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ION EXCHANGE, CHROMATOFOCUSING AND SIZE EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE HUMAN UTERINE PROGESTERONE RECEPTOR

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Summary—The human uterine progesterone receptor was subjected to high-performance liquid chromatography on size exclusion, anion exchange and chromatofocusing columns. For the rapid isolation of the receptor, recovery of [^{3}H]progesterone as well as protein from the columns was essential. The size exclusion columns (G2000 SW and G3000 SW) as well as Mono P HR 5/20 chromatofocusing column adsorbed [^{3}H]progesterone and thus were not useful for separation purposes. The anion exchange (polyanion SI-17) and chromatofocusing columns, AX500, and IEX 540 DEAE gave very good recoveries of protein (>90%) and [^{3}H]progesterone; 80, 66 and 88% respectively. These columns gave rapid and reproducible separation of the progesterone receptor from other cytosol proteins.

INTRODUCTION

Size exclusion high-performance liquid chromatography (HPLC) has been utilized to characterize estrogen and progesterone receptors [1, 2], and chromatofocusing HPLC techniques have been used to analyze estrogen receptor heterogeneity [3]. These methods offer considerable advantages in being able to resolve receptor forms within short periods of time with respect to conventional chromatography. HPLC is also characterized by improved resolution and high reproducibility. The aim of this report was to analyze different preparative HPLC columns which might be practical and efficient in isolating the progesterone receptor.

EXPERIMENTAL

Materials

[1,2-^{3}H]progesterone (52 Ci/mmol) was purchased from Amersham, Arlington Heights, IL. Polyanion SI-17 μm was obtained from Pharmacia, Uppsala, Sweden. Radioinert steroids were purchased from Sigma Chemical Co., St Louis, MO.

Preparation of cytosol

Human uterine specimens were diced, minced and then homogenized with 4 vol (w/v) of TETG buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 12 mM monothioglycerol and 10% v/v glycerol) in a Polytron (Brinkman) for three 5 s pulses. All procedures were at 4°C. The resulting homogenate was centrifuged at 10,000 *g* for 10 min and the supernatant further centrifuged at 100,000 *g* for 60 min. The cytosol preparation was then chromatographed on phosphocellulose (Whatman P-11). Three volumes of cytosol were applied to 1 vol of phosphocellulose (previously washed with 4 vol of buffer) and the drop through fraction collected; this procedure removed proteases from the preparation [4]. The phosphocellulose drop through was labeled by incubation with 20 nM [^{3}H]progesterone plus 2 μM cortisol in the presence or absence of 2 μM radioinert progesterone where indicated in the Results for 1–24 h at 4°C. The incubation was terminated by removing unbound steroid using a pellet derived from an equal volume of a 0.5% Dextran-coated charcoal suspension. The labeled cytosol was added to the charcoal pellet, vortexed, incubated for 5–10 min at 4°C and then centrifuged at 10,000 *g* for 10 min. This receptor preparation was then applied directly to the HPLC columns.

HPLC chromatography

Chromatography was performed on a Beckman Instruments Model 342 HPLC at 4°C. All buffers were filtered through a 0.2 μM filter before use. Size exclusion chromatography was performed on a Spherogel-TSK-G2000 SW column (7.5 × 300 mm, Beckman Instruments) and G3000 SW column (7.5 × 300 mm) in line fitted with a TSK-precolumn. Sample application was through a 1 ml sample loop. Isocratic elution was with 0.2 M phosphate buffer, pH 7.4 and 10% glycerol v/v.

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Chromatofocusing was performed on SynChropak AX500 (4.6 × 250 mm, Synchrom, Inc., Linden, IN), Bio-Sil TSK IEX 540 DEAE (5 × 300 mm, Bio-Rad, Richmond, CA) or Mono P HR 5/20 (Pharmacia, Uppsala, Sweden). The columns were equilibrated to pH 7.5 with 25 mM Tris–HCl, 10% glycerol v/v and the proteins eluted at 1.0 ml/min using Polybuffer 74 (Pharmacia) diluted 1:10 with 10% glycerol v/v, pH 4.0.

