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APPLICATION OF LIQUID–LIQUID PARTITION CHROMATOGRAPHY IN THE SIMULTANEOUS PURIFICATION OF SEX-HORMONE-BINDING GLOBULIN AND CORTICOSTEROID-BINDING GLOBULIN

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SUMMARY

Two human serum proteins, corticosteroid-binding globulin (CBG) and sex-hormone-binding globulin (SHBG), were purified to homogeneity by the application of a combination of three different modes of chromatography. Human pregnancy serum was fractionated with ammonium sulphate. SHBG (50% pellet) and CBG (80% pellet) were then purified by affinity chromatography on tresyl-activated Sepharose with 15-aminopentadecanoic acid (for SHBG) and 1,12-diaminododecane (for CBG) as spacers and 17ξ-aminoethyl-5α-androstan-3β,17-diol (for SHBG) and 17α-hydroxy-4-androsten-3-one-17β-carboxylic acid (for CBG) as specific ligands for these two proteins. The eluate was injected into a Mono Q anion-exchange column. Fractions containing SHBG or CBG were finally purified by liquid–liquid chromatography on Lipar-Gel 750. This chromatographic sequence clearly separated SHBG and CBG from other proteins, mainly serum albumin, without a loss of protein or binding activity.

INTRODUCTION

Corticosteroid-binding globulin (CBG) and sex-hormone-binding globulin (SHBG) are circulating plasma glycoproteins with molecular weights of about 70 000. They are both thought to be synthesized in the liver. CBG, a protein that binds corticosteroids and progesterone, was first first described in 1956[1,2]. Since that time, CBG has been extensively studied and has become more readily available following the use of affinity chromatography[3–5]. CBG was reported to consist of a single polypeptide chain with a carbohydrate content of about 30% and a molecular weight of 52 000[6,7]. In 1977, Stroupe and co-workers[8–10] described it as a protein consisting of two molecular variants, separable by gel electrophoresis.

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SHBG, a protein that binds estradiol and testosterone, was first described in 1966[11]. Since then, the protein has been purified to apparent homogeneity[12–14], but the structure, composition, and carbohydrate content varied considerably between different laboratories.

This paper describes a method for the simultaneous purification of SHBG and CBG to homogeneity in less than 16 h with a recovery of 61% for CBG and 44% for SHBG without a loss of binding activity towards steroids. We employed a new chromatographic technique that allows the clear separation of the different varieties of CBG and SHBG (due to carbohydrates) from albumin and other contaminants.

EXPERIMENTAL

Steroids

[^{3}H]Cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione, specific activity 85 Ci/mmol) and [^{3}H]dihydrotestosterone (DHT, 17β-hydroxy-5α-androstan-3-one, specific activity 118 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). The radiolabelled steroids were kept in benzene–ethanol (85:15, v/v) at 4°C and checked regularly for purity by thin-layer chromatography. Unlabelled steroids were purchased from Serva (Heidelberg, F.R.G.). The elemental analysis and IR spectra of the new compounds are in agreement with the stated structures.

Synthesis of 17α-hydroxy-4-androsten-3-one-17β-carboxylic acid N-hydroxysuccinimide ester

To a solution of 3.48 g (0.01 mol) of 17α-hydroxy-4-androsten-3-one-17β-carboxylic acid[15] and 1.15 g (0.01 mol) of N-hydroxysuccinimide in 50 ml of dioxane, 2.27 g (0.011 mol) of dicyclohexylcarbodiimide in 15 ml of dioxane were added over a period of 1 h. The reaction mixture was stirred at room temperature overnight, then 0.1 ml of acetic acid was added. After stirring for 30 min, the solid was filtered off. Crystallization from dioxane gave 3.5 g (81%) of the activated ester, m.p. 258–260°C.

Synthesis of 17ξ-aminomethyl-5β-androstane-3β,17-diol acetate

To a solution of 16.6 g (0.050 mol) of epi-androsterone and 5 g (0.05 mol) of trimethylsilyl cyanide, 50 mg of zinc iodide were added and the mixture was stirred at 60°C for 7 h. After evaporation of the solvent, the residue was suspended in 150 ml of anhydrous tetrahydrofuran and a suspension of 5.7 g (0.15 mol) of lithium aluminium hydride in diethyl ether was added dropwise over a period of 20 min. After stirring for 1 h at room temperature and refluxing for 1 h, the reaction mixture was cautiously decomposed with 20 ml of water and then diluted with an excess of water (about 200 ml). The tetrahydrofuran was evaporated *in vacuo* and the remaining aqueous suspension was extracted with diethyl ether. After washing with water, drying and evaporation of the solvents, the remaining solid was extracted with hot acetone and 5 ml of acetic acid were added. The crude product remaining after evaporation of the solvent was chromatographed on a column (500 × 20 mm I.D.) of silica gel 40 (Merck, Darmstadt, F.R.G.) with 2 l of ethanol as eluent. Recrystallization from methanol gave 9.9 g (52%) of the pure product, m.p. 210–211°C.

Synthesis of CBG-binding affinity gel

A 15-g amount of tresyl-activated Sepharose (Pharmacia, Uppsala, Sweden) was suspended in 150 ml of 1 m*M* hydrochloric acid and washed with 3 l of 1 m*M* hydrochloric acid. A solution of 1.5 g of 1,12-diaminododecane in 50 ml of ethanol plus 50 ml of 0.1 *M* sodium hydrogencarbonate–0.5 *M* sodium chloride was added to the gel and the mixture was rotated end-over-end for 16 h at 4°C. After washing with ethanol (3 l) a solution of 0.33 g of 17α-hydroxy-4-androsten-3-one-17β-carboxylic acid N-hydroxysuccinimide ester in 80 ml of dioxane and 70 ml of ethanol was added and the mixture was rotated for 16 h at 4°C. Washing with 400 ml of dioxane–ethanol (1:1, v/v) was followed by three washing cycles with acetate buffer (0.1 *M*, pH 4) containing 0.5 *M* sodium chloride and 0.1 *M* Tris buffer (pH 8) containing 0.5 *M* sodium chloride. The gel was stored in PENG buffer at 4°C.

