Journal of Chromatography, 327 (1985) 237–246
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 567

HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF STEROID HORMONE RECEPTORS

SALMAN M. HYDER, RONALD D. WIEHLE, DAVID W. BRANDT and JAMES L. WITTLIFF*
Hormone Receptor Laboratory, Department of Biochemistry, James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40292 (U.S.A.)

SUMMARY

The use of high-performance hydrophobic-interaction chromatography (HPHIC) on SynChropak 500 propyl columns has been evaluated for the first time in the analysis of estrogen receptors labeled with [^{125}I]iodoestradiol-17β. These receptors were extracted from reproductive tissues with 500 m*M* phosphate buffer and applied to the stationary phase. Utilizing an inverse phosphate gradient (500 to 10 m*M*), elution resulted in rapidly excluded components in the void volume followed by a second radioactive peak at 400 m*M* phosphate. Both peaks appeared to contain specific estrogen-binding components in that steroid association was inhibited by diethylstilbestrol and free ligand was eluted with a different retention time. A great deal of [^{125}I]iodoestradiol-17β was retained by the column. Inclusion of acetonitrile (20%) in the mobile phase resulted in the elution of [^{125}I]iodoestradiol–receptor complexes at a different position from free ligand. Distribution of specific estrogen-binding components appeared to be tumor-dependent. These preliminary results indicate that HPHIC may be useful for isolating various isoforms of steroid hormone receptors so that detailed information regarding their intrinsic properties may be ascertained.

INTRODUCTION

To understand the molecular mechanisms of steroid hormone actions considerable emphasis has been placed on the purification of the receptor proteins responsible for their physiologic effects. Some success has been achieved in this respect (*e.g.*, refs. 1–4). The majority of current procedures are lengthy and lead to a considerable overall loss of receptor during the purification process, presumably due to degradation or ligand dissociation[4]. In addition, analysis of purified estrogen receptors by centrifugation followed by DEAE-cellulose chromatography (*e.g.*, ref. 5) or by sodium dodecyl sulfate–polyacrylamide gels[6,7] indicated a heterogeneous population of receptors. The latter point has been supported by the finding that steroid receptors exhibit polymorphism, as demonstrated by high-performance liquid chromatography (HPLC) in a variety of modes[8–12]. These HPLC methods include size-exclusion[9], ion-exchange[10], and chromatofocusing[8]. These procedures are rapid, efficient, and reproducible, giving recoveries of 75–100%[4].

0021-9673/85/$03.30

Recent reports have demonstrated the usefulness of high-performance hydrophobic-interaction chromatography (HPHIC) in the separation of proteins based on their respective hydrophobicity[13–15]. Using this method, one should be able to separate different populations of estrogen receptors, assuming that these differ in their surface hydrophobicity. This should aid in the purification of the various isoforms of sex hormone receptors. The hydrophobic nature of the estrogen receptor from various sources has been documented previously by open column chromatography[16,17]. A recent report has indicated separation of two forms of previously undetected human complement factor H, based on their hydrophobic properties[18].

We have conducted preliminary experiments with human breast cancer and reproductive tissues, using soluble estrogen receptors and the mildly hydrophobic SynChropak 500 propyl column in the HPLC mode. For these studies the receptor was extracted with buffers, containing 500 m*M* potassium phosphate. Such a high-ionic-strength buffer would favor the extraction of receptor from both nuclear and cytoplasmic compartments of the cell, dissociate receptor into its various subunits, minimize non-specific interactions and favor the interaction of the protein isoforms with the hydrophobic column matrix. Reverse salt-gradients were then used in an attempt to release the receptor from the column. This paper describes our preliminary results and some of the problems encountered and suggests alternative approaches which may prove useful in future studies.