Anion exchange chromatography was performed on Polyanion S-17 packed in a 600 × 10 mm column. The column was equilibrated with TETG buffer pH 7.0 and cytosol (30–40 ml) was applied by an Altex 6-way valve. Proteins were eluted with a gradient of TETG buffer, pH 7.0 with 0.5 M NaCl.

Post-column sample collection was made with a Pharmacia FRAC 100 fraction collector and protein was measured by the method of Bradford[5].

Photoaffinity labeling

Preparations were incubated with 10 nM [^{3}H]R5020 and 1 μM cortisol with or without 1 μM progesterone for 2–18 h at 4°C. Prior to irradiation excess free steroid was removed by treating the cytosol with a pellet derived from an equal volume of dextran-coated charcoal (0.5% charcoal, 0.05% dextran). The sample was then placed in a Pyrex tube in a 4°C water bath and irradiated with a 100 W mercury-vapor immersion lamp (Ace Glass, Vineland, NJ) enclosed in a water-jacketed photochemical immersion well. The sample was 5 cm from the light source and irradiation was for 20 min.

The cytosol preparation was then subjected to sodium dodecylsulfate gel electrophoresis as described by Laemmli[6]. The running gels used contained 10% acrylamide and 0.27% *N,N*-methylene-*bis*-acrylamide (*bis*); the stacking gel contained 4.5% acrylamide and 0.12% *bis*. The gels used were 3 mm thick and 200 μg protein per lane was loaded. The molecular weight markers used were supplied by Bio-Rad (Richmond, CA) and visualized by staining with Coomassie brilliant blue. Individual lanes were sliced into 2 mm fractions and 0.5 ml NCS tissue solubilizer added and heated for 2 h at 55°C. The samples were counted in 5 ml scintiverse (Fisher Scientific, Pittsburgh, PA) after equilibrating for 2 days in the dark at RT. The efficiency of photoaffinity labeling was 2–5%.

RESULTS AND DISCUSSION

Size exclusion HPLC

Size exclusion HPLC revealed two peaks of specifically labeled progesterone receptor (PR) from cytosol preparations (Fig. 1). One form of the PR eluted at or near the void volume of the column and the other at 28 K. A similar profile was reported by Pavlik *et al.*[1] for the PR in breast tumor cytosol. However, the recovery of macromolecular bound [^{3}H]progesterone decreased with each successive use of the column, from 62% (Fig. 1) to 8% on the third chromatographic separation. Addition of 1 nM [^{3}H]progesterone to the columns resulted in only a 1–5% recovery of the ^{3}H applied, the remaining activity could be recovered with a methanol wash of the column. Inclusion of an excess of cortisol (1 μM) in the column buffer did not improve recovery. Also, addition of 1% propanol (which does not inhibit progesterone binding to its receptor) to the column buffer did not increase the recovery. The yield of protein from the column was >90%. Post-labeling of the eluted fractions revealed the same profile as shown in Fig. 1 (data not shown). However, because [^{3}H]progesterone preferentially adsorbed to the column, this procedure was not further utilized as it would necessitate post-labeling to accurately quantitate progesterone receptor fractions, and the time involved would not make size-exclusion HPLC beneficial to an isolation protocol. In addition, Hutchens *et al.*[7] have reported significant differences in the apparent size/shape distribution of estrogen binding proteins when comparing Sephacryl S-300 open-column size exclusion with high-performance size-exclusion chromatography on TSK 3000SW columns.

Anion exchange HPLC

Volumes of 30–40 ml of cytosol (approx 100 mg protein) are routinely chromatographed on the polyanion SI-17, protein recoveries were >90%. The average recovery of [^{3}H]progesterone from a cytosol preparation was 80 ± 7% from 7 chromatographic