Synthesis of SHBG-binding affinity gel

15-Aminopentanoic acid was bound as a spacer to tresyl-activated Sepharose. Then 17ξ-aminomethyl-5α-androstane-3β,17-diol was combined via the spacer with the Sepharose[16].

Human pregnancy serum

Human pregnancy serum was pooled, stripped of endogenous steroids with charcoal (2 g per 100 ml for 2 h at 23°C) and kept at −30°C. The serum was used as a source of SHBG and CBG.

Quantification of specific steroid-binding sites

The binding capacities were determined by multi-point titration and Scatchard analysis of the data[17]. Human pregnancy serum or aliquots of the purified proteins were diluted (1:40 for human pregnancy serum, 1:200 for purified proteins) in PENG buffer (10 m*M* KH_2PO_4–10 m*M* K_2HPO_4–1.5 m*M* EDTA–3 m*M* NaN_3–10% glycerol, pH 7.5). For the dilution of the purified proteins 0.1% bovine serum albumin was added to the PENG buffer. The diluted samples were incubated for 16 h at 4°C with various concentrations of [^{3}H]cortisol (for the measurement of CBG-binding capacity) or [^{3}H]dihydrotestosterone (for the measurement of SHBG-binding capacity) over a range calculated to saturate the binding capacity (1–16 nmol/l). Additional samples were incubated with tritiated ligands in the presence of a 200-fold excess of unlabelled ligands in order to determine the extent of non-specific binding. Unbound ligand was removed from protein-associated ligand with 500 μl of dextran-coated charcoal (DCC). For the preparation of DCC, 5 g of Norit A (Serva) were suspended in doubly distilled water. The charcoal particles were repeatedly allowed to settle and the fines were removed. The pelleted charcoal was finally resuspended in 1 l of PENG buffer and 0.5 g of dextran T 500 (Pharmacia) was added. Additionally, SHBG concentrations were determined with an immunoradiometric assay kit (Farmos Diagnostica, Oulunsalo, Finland) and CBG concentrations with a radioimmunoassay kit (IRE Diagnostica, Düsseldorf, F.R.G.).

Protein determinations

The protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, U.S.A.) using bovine serum albumin as a standard.

High-performance liquid chromatography

Chromatography was carried out with the Pharmacia FPLC System, connected to an LB 510 two-channel UV Radioactivity Chromatography System (Berthold, Wildbad, F.R.G.) by one of the following two methods.

(1) A pre-packed Mono Q anion-exchange column (HR 5/50) (Pharmacia) was equilibrated in T buffer [20 m*M* Tris–HCl (pH 7.5)] at room temperature and, after sample injection, eluted at a flow-rate of 2 ml/min with a sodium chloride gradient, formed by T buffer and TN buffer [20 m*M* Tris–HCl (pH 7.5)–1 *M* sodium chloride]; gradient, 0–4 ml 0% TN buffer, 4–80 ml 0–30% TN buffer, 30–36 ml 30–100% TN buffer, 36–40 ml 100% TN buffer.

(2) A pre-packed Superose 12 column (Pharmacia) was equilibrated in PBS buffer (phosphate-buffered saline, pH 7.5) at room temperature and eluted at a flow-rate of 1 ml/min with 30 ml of PBS buffer. Proteins were monitored at 280 nm. Fractions of 500 μl were collected. The radioactivity was monitored in 30 μl of each fraction by liquid scintillation counting (Ready-Solv HP scintillation cocktail and LS 6800 scintillation counter; Beckman, Munich, F.R.G.). For the characterization of SHBG and CBG in human pregnancy serum, the radioactivity was measured with the Berthold LB 510 system.

Liquid–liquid partition chromatography

The poly(ethylene glycol) (PEG)–dextran system contained 9.8% (w/w) of dextran T 40 (Pfeiffer & Langen, Dormargen, F.R.G.) and 5.4% (w/w) of PEG 6000 (Serva)[18,19] in PD buffer [phosphate buffer, 10 m*M* NaH_2PO_4–10 m*M* Na_2HPO_4 (2:3, v/v), containing 1 m*M* EDTA and 1 m*M* NaN_3 (pH 7.3)].

For preparing 10 kg of upper phase, 930 g of PEG 6000 and 130 g of of dextran T 40 were added to 8940 ml of PD buffer. The mixture was stirred until the solids had dissolved, and the turbid fluid was stored for 1 week at room temperature. The clear upper phase was separated from small amounts of lower phase (1–3%) and stored at room temperature until used. For preparing 500 ml of lower phase, 92 g of dextran T 40 and 8 g of PEG 6000 were dissolved in 400 g of PD buffer. The turbid fluid was stored for 1 week at 37°C and then used for coating the support particles. The support particles (Lipar-Gel 750; Merck) were suspended in about three volumes of lower phase and packed at 20°C and a pressure of 0.05 MPa into an HR 10/300 column (Pharmacia), equipped with a heating mantle. The column was then equilibrated with the upper phase (containing 65 m*M* potassium bromide–3 m*M* lithium citrate for the purification of SHBG and 60 m*M* potassium bromide–5 m*M* lithium citrate for the purification of CBG) at a flow-rate of 0.2 ml/min until the eluate was clear. This indicated that excess of lower phase had been removed from the column.

In the sample solutions of CBG or SHBG to be fractionated, sufficient solid PEG 6000 was dissolved to reach the same concentration as was present in the mobile phase. A 1-ml volume of sample was injected into the column, which was installed in the Pharmacia FPLC system. The column was eluted with 60 ml of upper phase at a flow-rate of 0.2 ml/min. Fractions were collected with a FRAC 100 fraction collector (Pharmacia) in the peak collection mode and were assayed for concentrations of CBG and SHBG.