EXPERIMENTAL

Materials

The ligand, [16α-^{125}I]iodoestradiol-17β (1500–2200 Ci/mmol), was obtained from New England Nuclear/DuPont (Boston, MA, U.S.A.). Disodium ethylenediaminetetraacetic acid (EDTA) and glycerol were purchased from Fisher Scientific (Louisville, KY, U.S.A.). Unlabelled diethylstilbestrol which was used as an estrogen inhibitor, Norite A, Dextran T-70, and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). Fisher Scientific provided the organic solvents used in the HPLC experiments.

Human breast tumor and uterine tissue from patients were provided by the various surgeons and pathologists at the local hospitals, cooperating with the Hormone Receptor Laboratory. The tissues were brought to the laboratory on dry ice and were kept frozen at −86°C until analyzed. Only residual tissue from clinical receptor analyses was used in this study.

Preparation and radioactive labeling of soluble estrogen receptors

All procedures in this section were carried out at 4°C. Human tissues were homogenized at 300–400 mg/ml in P_{500}EDG (500 m*M* phosphate, 1.5 m*M* EDTA, 1 m*M* DTT and 10%, v/v, glycerol) in two 10-sec bursts from a Brinkmann Polytron homogenizer (Westbury, NY, U.S.A.). The same buffer was used for homogenization, since the starting mobile phase for hydrophobic-interaction chromatography must be of high ionic strength in order to promote interaction of receptor with the hydrophobic column.

Soluble fractions were prepared by centrifugation of the homogenate for 30 min at 50 000 rpm in a Beckman Ti 70.1 rotor (Palo Alto, CA, U.S.A.). Supernatant

was then carefully removed, avoiding the layer of fat at the top. The soluble fractions were then labeled with 2–3 n*M* [16α-^{125}I]iodoestradiol-17β in the presence and absence of a 200-fold excess diethylstilbestrol for 2–4 h at 4°C. The reaction was terminated by removing unbound steroid in a pellet, derived from dextran-coated charcoal suspension (1% charcoal, 0.5% dextran). The labeled cytosol was applied to the charcoal pellet, mixed, and allowed to stand for 5 min at 4°C. Dextran-coated charcoal was then removed by centrifuging the sample for 5 min at 1000 *g*. Cytosol protein concentrations were determined by the method of Waddell[19], using bovine serum albumin as the standard. The protein concentrations generally ranged from 8 to 12 mg/ml.

HPLC in the hydrophobic-interaction mode

All chromatographic procedures were performed at 4°C in a Puffer-Hubbard (Ashville, NC, U.S.A.) cold box. Buffers were filtered under vacuum through Millipore 0.45-μm HAWP filters (Bedford, MA, U.S.A.) before use. Filters of 0.5-μm pore size (Type FHLP, also from Millipore) were substituted when buffers containing organic solvents were filtered. Free steroid or estrogen-receptor complexes were applied to the SynChropak 500 propyl (250 × 4.6 mm I.D.) hydrophobic column (SynChrom, Linden, IN, U.S.A.) with an Altex Model 210 sample injection valve. Elution was carried out with a Beckman 112 solvent delivery module, including a Model 421 system controller.

Several combinations of buffers were tested, and two different column elution programs were used. The column was equilibrated with the high-ionic-strength buffer (P_{500}EDG) and eluted with a reverse salt gradient (500 to 10 m*M* phosphate). Initially, a long program (105 min) of the reverse phosphate gradient was used with a flow-rate of 0.2 ml/min to allow greater contact time of the receptor with the stationary phase. After 10 min, the flow-rate was increased to 1 ml/min for the next 75 min, during which the reverse salt gradient was developed. The column was washed for 20 min with a similar buffer, except that the phosphate concentration was 10 m*M*. Later in the study, the elution program time was reduced to 60 min, consisting of an initial flow-rate of 1 ml/min for 5 min and then a descending salt gradient elution for 20 min. At the end of the elution program, the column was washed with either water or P_{10}EDG buffer (10 m*M* phosphate, 1.5 m*M* EDTA, 1 m*M* DTT, and 10%, v/v, glycerol) for an additional 35 min. This latter step was followed by washing of the column with methanol–P_{10}EDG buffer (50:50). Eluted steroid (free and protein-bound) was collected as 1-ml fractions and detected radiometrically in a Micromedics 4/600 gamma counter (Rohm & Haas, Cleveland, OH, U.S.A.). The counting efficiency was 65%.