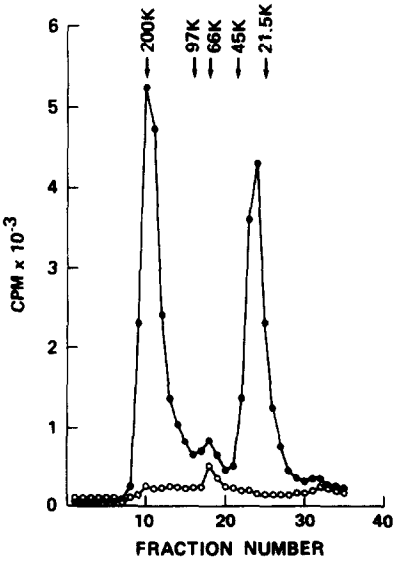

Fig. 1. Size exclusion HPLC of the progesterone receptor. Chromatography was performed on Sepherogel TSK-G2000 SW and G3000 SW columns in line with a TSK-precolumn. Elution buffer was 0.2 M phosphate buffer, pH 7.4 and 10% glycerol. Flow rate 0.5 ml/min, fraction size 1.0 ml and 0.5 ml cytosol applied. [^{3}H]progesterone (20 nM) without (●) or with (○) 2 μM progesterone; recoveries were 64 and 42% respectively.

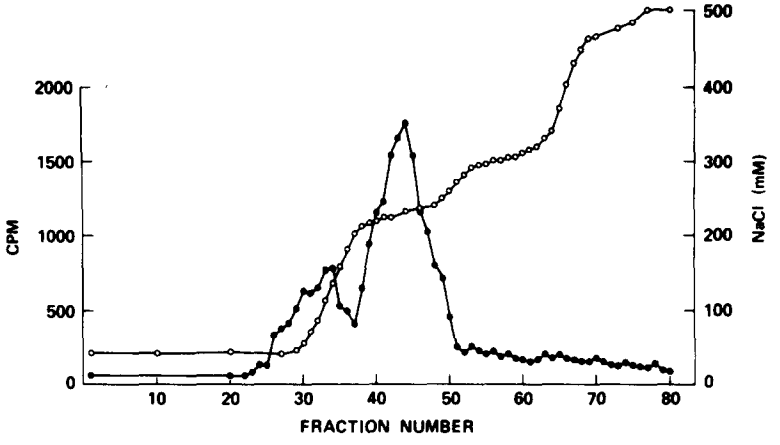

Fig. 2. Anion exchange HPLC of the progesterone receptor. Chromatography was performed on a 60 × 1 cm polyanion SI-17 column. Recovery of [^{3}H]progesterone was 85%. Column equilibration buffer was TETG pH 7.0 (B) and proteins were eluted with a gradient of TETG pH 7.0 with 0.5 M NaCl (A). Program; 80% B in 5 min hold 25 min; 60% B in 10 min hold 20 min; 40% B in 10 min hold 20 min; 0% B in 10 min, hold. Thirty ml of cytosol was applied, flow rate 1.5 ml/min and fraction size 3.0 ml (cpm of an 0.2 ml aliquot of each fraction ●——●; NaCl ○——○).

runs. Free [^{3}H]progesterone appears in the void volume of the column. The progesterone receptor eluted as a sharp peak at 220 mM NaCl, Fig. 2. Photoaffinity labeling of this fraction revealed 2 molecular weight forms of the receptor of 120 and 84 K (Fig. 3). The proportion of the 120 K and 84 K PR's does vary from each preparation, unlike other PR's [8, 9]. Another peak of macromolecular bound [^{3}H]progesterone also elutes at 65–100 mM NaCl, corresponding to the position albumin elutes from the column. However, when this fraction was photoaffinity labeled there was also evidence of the 120 and 84 K receptors being present (data not shown).

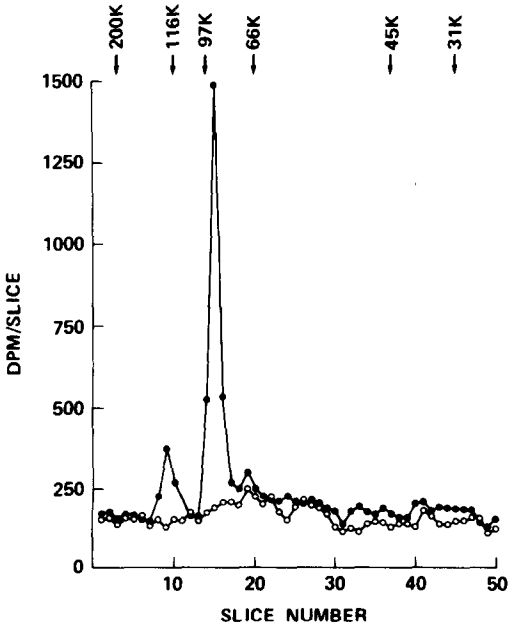

Fig. 3. Photoaffinity labeling of the 220 mM peak from Fig. 2 10 nM [^{3}H]R5020 without (●) or with (○) 1 μM progesterone.