Purification of CBG and SHBG

Volumes of 200 ml of human pregnancy serum were fractionated by ammonium sulphate precipitation. The 50% pellet and the 80% pellet were dissolved in 1 l of PENG buffer and incubated with 5 g of affinity gel (wet weight) (50% pellet for SHBG and 80% pellet for CBG purification) for 2 h at 4°C. The gel was then washed on a sintered glass funnel with 200 ml of PENG buffer and eluted with a high molar excess of DHT (for SHBG, 10 ml of $3 \cdot 10^{-4}$ *M* solution) and cortisol (for CBG, 10 ml of $3 \cdot 10^{-4}$ *M* solution). Aliquots (100–200 mg) of the gels were eluted with 2 ml of tritiated hormones (64 n*M*). The mixture of both eluates was chromatographed on a Mono Q column. Fractions containing bound radioactivity were pooled, concentrated with Centricon 10 (Amicon, Witten, F.R.G.) and further purified by liquid–liquid partition chromatography. Separation of SHBG or CBG from dextran and PEG was achieved by anion-exchange chromatography (Mono Q).

Isoelectric focusing and gel electrophoresis

Isoelectric focusing (IEF) and gel electrophoresis were carried out with the Phast Gel system and prefabricated gels (Pharmacia). For isoelectric focusing, gels of pH 3–9 were used and calibrated with Pharmacia calibration proteins for IEF from pH 3 to 10. Gel electrophoresis was carried out on gradient gels [8–25% polyacrylamide for native and 10–15% polyacrylamide for sodium dodecyl sulphate (SDS)-PAGE]. For SDS-PAGE, samples were heated for 10 min at 100°C in buffer

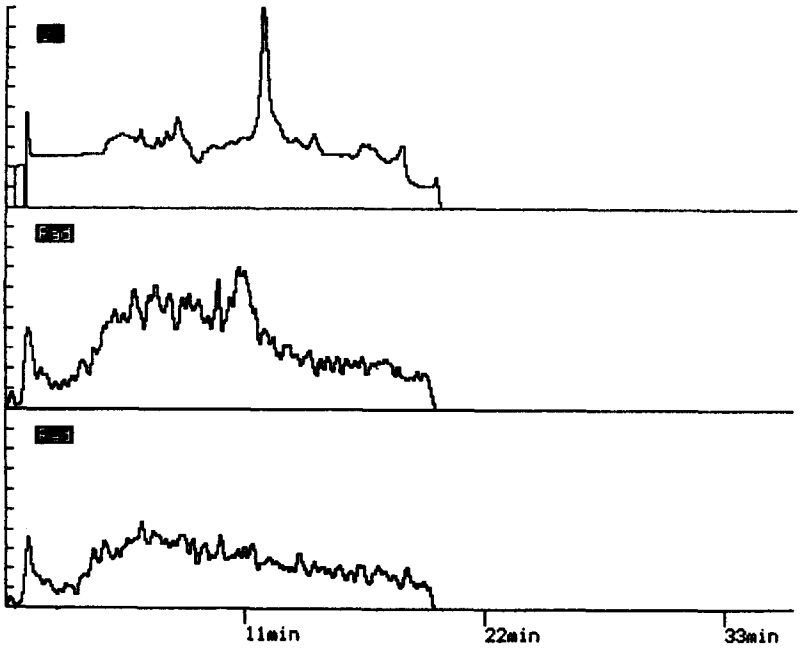

Fig. 1. FPLC anion-exchange chromatography of human pregnancy serum. Serum was diluted 1:10 and incubated with [^{3}H]cortisol (16 nmol/l) with (lower part) or without (middle part) a 200-fold excess of unlabelled cortisol for 2 h at 40°C. After filtration, the samples were injected on to a Mono Q column and eluted as described under Experimental. Protein was monitored at 280 nm (upper part).

containing 5% of β-mercaptoethanol and 20% of SDS. Gradients were calibrated with low-molecular-weight standard proteins (SDS-PAGE) and high-molecular-weight standard proteins. The conditions for all runs and titration curves were chosen according to the manual for the Pharmacia Phast Gel system and a silver staining was also performed according to the manual. Desialylated CBG and SHBG were prepared as described by Hammond *et al.*[20] with neuraminidase (Boehringer, Mannheim, F.R.G.).

RESULTS

Binding of [^{3}H]cortisol and [^{3}H]DHT in human pregnancy serum

Figs. 1 and 2 illustrate the FPLC anion-exchange chromatography of differently labelled human pregnancy serum. Unbound [^{3}H]DHT was eluted with the washing buffer, whereas free [^{3}H]cortisol was eluted only partly with the washing buffer but mainly in the salt gradient. Specifically bound [^{3}H]DHT was detectable at a sodium chloride concentration of 0.15 *M* and specifically bound [^{3}H]cortisol at 0.18 *M* sodium chloride. The protein eluted at 0.2 *M* sodium chloride could be shown to be serum albumin (LC-Partigen albumin immunodiffusion; Behring Werke, Marburg, F.R.G.).