RESULTS AND DISCUSSION

Past experience has indicated that it is important to characterize the behavior of unbound steroid ligands on the various HPLC columns in order to ascertain steroid–receptor complexes. For our initial studies, we used an elution programme of 105 min (see Experimental). The starting buffers, all pH 7.4, were of high ionic strength and included P_{10}EDG buffer containing either 3 *M* or 1 *M* potassium chloride and P_{500}EDG with either 10 or 40% glycerol. The final buffer concentration

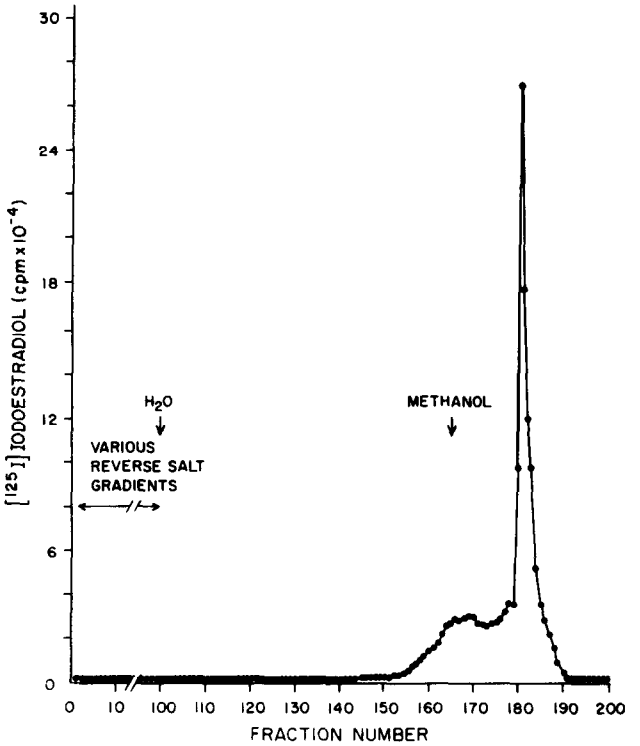

Fig. 1. Behavior of free [^{125}I]iodoestradiol-17β on SynChropak 500 propyl column. The column was developed for 105 min with various reverse salt gradients, as described in the Experimental section. The reverse salt gradients evaluated during the first 100 min included (a) P_{10}EDG buffer–glycerol (90:10) containing either 1 *M* or 3 *M* potassium chloride, and (b) P_{500}EDG buffer with either 10% or 40% glycerol. After chromatography, the column was washed with water, and further elution was carried out with methanol. The flow-rate was 1 ml/min, and the recovery of [^{125}I]iodoestradiol-17β was 100%.

was always P_{10}EDG, containing 10 or 40% glycerol, depending on the nature of the experiment (low-ionic-strength buffers).

Fig. 1 shows the behavior of unbound [^{125}I]iodoestradiol-17β under these conditions when the SynChropak 500 propyl column was used. When the various reverse salt gradients were applied, no steroid was released. Furthermore, only a small quantity of steroid was released when the column was washed with either water or P_{10} buffer for 1 h. A 100% recovery of steroid was achieved when the column was washed with methanol. These data suggest that if labeled steroid was observed in elution fractions obtained during reverse salt gradients, they should be attributed to protein-bound [^{125}I]iodoestradiol-17β.

When preparations of estrogen–receptor complexes were applied to the propyl 500 column, no labeled steroid, either free or protein-bound, was eluted with reverse phosphate gradients in the 105-min program. We could not ascertain whether (1) the stationary phase was stripping the labeled steroid from the receptor allowing the unliganded species to be eluted, (2) the steroid–receptor complexes were tightly bound to the hydrophobic column, or (3) the receptor protein was denatured, releasing the

steroid to interact with the column matrix as a result of the high pressure used in this method.