Chromatofocusing HPLC

Initial experiments were performed on the Mono P column. However, the same problems encountered with the size exclusion columns occurred. Although the receptor eluted at pH 5.09 the recovery of [^{3}H]progesterone was very low (20% first run) and diminished with every chromatographic run. The [^{3}H]progesterone adsorbs to this column material in preference to the receptor, thus other columns were sought. With the AX 500 column (Fig. 4) protein recovery was >90% and recovery of [^{3}H]progesterone was 66 ± 10% (n = 7). The free [^{3}H]progesterone elutes in the void volume, fractions 6–8. The progesterone receptor eluted at pH 6.95, range 6.90–7.0. Utilization of the IEX 540 DEAE column

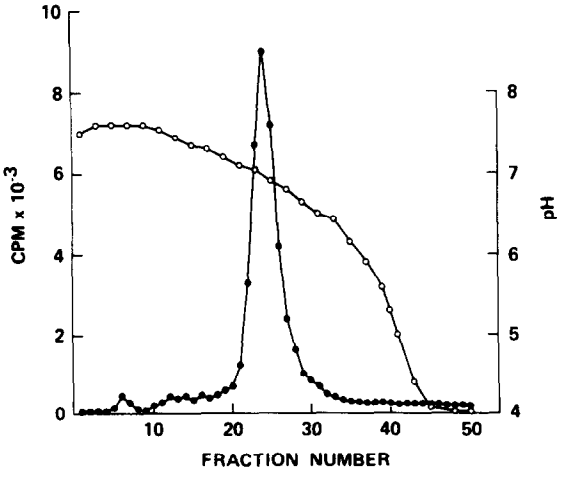

Fig. 4. Chromatofocusing of the progesterone receptor on AX 500. A 1.0 ml sample was applied, starting buffer 25 mM Tris–HCl, pH 7.5, 10% glycerol, elution buffer 1:10 dilution of polybuffer, pH 4.0. Flow rate 1.0 ml/min, fraction size 1.0 ml, [^{3}H]progesterone recovery 62%. (cpm of a 0.2 ml aliquot of each fraction ●——●; pH ○——○).

Table 1. Purification of the human progesterone receptor by HPLC

Purification step	Total protein (mg)	Specific activity (cpm/mg)	Recovery of counts (%)	Purification (fold)
Cytosol	180	21.6×10^3	100	1
Polyanion S-17 HPLC	8.2	40.3×10^4	70	19
IEX 540 DEAE	0.23	90.7×10^5	95	420

for chromatofocusing also gave protein recoveries of >90%; the receptor eluted at pH 6.95 and the recovery of [^{3}H]progesterone was 86 and 90% from the two chromatographic separations. The polyanion SI-17 packing material can also be used in a chromatofocusing mode. However, the polybuffer concentration had to be increased to a 1:5 dilution to achieve a pH gradient from 7.5 to 4.0. Photoaffinity labeling of the receptor peak from the AX 500 column (Fig. 4) revealed the 120 and 84 K mol. wt forms of the PR (data not shown).

In conclusion, the anion exchange column can be utilized to process 40 ml of cytosol with a turn around time of 4 h. The recovery of protein and [^{3}H]progesterone is very good. Similarly the AX 500 and IEX 540 DEAE columns for chromatofocusing gave rapid and reproducible recovery of both receptor and [^{3}H]progesterone. In this preliminary report purification of the receptor from both anion exchange and chromatofocusing HPLC was 420-fold (Table 1). The polyanion S-17 anion exchange column and the IEX 540 DEAE column for chromatofocusing proved to be the best combination for both recovery of counts and protein.

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