Purification of CBG and SHBG

In order to prevent partial degradation of CBG and SHBG by protease activ-

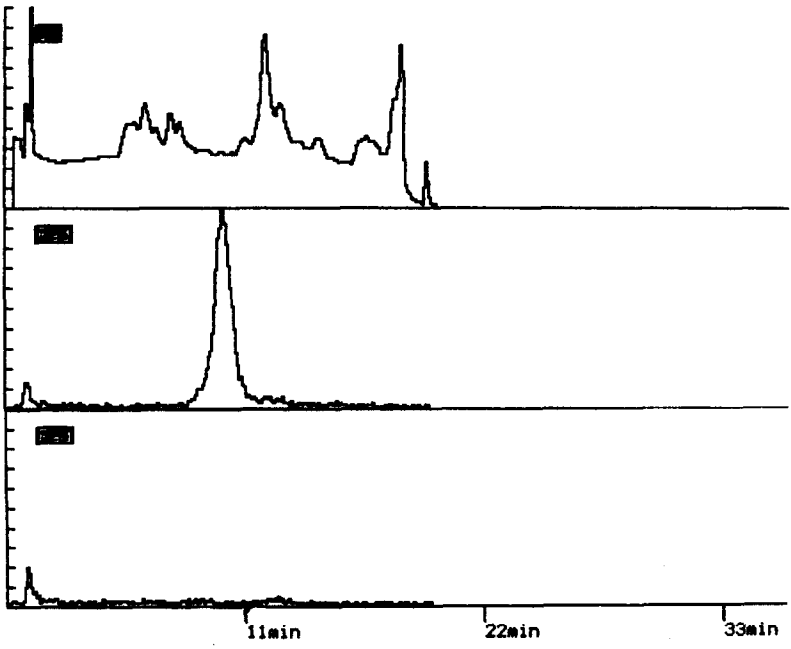

Fig. 2. FPLC anion-exchange chromatography of human pregnancy serum. Serum was diluted 1:10 and incubated with [^{3}H]DHT (16 nmol/l), with (lower part) or without (middle part) a 200-fold excess of unlabelled DHT for 2 h at 4°C. After filtration, the samples were injected on to a Mono Q column and eluted as described under Experimental. Protein was monitored at 280 nm (upper part).

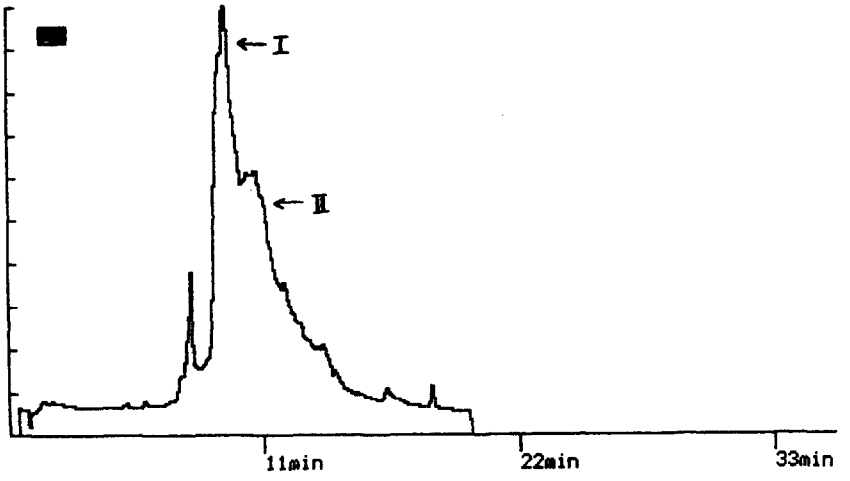

Fig. 3. After incubation with the dissolved 80% pellet from ammonium sulphate precipitation, the affinity gel was incubated with cortisol and a small batch was incubated with [^{3}H]cortisol. After separation from the solids, the two eluates were mixed and injected on to a Mono Q column. Fractions containing bound radioactivity were pooled and further purified.

ity, it was important that the purification be carried out as fast as possible. CBG and SHBG were purified by a combination of affinity chromatography, FPLC anion-exchange chromatography and liquid–liquid partition chromatography. Ammonium sulphate precipitation and affinity chromatography achieved a 100-fold purification of both proteins. Subsequent anion-exchange chromatography, the second chro-

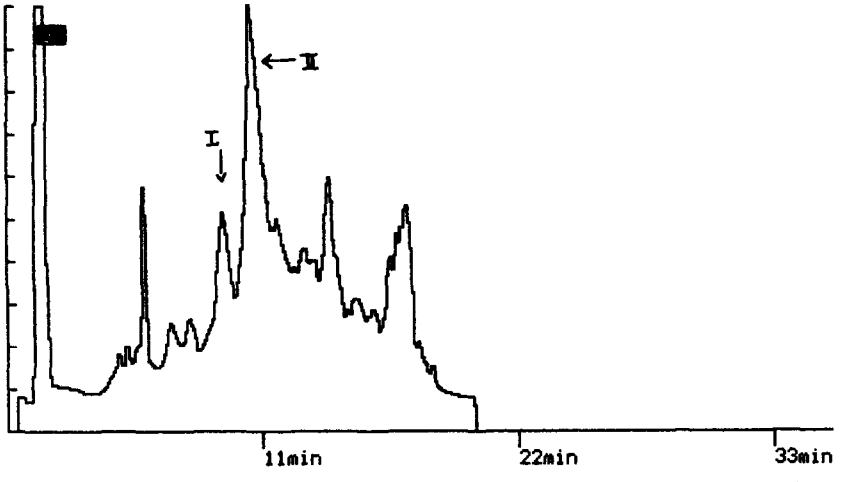

Fig. 4. After incubation with the dissolved 50% pellet from ammonium sulphate precipitation, the affinity gel was incubated with DHT and a small batch was incubated with [^{3}H]DHT. After separation of the solids, the two eluates were mixed and injected on to a Mono Q column. Fractions containing bound [^{3}H]DHT were pooled and further purified.