The high pressure does not seem to be the cause, since, following other modes of HPLC, virtually all the receptor activity was recovered[4,8–12]. In this respect, other modifications of the gradient were tested, including a combination of descending salt concentration and ascending glycerol concentrations. In all cases, no release of protein bound steroid was observed during the elution time.

We reasoned that if the steroid was dissociated from the receptor by interaction with the hydrophobic column during chromatography, perhaps a shorter program, consisting of a shorter reverse salt gradient, would assist in the isolation of receptor isoforms on the basis of their hydrophobicity.The elution program was reduced to 60 min (see Experimental) with a reduction in the initial contact and washing times. The free [^{125}I]iodoestradiol behaved somewhat differently under these conditions (Fig. 2). Again no free steroid was eluted during the reverse salt gradient, which took approximately 30 min. However, immediately after the gradient, labeled iodoestradiol-17β began to be eluted gradually. Thus we established that, if any [^{125}I]iodoestradiol radioactivity appeared during the reverse salt gradient when a receptor preparation was applied to the SynChropak 500 propyl column, it should be attributed to receptor-bound steroid. Our preliminary investigations indicated that the behavior of [^{3}H]estradiol-17β is different from that of iodoestradiol-17β in that approximately 70% of the free steroid was relased within the gradient elution program with a peak at fraction 20 under the same conditions as shown in Fig. 2. This indicates that the iodoestradiol-17β molecule is more hydrophobic than native estra-

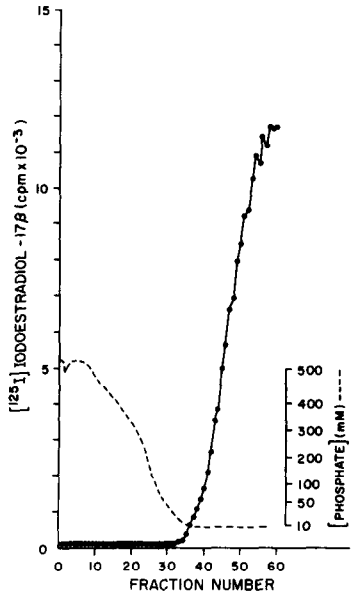

Fig. 2. Release of free [^{125}I]iodoestradiol-17β from SynChropak 500 propyl column, following a short-time reverse phosphate gradient (dashed line). The column was developed for 60 min with a phosphate gradient from 500 mM to 10 mM as described in the Experimental section. Release of free steroid towards the end of the short elution program (fractions 35–60) represents 16% of total applied radioactivity.

diol-17β. As a result of this finding, we have concentrated on the use of iodoestradiol-17β as ligand to identify receptor isoforms.

To evaluate hydrophobic-interaction chromatography, tissue extracts labeled with [^{125}I]iodoestradiol were applied to the SynChropak 500 propyl column and eluted with the 60-min program described earlier. Fig. 3 is a representative profile of estrogen-binding components, separated by this procedure. Two peaks of radioactivity were detected, each of which appeared to contain specific protein-bound iodostradiol-17β. As noted, when unlabeled diethylstilbestrol (inhibitor) was incubated with labeled steroid and tissue extracts, the binding of [^{125}I]iodoestradiol was greatly diminished, indicating the specificity of the interaction. One of the components (peak

