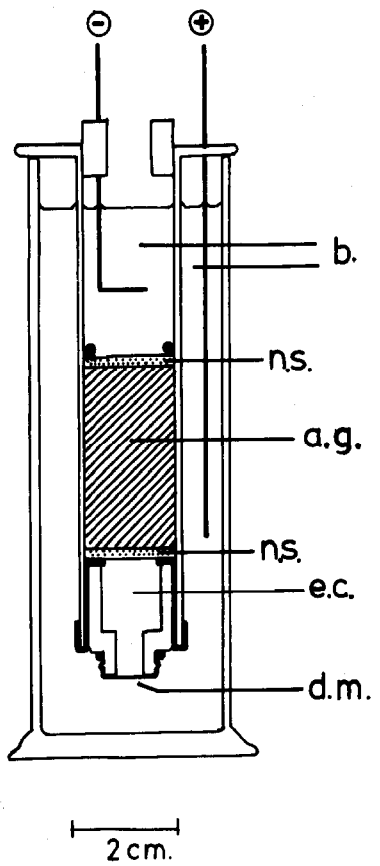

Fig.1. Diagram of electrophoresis cell. b., buffer compartment; n.s., porous nylon support; a.g., affinity gel; e.c., elution chamber; d.m., dialysis membrane.

Amersham). One of each of the duplicates were cut into 3 mm segments and counted for radioactivity and the remainder double stained: first for glycoproteins as in [10], then using Coomassie brilliant blue.

Protein determinations were made using a Bio-Rad (Bio-Rad, Watford) protein assay system based on the method in [11].

Scintillation counting was done in a Wallac 8100 liquid scintillation counter using a 3:2 toluene:Triton X-100 scintillant containing 3 g/l PPO.

Sepharose 4B was obtained from Pharmacia, 5α-dihydrotestosterone from Sigma Chemical Co. and electrophoresis reagents from Bio-Rad Laboratories, Watford, England. The 5α-androstane-3β,17β-diol-3-hemisuccinate (m.p. 214°C) was a gift from Steranti Res. Ltd, St Albans, England.

3. Results

After electrophoretic desorption of the affinity gel the elution chamber fluid had protein conc. 0.33 mg/ml. The elution chamber had total vol. 3.8 ml. Scatchard analysis of the elution chamber fluid gave a binding capacity for 5α-DHT of 0.738 nmol/ml and an association constant of 0.46×10^7 l/mol. Table 1 shows the binding capacity for 5α-DHT in the original plasma and the elution chamber fluid, the specific activity of the proteins in terms of binding capacity/mg protein and the purification factor.

Staining of the analytical PAGE gels using the

Table 1
Comparison of binding data and protein concentrations for original plasma and electrophoretically desorbed protein

	Protein conc. (mg/ml)	Binding capacity (nmol/ml)	Spec. act. (nmol/mg)	Purification factor
Original plasma (5 ml)	119.0	0.238	0.002	–
Elution chamber fluid (3.8)	0.33	0.738	2.24	1118

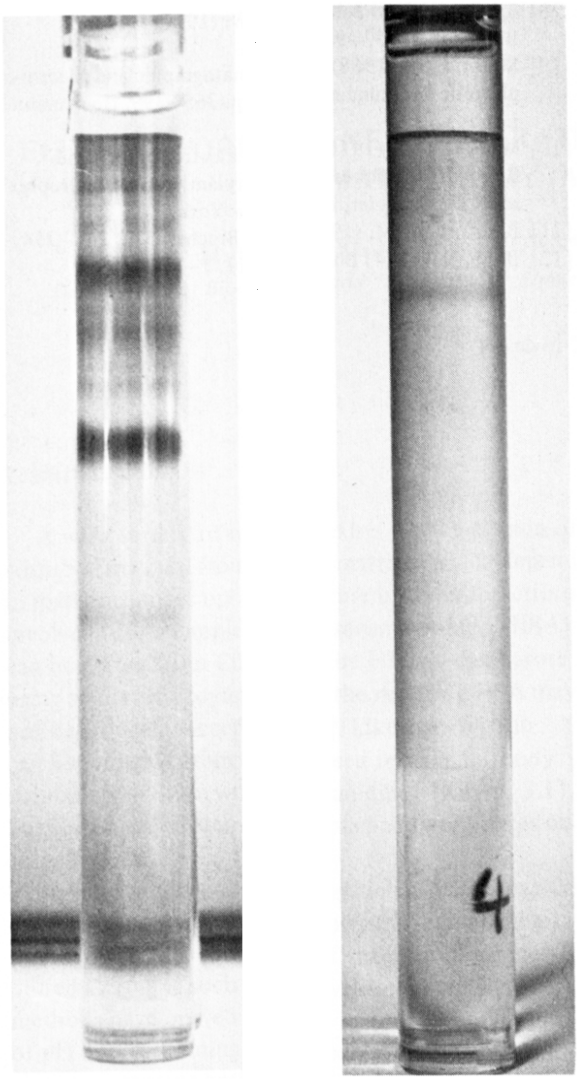

Fig.2. Polyacrylamide gel electrophoresis of original plasma and elution chamber fluid.

glycoprotein specific stain demonstrated 13 bands for the original plasma and a single sharp band for both the elution chamber fluid samples. Secondary staining with Coomassie brilliant blue resulted in 25 observable bands for the plasma sample and only a single band for the purified material (R_F relative to bromophenol blue front was 0.214), fig.2. Determination of the radioactivity in the gel slices for the original plasma and elution chamber fluid samples gave single, sharp peaks with R_F values of 0.220, 0.200 and 0.210, respectively.

4. Discussion

The results show how a small scale preparation of sex hormone-binding globulin (SHBG), a 17-hydroxy-C19-steroid-binding glycoprotein (mol. wt 98 000–100 000) present at ca. 100 mg/l in human pregnancy plasma [11] can be achieved in a single step electrophoresis using an agarose-based affinity matrix. The resultant protein was purified 1118-fold in the above procedure and total 1.25 mg protein obtained from a 5 ml plasma sample. This figure for placental plasma is in reasonable agreement with the estimated value for pregnancy plasma obtained [11] using the Laurell rocket technique. For SHBG the cell was capable of separating sufficient quantities of protein for an immunisation programme in rabbits to elicit antisera for the quantitative radioimmunological determination of SHBG in clinical samples. However from the binding capacity data in table 1 it may be estimated that the electrophoretically desorbed protein has about 0.2 DHT binding site/mol. Evidently the conditions for the electrophoretic desorption of the protein specifically immobilised through its binding site need to be more fully explored to obtain a fully active protein as the figure for binding site per mole and the K_a value calculated from Scatchard analysis were lower than expected and indicate a degree of denaturation of the protein binding site.

The procedure described has general application in the purification of proteins whose elution from affinity matrices has proved difficult and the use of diluting volumes of a competing ligand solution or a chaotrophic buffer needs to be avoided. In relation to this the authors have experimented on the desorption of glycoproteins from concanavalin A–Sepharose and human serum albumin from Cibacron blue–Sepharose exploring the use of the cell in conjunction with varying molecular weight cut-off membrane filters with a closed loop ultra violet detection system coupled to the elution chamber to enable sampling and investigation of selective desorption in the former case.

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