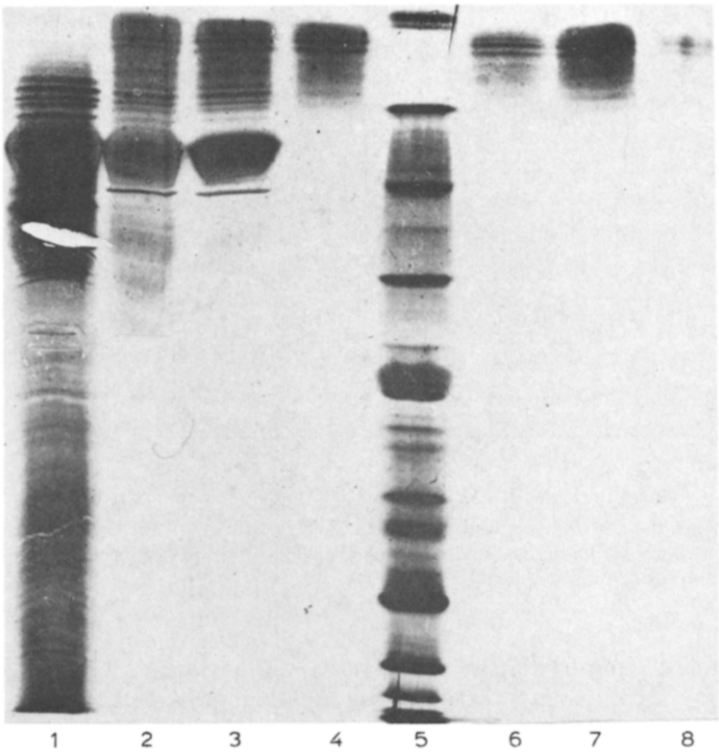

Fig. 5. IEF (pH 3–9) of human pregnancy serum (lane 1), affinity eluate CBG (lane 2), Mono Q eluate CBG (lane 3), fractions from liquid–liquid partition chromatography containing CBG (lanes 4, 6, 7 and 8) and Pharmacia "calibration proteins pH 3–10".

matographic step, resulted in a further 15-fold purification (see Figs. 3 and 4). The recovery was 48% for SHBG and 69% for CBG. After anion-exchange chromatography we could still detect a few contaminants (Figs. 5 and 6), mainly serum albumin. Only after separating the CBG- and SHBG-containing fractions by means of liquid–liquid partition chromatography were no impurities detectable (Figs. 5 and 6). Fig. 7 (top) shows the separation of the fractions with bound [^{3}H]cortisol, eluted from the Mono Q column. All peaks were assayed for their CBG concentration. Peaks I and II contained CBG, as verified by multi-point titration with [^{3}H]cortisol as ligand. Peak III could be identified as serum albumin (LC-Partigen immunodiffusion). Fig. 7 (bottom) shows the analogous separation of the SHBG-containing fractions eluted from the Mono Q column. Multi-point titration with [^{3}H]DHT showed that only peak I contained SHBG. Peak II was identified as albumin. Table I summarizes the purification. A 200-ml volume of serum contained 10400 mg of protein. The concentrations of CBG and SHBG were 0.44 μg/mg (total 4.6 mg) and 0.37 μg/mg (total 3.85 mg), respectively. After the last purification step the total amounts of protein were 2.7 mg (for CBG purification) and 1.55 mg (for SHBG purification, and immunologically 2.8 mg of CBG (recovery 60.9%) and 1.69 mg of SHBG (recovery 43.9%) were detectable. Derived from these data, a 2272-fold purification for CBG and a 2648-fold purification for SHBG were achieved.

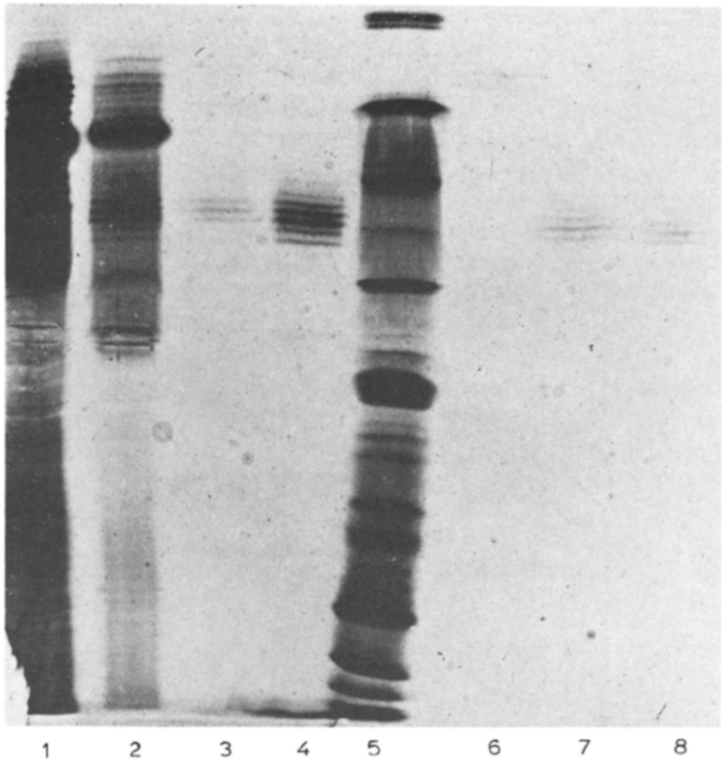

Fig. 6. IEF (pH 3–9) of samples from different purification steps (SHBG): human pregnancy serum (lane 1), affinity eluate (lane 2), fractions from liquid–liquid partition chromatography containing SHBG (lanes 3, 4, 7 and 8) and Pharmacia "calibration proteins pH 3–10".

After removal of PEG and dextran by means of anion-exchange chromatography (Mono Q), the purified proteins were characterized and compared with CBG and SHBG in crude serum. The binding constants for purified CBG and SHBG (CBG, $K_D = 5.86 \cdot 10^{-9}$; SHBG, $K_D = 1.27 \cdot 10^{-9}$) were nearly identical with the binding constants for CBG and SHBG in crude serum (CBG, $K_D = 4.97 \cdot 10^{-9}$; SHBG, $K_D = 1.1 \cdot 10^{-9}$). A comparison of purified CBG, SHBG and human serum albumin by FPLC gel filtration (Fig. 8) shows the similarities of their molecular weights (Stokes radii). The Stokes radii of CBG (37 Å) and SHBG (38 Å) are similar to that of albumin (36 Å). Native PAGE (Fig. 9) reveals a double band for CBG (molecular weight, M_r = 66000–70000) and a single band for SHBG (M_r = 140000). In some of the SHBG preparations an additional band with a molecular weight of 70000 could be observed (data not shown). SDS-PAGE of the same samples after treatment with 20% SDS and β-mercaptoethanol at 100°C is shown in Fig. 10. CBG still appears as a double band with a molecular weight similar to that of albumin (65000–67000). SHBG migrates as a main band (M_r = 45000) and a faint band (M_r = 43000). IEF of native proteins clearly shows the heterogeneity of CBG [Fig. 5, isoelectric point (p*I*) 3.5–4.4] and SHBG (Fig. 6, p*I* 5.2–5.7). Desialy-

TABLE I

PURIFICATION OF HUMAN CBG AND SHBG

Concentrations of CBG and SHBG were determined immunologically.