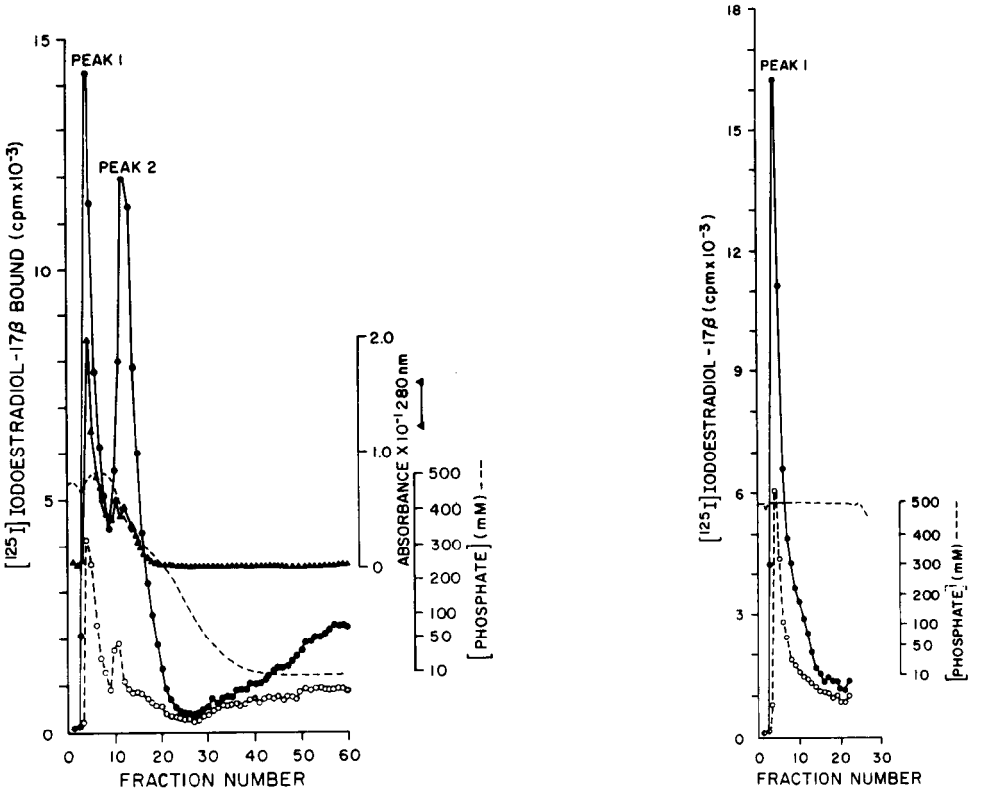

Fig. 3. Elution of estrogen receptor–[^{125}I]iodoestradiol-17β complexes from SynChropak 500 propyl column with a reverse phosphate gradient. The 60-min elution program was used (– – –) (see Experimental). Labeled soluble estrogen receptors in P_{500}EDG buffer were applied to the column, following removal of free steroid with a pellet of dextran-coated charcoal. Chromatographic separation was then employed. Total binding is indicated by ● while non-specific binding is indicated by ○. There was a 12% recovery of specifically bound steroid before fraction 35, where free steroid began to be eluted. Peaks 1 and 2 were shown to contain protein-bound radioactivity by exposing an aliquot to a pellet of dextran-coated charcoal (0.5%, w/v, final concentration). The recovery of soluble protein was 85%.

Fig. 4. Hydrophobic nature of peak 2 in Fig. 3. The same radioactively labeled soluble fraction as described in Fig. 3 was applied to the column. Instead of initiating the salt gradient, the phosphate concentration was kept constant at 500 m*M* for 20 min. Of the total radioactivity applied to column, only 7% was recovered in fractions 1–22. Total binding is indicated by ●, while non-specific binding is indicated by ○.

1) was eluted in the void volume, while the other were eluted at 350–450 mM phosphate (peak 2). It should also be noted that peak 1 was eluted at a position where the majority of proteins were detected by their absorption at 280 nm (Fig. 3).

Using the same column and an identical receptor preparation, a further study was conducted to show the hydrophobic nature of peak 2. In this study, the labeled estrogen receptors were applied to the stationary phase and the column was eluted with a continuous wash of P_{500}EDG buffer without a phosphate gradient. As shown in Fig. 4, only the volume-dependent peak 1 was eluted, *i.e.*, no steroid-binding components identified as peak 2 by phosphate gradient elution (Fig. 3) were observed. This finding substantiates the suggestion that estrogen-binding components in peak 2 exhibit hydrophobic properties.

