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RAPID, HIGH-RESOLUTION PROCEDURE FOR ASSESSMENT OF ESTROGEN RECEPTOR HETEROGENEITY IN CLINICAL SAMPLES

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SUMMARY

Approximately two-third of the women with breast cancer may benefit from hormone therapies if the lesion contains estrogen receptor in reasonable levels (> 10 fmol/mg cytosol protein). Our lab and others have suggested that not only the concentration, but the various molecular form(s) of the receptor may be an important factor in predicting patient responsiveness. Until now, this heterogeneity has been determined by analysis on sucrose density gradients requiring 16 h of centrifugation. Other methodologies which can disclose the profile of receptor isoforms are compromised by irreproducibility, poor recovery, or the length of time required to perform the analysis. We believe that high-performance liquid chromatography (HPLC) in the anion-exchange and chromatofocusing modes may be able to supply more insight into receptor structure than currently available by any other method. Utilizing the high activity ligand [16α-^{125}I]iodoestradiol-17β (2200 Ci/mmol) and flow-through equipment to monitor conductivity, pH, and radioactivity, we are able to describe the distribution of ionic isoforms in crude cytosolic preparations of breast cancer and uterus. This "on-line" technology is rapid (chromatogram is complete within 120 min), sensitive (receptor isoforms equivalent to 1 fmol are apparent), efficient (columns typically return > 90% of applied radio-labeled receptor) and reproducible (due to the sophistication of modern HPLC equipment). It is clear these techniques may be used in the clinical setting to better describe the profile of estrogen receptor isoforms and should be explored as a method of correlating receptor structure with hormone function in responsive tissues.

INTRODUCTION

The presence of receptor proteins for estrogen or both estrogen and progestin is a predictive index of response to hormone therapy in women with breast cancer[1–3]. Not only is quantification of receptor levels essential (*e.g.*, refs. 2 and 3), but the presence of certain receptor isoforms also appears important in the selection of patients most likely to respond to endocrine manipulation (*e.g.*, refs. 3–6).

Our laboratory has utilized a number of methods to explore receptor heterogeneity. These include sucrose density gradient centrifugation[3,4], ion-exchange chro-

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matography[7], isoelectric focusing[8], size-exclusion chromatography[9], and DNA-cellulose binding[10]. Of these, only sucrose density gradient centrifugation has proved to be useful for routine analyses of the molecular properties of these steroid receptors in the clinical setting. Conventional open column ion-exchange chromatography and isoelectric focusing are time-consuming and often give poor recoveries. Although open-column size-exclusion chromatography may be used in an analytical fashion, it is also tedious and slow to be employed routinely with labile receptor proteins. These techniques also are not readily amenable to multiple assays.

Recently we have employed high-performance liquid chromatography (HPLC) methodologies in the size-exclusion[11], ion-exchange[12], and chromatofocusing[13] modes to examine estrogen receptor isoforms on the basis of shape and surface charge. These HPLC methods are also useful for characterizing progestin receptors[14]. High-performance chromatofocusing (HPCF) and high-performance ion-exchange chromatography (HPIEC) in particular have proved to be rapid, reproducible, and afford high recoveries[15].

In this report, we extend our use of HPCF and HPIEC to include on-line methods of analysis. To quickly ascertain receptor isoform distribution a Beckman Model 170 flow-through radioisotope detector has been connected to the low-pressure column outlet line between either a pH or conductivity flow cell and fraction collector. Analyses are complete within 60 min (HPIEC) or 90 min (HPCF) following a 2-h incubation period with the radiolabeled steroid. Additionally, the use of the high activity ligand [16α-^{125}I]iodestradiol-17β (2200 Ci/mmol) enables the identification of small quantities (0.5–1.0 fmol) of individual receptor isoforms. This high resolution, sensitive technique is readily adaptable to the routine analysis of receptor polymorphism in clinical specimens.

EXPERIMENTAL

Materials

The ligand [16α-^{125}I]iodoestradiol-17β (2200 Ci/mmol) was obtained from New England Nuclear/DuPont. Disodium ethylenediaminetetraacetic acid (EDTA), potassium phosphate, glycerol, dithiothreitol (DTT), unlabeled diethylstilbestrol, Norit A, and Dextran T-70 were obtained from Sigma. Polybuffer 96 and Polybuffer 74 were purchased from Pharmacia.