*Sample**	*Total protein (mg) in preparation of*		*Protein (mg/ml) in preparation of*		*Concentration (μg/mg)*		*Total amount (mg)*		*Recovery (%)*		*Purification (n-fold)*	
	CBG	*SHBG*	*CBG*	*SHBG*	*CBG*	*SHBG*	*CBG*	*SHBG*	*CBG*	*SHBG*	*CBG*	*SHBG*
1	10 400	10 400	52	52	0.44	0.37	4.6	3.85	100	100	–	–
2	2.7	1.55	0.77	0.52	1000	1000	2.8	1.69	60.9	43.9	2272	2648

* 1 = Human pregnancy serum; 2 = last purification step.

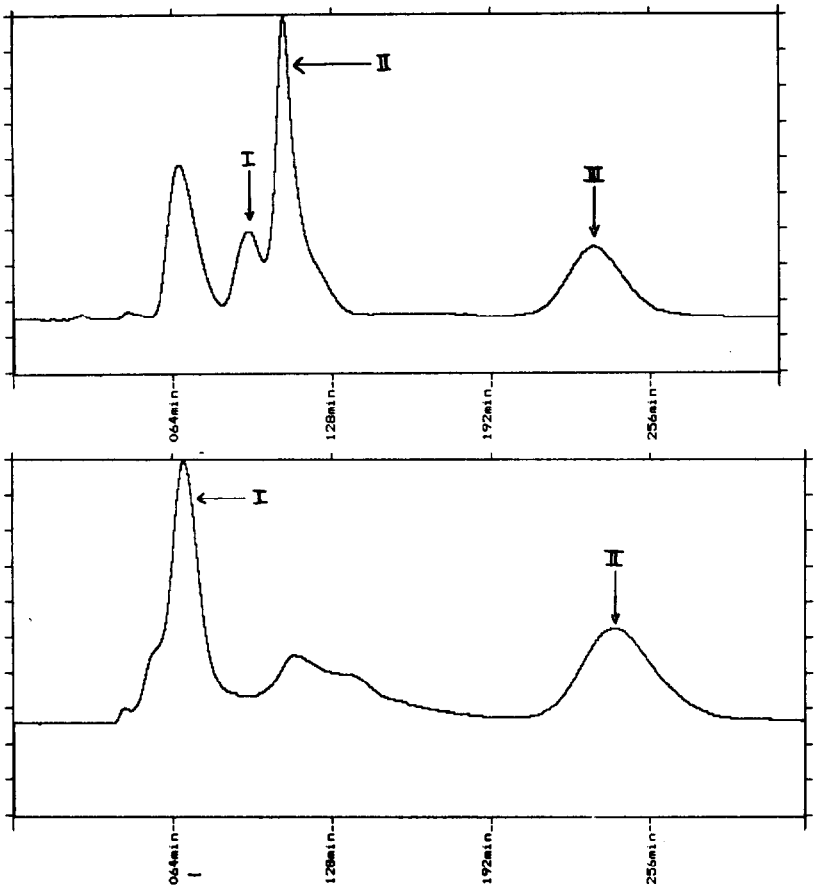

Fig. 7. Fractions eluted from the Mono Q column with bound [^{3}H]cortisol (top, CBG purification) or with bound [^{3}H]DHT (bottom, SHBG purification) were pooled, concentrated and further purified by liquid–liquid partition chromatography.

lation of purified CBG and SHBG led to different p*I*s (CBG, 5.0–5.9; SHBG, 5.4–6.0), but they still showed the same heterogeneity, with at least 14 different bands for CBG (see Fig. 11). The electrophoretic titration curves reflect the elution behaviour of SHBG, CBG and albumin in anion-exchange chromatography (data not shown).

DISCUSSION

One of the basic conditions for a successful purification of proteins, present in small amounts, is a highly specific and fast purification method based on affinity chromatography. The affinity gel must satisfy the following demands: (1) the ligand coupled with the gel matrix must interact in a highly specific manner with the acceptor site of the protein and (2) the linkage of the ligand with the gel matrix must be stable and covalent, so that the ligand cannot be separated during the incubation of the affinity gel with a crude protein mixture.