Radioactivity, bound by components in peaks 1 and 2, represent 3–12% of that applied, suggesting that the SynChropak 500 propyl matrix is either stripping the ligand from receptor or has retained other estrogen-binding components. As shown in Fig. 5, all of the remaining [^{125}I]iodoestradiol-17β applied may be eluted from the column by a methanol wash. Sequential assays of aliquots from the same soluble fraction gave reproducible results (within 1% of the total radioactivity recovered in peaks 1 and 2). Currently, we are investigating different types of eluting ions under conditions which stabilize these receptors, *e.g.*, sodium molybdate[4,8,11].

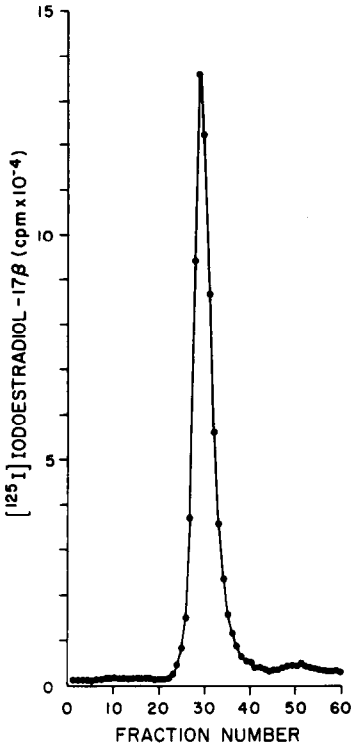

Fig. 5. Recovery of steroid bound to the SynChropak 500 propyl column. Following chromatography (Fig. 3), the column was washed with water for 30 min and then eluted with methanol–P_{10}EDG buffer (50:50). Of the remaining 72% of the labeled iodostradiol-17β, bound to the column, 64% was eluted.

It is not possible to ascertain presently if the remaining labeled ligand was bound to estrogen receptors interacting tightly with the SynChropak 500 propyl column.

To support further the assumption that the labeled steroid in peaks 1 and 2 were protein-bound, fractions of each component were treated with pellets of dextran-coated charcoal to remove free steroid. We observed that 75–80% of peak 1 and 60–65% of peak 2 contained protein-bound radioactivity, suggesting the presence of receptor isoforms. Certainly, this is an underestimation, since dextran-coated charcoal is known to strip steroid ligands from their receptors under high-ionic-strength conditions[4,21]. Due to the elution position, peak 2 exhibited hydrophobic properties unlike peak 1, as discussed earlier.

Using conventional open column hydrophobic chromatography, other workers have recognized that certain estrogen receptors from carcinogen-induced mammary tumors of rats[16,17] as well as androgen receptors from rat prostate[3] display hydrophobic characteristics. Maggi *et al.*[20] have used the latter procedure to study affinity-labeled progestin receptors from chick oviduct. Currently, the specific chemical basis of the hydrophobic interaction common to these receptors is unknown. However, the report of Tenenbaum and Leclercq[17] may shed light upon this question, since they suggest the estrogen receptor in the liganded and unliganded state behaves differently on a hydrophobic column (Blue Sepharose CL-6B).

Moderate concentration of organic solvents, such as methanol and acetonitrile, have been used successfully in reversed-phase HPLC for the purification of various proteins[22]. In this study, we have evaluated the use of various organic solvents to retard the interaction of iodoestradiol-17β with the stationary phase and to isolate labeled receptor as intact isoforms. Our results with acetonitrile–phosphate buffer (20:80) show that [^{125}I]iodoestradiol-17β was eluted with a retention time of approximately 15 min (Fig. 6). It was observed that free steroid now was eluted within the phosphate gradient in the presence of acetonitrile.