The human breast or uterine tissues used in this study were obtained from women through the aegis of surgeons and pathologists at local hospitals. Specimens were frozen and brought to the laboratory on dry ice or were placed on ice after surgery and brought to the laboratory. In either case, tissues were frozen and kept at -86°C until use.

Preparation of cytosolic estrogen receptors

All procedures were carried out at 0–4°C. Tissues were sliced thinly by hand and then homogenized using a Brinkman Polytron (two 10-sec bursts) in 2–4 volumes of the respective column equilibration buffer. Cytosols were prepared by centrifugation of the homogenates for 20 min at 100 000 rpm in a Beckman TLA 100.2 rotor and TL-100 tabletop ultracentrifuge. In the case of breast tissue, the supernatant lipid was separated from the cytosol layer. Cytosols were incubated at 4°C for 2 h

with 5 n*M* [^{125}I]iodoestradiol-17β in the presence (non-specific binding) or absence (total binding) of a 200-fold molar excess of an unlabeled competitor. The incubations were terminated by removing unbound steroid with a pellet derived from an equal volume of a 1% dextran-coated charcoal suspension (1% charcoal, 0.5% dextran). Cytosol protein concentrations were determined by the method of Waddell[16]. Specific binding capacity was expressed as femtomoles of steroid bound per milligram of cytosol protein.

HPLC and flow-through instrumentation

All chromatographic separations were performed at 0–4°C with an Altex Model 344 liquid chromatograph using Model 112 solvent pumps and a Model 210 injection valve[11–15]. The radioactivity and conductivity or pH profiles of the column eluate were recorded simultaneously on a Kipp and Zonen BD-41 recorder. To accomplish this, a Beckman Model 170 flow-through radioisotope detector with an Epson Rx-80 printer and a Bio-Rad conductivity meter with flow cell or Pharmacia pH monitor with flow-through pH electrode were used. The components were connected on-line to the low pressure line 30 cm from the column outlet to measure continuously occurring changes in the column effluent. The combination of low flow cell dead volumes (2 μl for conductivity flow cell, <30 μl for flow-through pH electrode) and the direct attachment of the Model 170 detector to either flow cell yielded simultaneous, reproducible tracings for radioactivity and either conductivity or pH.

The efficiency of the Model 170 radioisotope detector was measured statically by counting a known quantity of [^{125}I]iodoestradiol-17β[13] within the active counting area (total volume of the cell) and was determined to be 40%. Values obtained from the on-line recordings of either the conductivity or pH gradients typically differed less than 3% and 8% maximally. The volume of the flow cell for the gamma detector is determined by the length of tubing chosen and must be less than or equal to one-fifth the volume of the smallest peak. The following parameters were used in all experiments. A flow cell of 27 cm × 1.86 mm I.D. thin-walled polyethylene tubing (0.73 ml total cell volume) with 66.4 cm exit length was used. A background value of 0.5 kcpm (500 cpm) was determined and employed in all experiments. Only the full scale value in kcpm was altered in each experiment.

Using a Buchler LC-200, 1-ml fractions were collected dropwise (19 drops/ml) which corresponded to a fraction time of 1 min. Individual fractions were measured radiometrically for the presence of [^{125}I]iodoestradiol-labeled receptor complexes, non-specific binding components, and free steroid in each fraction with a Micromedics 4/600 automatic gamma counter. The counting efficiency of this instrument averaged 65% determined by reference to independent determinations per minute using a Beckman 4000 two-channel gamma counter[13].

High-performance ion-exchange and chromatofocusing

All chromatography was performed in a Puffer-Hubbard cold box at 4°C. Buffers were filtered under vacuum through Millipore 0.45-μm HA-type filters before use. A flow-rate of 1.0 ml/min was used for all experiments. Free steroid or the estrogen-labeled cytosols (10–200 μl) were applied with a Hamilton syringe to the silica-based polyamine-coated SynChropak AX-500 (HPCF) or AX-1000 (HPIEC) anion-exchange columns (250 × 4.1 mm I.D.) from SynChrom[12,13].

HPIEC was performed on an AX-1000 column equilibrated with buffer A: water, containing 10 m*M* potassium phosphate, 1.5 m*M* EDTA, 1 m*M* DTT 10% (v/v) glycerol, pH 7.4 at 4°C. The composition of buffer B was identical to that of buffer A except for 500 m*M* potassium phosphate.