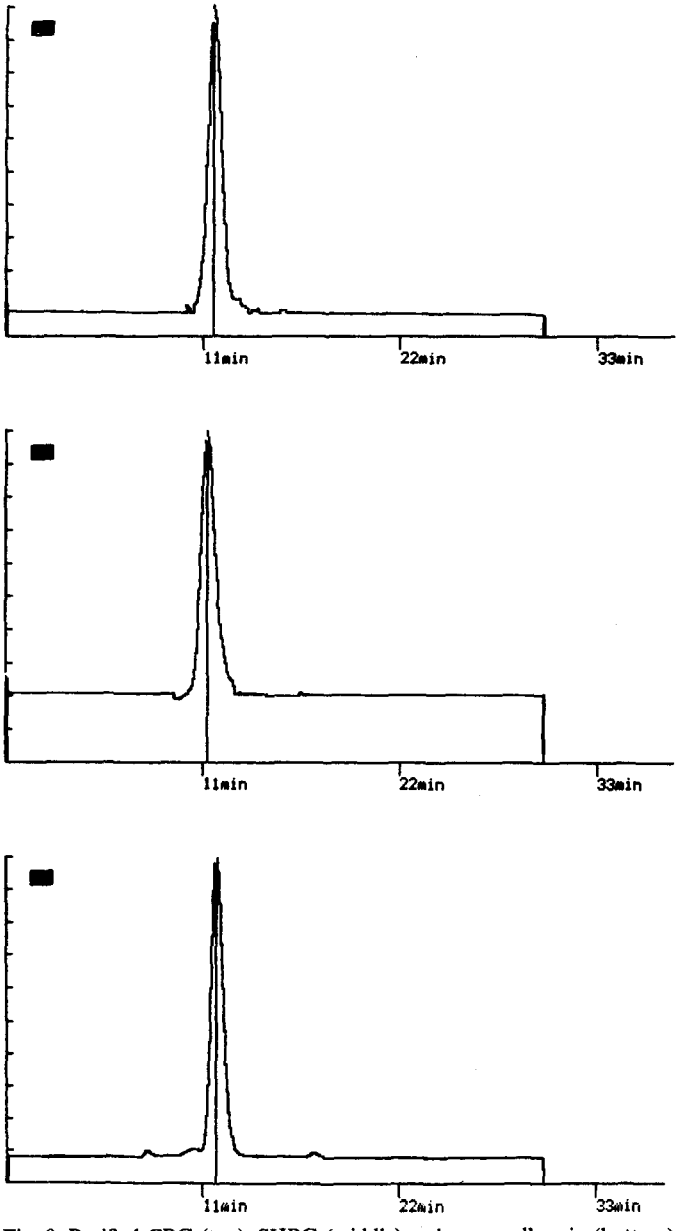

Fig. 8. Purified CBG (top), SHBG (middle) and serum albumin (bottom) analysed by gel filtration on a Superose 12 column. The column was eluted as described under Experimental. The cursor shows the peak maximum at 280 nm; top: 11 min 35 s, middle: 11 min 15 s, bottom: 11 min 45 s.

We used tresyl-activated Sepharose 4B as gel matrix. Tresyl-activated Sepharose allows easy and reproducible coupling and chemically stable bonds, which makes the gel useful for low-molecular-weight ligands. A 5-g amount of gel (wet weight) bound more than 95% (5 mg) of the protein that had to be purified. The affinity gel

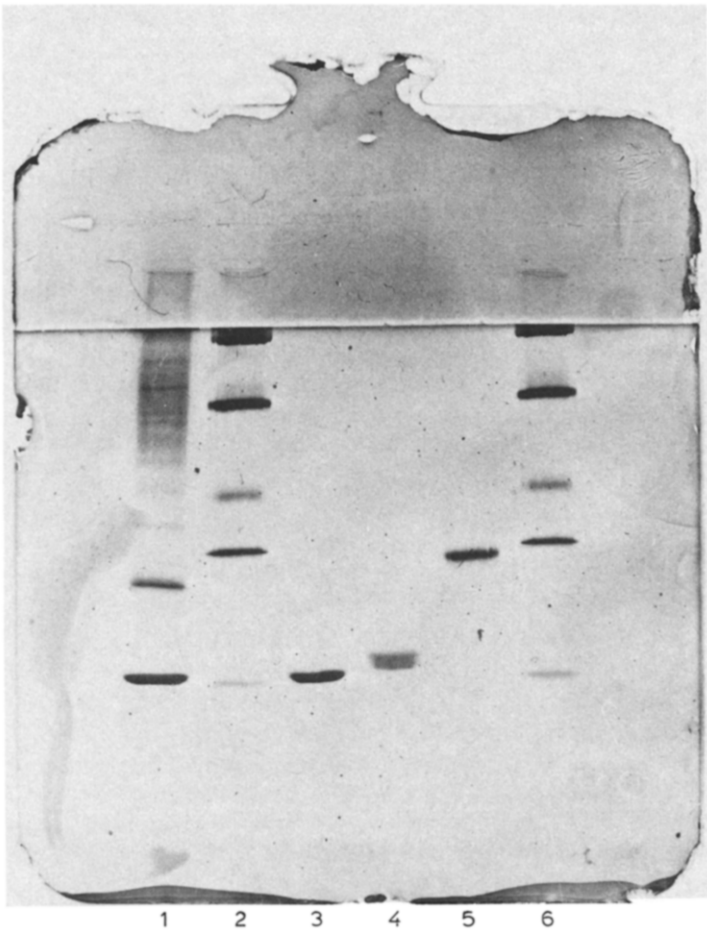

Fig. 9. Native-PAGE (8–25% PAA) of purified proteins. Human pregnancy serum (lane 1), Pharmacia "high-molecular-weight standard proteins" (lane 2), human serum albumin (lane 3), CBG (lane 4), SHBG (lane 5) and standard proteins (lane 6).

can be recycled more than five times without a significant loss of binding activity. It is stable against short washes with 1 *M* sodium hydroxide solution, 50% acetic acid and 100% ethanol. Many methods have been described for the purification of SHBG and CBG[12–14,20–26] on the basis of affinity chromatography with different types of columns, used as the second purification step, *e.g.*, hydroxylapatite, anion exchangers, Cibachron Blue Sepharose, gel filtration and preparative isoelectric focusing as a second or third purification step. Most purification procedures lead to an apparent homogeneity with a molecular weight, determined by SDS-PAGE, of 60 000–70 000 for CBG (double band) and 40 000–52 000 for SHBG (either a single or double band). We obtained results by SDS-PAGE that are in accord with those reported by other groups. The results obtained by native-PAGE (M_r 140 000 for SHBG and 66 000–70 000 for CBG) are in reasonable agreement with previous estimates (90 000–115 000 for SHBG and 52 000–71 000 for CBG)[6–10,13,22,26,27]. In some preparations of SHBG,