After establishing the elution position of free iodoestradiol-17β, we investigated the separation of labeled estrogen receptors by HPHIC (Fig. 7). Two [^{125}I]iodoestradiol-binding components were observed in the presence of 20% acetonitrile. Both of these components appeared to be associated with labeled estrogen in a specific fashion, since unlabeled diethylstilbestrol diminished binding (Fig. 7). The first peak of radioactivity was eluted in the same position as unbound steroid (Fig. 6). However, the inhibition of binding by diethylstibestrol suggests that peak 1 may consist of both protein-bound and free iodoestradiol-17β. The peak eluted at approximately 50 m*M* phosphate appears to consist of specific estrogen-binding components, since ligand association was diminished by inhibitor, and the elution position was different from that of free steroid. It should be noted that HPHIC profiles of estrogen receptors in human breast cancer were patient-dependent, suggesting individual tumor variation. Separation profiles should reflect differences in the intrinsic properties (such as amino acid sequence) of estrogen-binding components. The physiological significance of this will have to await progress in the application of HPHIC to steroid hormone receptors.

Thus far, the use of hydrophobic-interaction chromatography in the separation and characterization of estrogen receptors has met with limited success. It appears that the application of HPHIC with buffers containing highly polar organic solvents may be useful for discerning various receptor isoforms in the active and inactive states. Further investigations are needed to pursue these questions.

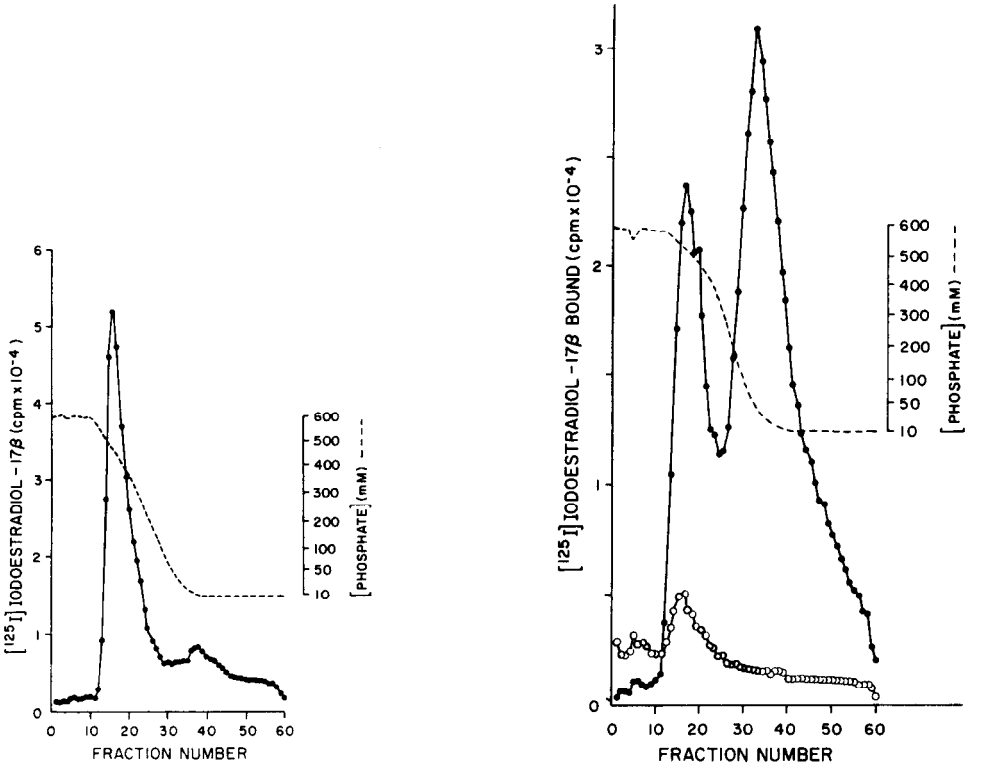

Fig. 6. Behavior of free [^{125}I]iodoestradiol-17β on a SynChropak 500 propyl column. The conditions of chromatography were the same as those described in the legend for Fig. 2, except that 20% acetonitrile was present in both high- and low-salt buffers. Recovery of the applied radioactivity was 86%.