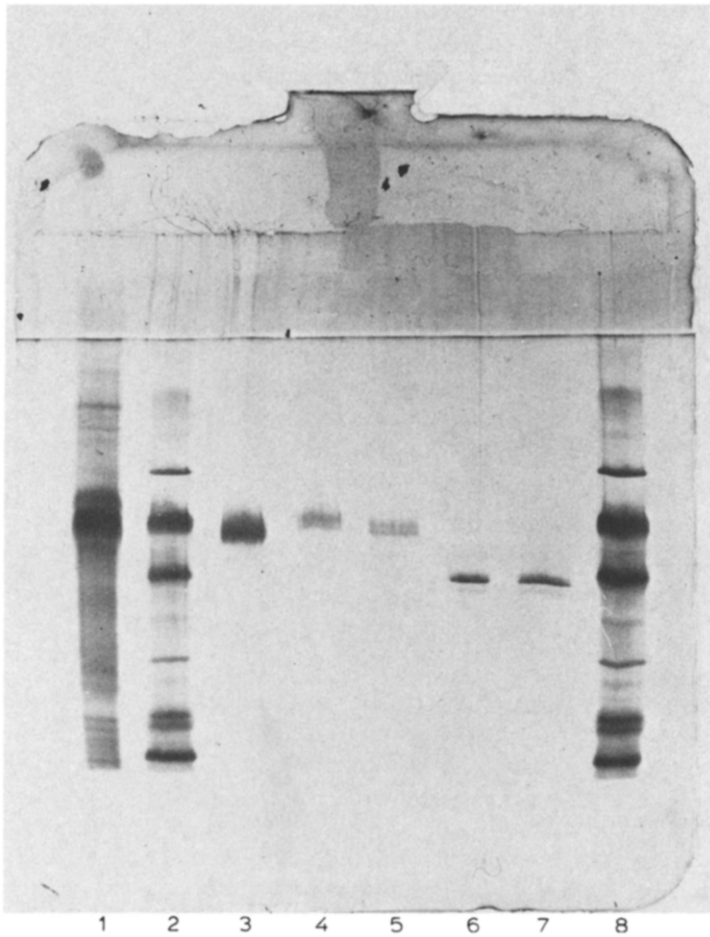

Fig. 10. SDS-PAGE (10–15% PAA) of purified proteins. Human pregnancy serum (lane 1), Pharmacia "low-molecular-weight standard proteins" (lane 2), human serum albumin (lane 3), CBG (lanes 4 and 5), SHBG (lanes 6 and 7) and standard proteins (lane 8).

we observed a second band with a molecular weight of 70 000, which could be demonstrated only by silver staining. Various explanations for this band are possible:

(1) This band could be connected with the observation of the light protomer chain (M_r 43 000), first described by Cheng *et al.*[22], in SDS-PAGE. This would mean that the M_r 43 000 band in SDS-PAGE is derived from the M_r 70 000 band in native-PAGE.

(2) The M_r 70 000 band could represent one component of the M_r 140 000 protein. This would agree with our gel filtration results, which showed only a protein with a molecular weight of 70 000. From this it could be concluded that the M_r 140 000 protein is an aggregate of two smaller molecules.

(3) The M_r 70 000 protein could be a contaminant. This is unlikely, based on the results of SDS-PAGE and IEF.

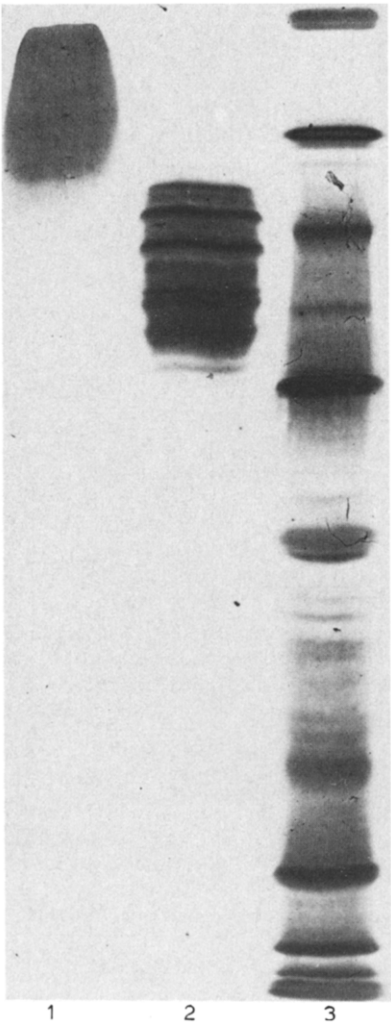

Fig. 11. IEF (pH 3–9) of CBG (lane 1), desialylated CBG (lane 2) and Pharmacia "calibration proteins pH 3–10" (lane 3).

In this study, we observed a microheterogeneity of CBG and SHBG which had not been described before. IEF shows a diffuse pattern for CBG over a wide pH range. Treatment of CBG with neuraminidase led to at least 14 bands at higher pH. In SHBG we observed at least six different bands at pH 5.2–5.7 which migrated in a pH range from 5.4 to 6.0 after desialylation, but still showed heterogeneity. The microheterogeneity pointed out in this paper is probably due to the purification procedure. We could obtain apparent homogeneity of the purified proteins using only those one or two fractions of the Mono Q eluate with maximum bound radioactivity, but as the purified molecules are so heterogeneous with respect to their charge and carbohydrate content, a loss of protein and microheterogeneity had to be accepted. Liquid–liquid partition chromatography, our third purification step, allows a clear

separation of SHBG and CBG from other proteins, mainly albumin. Therefore, we could use all fractions eluted from the Mono Q column showing steroid binding activity for further purification.

Aqueous two-phase systems have been used successfully for measuring the binding of steroids to SHBG[28–31]. In preliminary experiments, different aqueous two phase systems were used for the determination of the partition coefficient of the proteins that had to be separated (data not shown). These data were important in establishing the composition of the lower and upper phases used in chromatography.

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