Fig. 7. HPHIC separation of estrogen receptors from human breast cancer Conditions were the same as those described in the legend for Fig. 3, except that all buffers contained 20% acetonitrile. Of the applied radioactivity 90% was recovered, of which 30% was located in the first peak (fractions 11–24) and 70% in the second peak (fractions 24–60). Total binding is indicated by ● while non-specific binding is indicated by O.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Mr. Phillip McCauley. Our appreciation is extended to Ms. Dana Gibson for her excellent efforts in the preparation of the typescript. We also wish to thank Drs. George Barrows, Greg Kuhns, Stuart Wolfson and other pathologists cooperating in the collection of the tissue biopsies. This research was supported in part by the Phi Beta Psi Sorority, by USPHS Grant CA-32102 from the National Cancer Institute and by the Marie Overbey Memorial Grant from the American Cancer Society.

REFERENCES

1 V. Sica and F. Bresciani, *Biochemistry*, 18 (1979) 2369.
2 W. T. Schrader, M. E. Birnbaumer, M. R. Hughes, N. L. Weigel, W. W. Grody and B. W. O'Malley, *Rec. Prog. Hormone Res.*, 37 (1981) 583.
3 N. Bruchovsky, P. S. Rennie and T. Comeau, *Eur. J. Biochem.*, 120 (1981) 399.
4 J. L. Wittliff and R. D. Wiehle, in V. P. Hollander (Editor), *Hormonally Sensitive Tumors*, Academic Press, NY, 1985, in press.
5 T. E. Kute, P. Heidemann and J. L. Wittliff, *Cancer Res.*, 38 (1978) 4307.
6 G. A. Puca, E. Nola, V. Sica and F. Bresciani, *Biochemistry*, 10 (1971) 3769.
7 T. W. Hutchens, D. B. Lubahn, R. D. Wiehle, K. S. McCarty, Jr., K. S. McCarty, Sr. and J. L. Wittliff, submitted for publication.
8 T. W. Hutchens, R. D. Wiehle, N. A. Shahabi and J. L. Wittliff, *J. Chromatogr.*, 266 (1983) 115.
9 R. D. Wiehle, G. E. Hofmann, A. Fuchs and J. L. Wittliff, *J. Chromatogr.*, 307 (1984) 39.
10 R. D. Wiehle and J. L. Wittliff, *J. Chromatogr.*, 297 (1984) 313.
11 N. A. Shahabi, T. W. Hutchens, J. L. Wittliff, S. D. Halmo, M. E. Kirk and J. A. Nisker, *Prog. Cancer Res. Therap.*, 31 (1984) 63.
12 A. Van der Walt and J. L. Wittliff, *J. Chromatogr.*, submitted for publication.
13 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
14 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 298 (1984) 407.
15 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. E. Regnier, *Anal. Biochem.*, 137 (1984) 464.
16 A. Tenenbaum and G. Leclercq, *J. Steroid Biochem.*, 13 (1980) 829.
17 A. Tenenbaum and G. Leclercq, *Acta Endocr. Suppl.*, 225 (1979) 272.
18 J. Ripoche, A. Al Salihi, J. Rousseaux and M. Fontaine, *Biochem. J.*, 221 (1984) 89.
19 W. J. Waddell, *J. Lab. Clin. Med.*, 48 (1956) 311.
20 A. Maggi, J. G. Compton, M. Fahnstock, W. T. Schrader and B. W. O'Malley, *J. Steroid Biochem.*, 15 (1981) 63.
21 E. J. Peck and J. H. Clark, *Endocrinology*, 101 (1977) 1034.
22 J. W. Wilks and S. S. Butler, *J. Chromatogr.*, 298 (1984) 123.