A programmed gradient elution was carried out after injection of sample at time $t = 0.5$ min in the following manner: 100% buffer A from 0 to 10 min, 0 to 60% buffer B from 10 to 35 min, 60 to 100% buffer B from 35 to 45 min, 100% to 0% buffer B from 47 to 49 min, 100% buffer A from 49 to 55 min. Potassium phosphate concentrations were determined by comparison with conductivity measurements performed with identical buffer solutions containing varying amounts of phosphate, pH 7.4 at 4°C.

In the case of HPCF on the AX-500 column, we used water containing 25 m*M* Tris–HCl, 1 m*M* DTT and 20% (v/v) glycerol adjusted to pH 8.3 at 4°C as equilibration buffer. At $t = 2$ min (sample injection $t = 0.5$ min), the mobile phase to generate the pH gradient was started. This solution was a 30:70 mixture of Polybuffers 96 and 74 diluted 1:10 with water–glycerol (80:20) containing 1 m*M* DTT, filtered and adjusted to pH 3.0 at 4°C. When the pH = 4.0, columns were regenerated to their starting pH (8.3) with column equilibration buffer at 1–2 ml/min. The pH of every third fraction was determined at 0–4°C using a Beckman Model 3500 pH meter with a combination glass electrode.

RESULTS AND DISCUSSION

The purpose of this study was to develop a rapid, sensitive, and reproducible means of assessing estrogen receptor heterogeneity in clinical samples. HPLC is especially appropriate since this technology has proven useful for efficient separations of proteins and other biopolymers[17].

In this study we have evaluated the combined use of HPLC methods for the separation of estrogen receptors in human breast tumors and uteri with on-line instrumentation to monitor radioactivity, conductivity, and pH. Fig. 1 shows the separation of different ionic isoforms of the estrogen receptor in cytosol from human breast cancer by HPIEC. This figure compares the radioactivity of profiles obtained by external manual measurements (Fig. 1A) with a continuous recording of radioactivity using on-line instrumentation (Fig. 1B). Except for differences in isotope-counting efficiency observed with the two methods, the profiles of bound radioactivity were identical with respect to the types and amounts of receptors isoforms separated. However, the profile shown in Fig. 1B was generated within 2 h in a continuous fashion during separation.

Four peaks of bound radioactivity were described by HPIEC for this sample of human breast tissue. Two non-specific binding components, as defined by the inhibition of [^{125}I]iodoestradiol-17β binding in the presence of diethylstilbestrol, were eluted just after the void volume. Free [^{125}I]iodoestradiol-17β may have contributed to the larger, second non-specific component as we reported earlier[12]. Fig. 1 also illustrates two isoforms of estrogen receptor eluting between 120–140 m*M* (retention time, 22.5 min) and 250–275 m*M* (retention time, 33.3 min) phosphate concentration in this patient sample. These are equivalent to 91 fmol (26% of total) and 258 fmol (77% of total) of receptor, respectively. The integration of the areas under the peaks

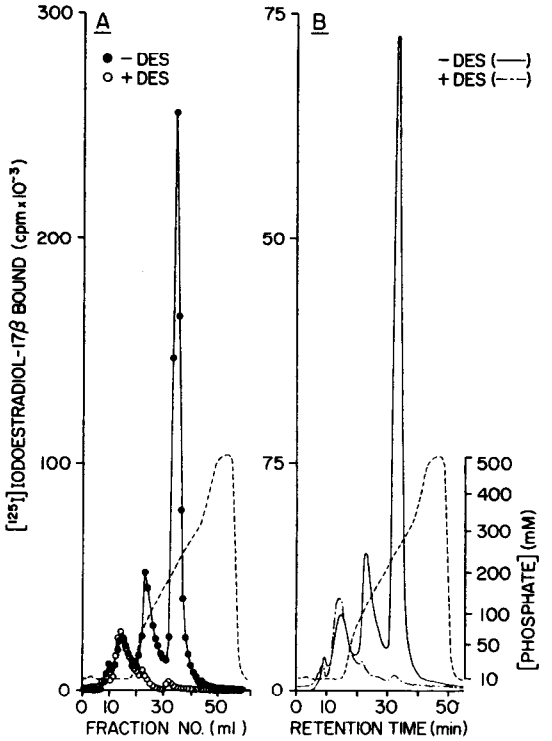

Fig. 1. HPIEC separation of ionic isoforms of estrogen receptors from human breast cancer. Cytosol was prepared as described in Materials and methods and incubated for 2 h with 5 n*M* [^{125}I]iodoestradiol-17β in the presence or absence of 200-fold excess diethylstilbestrol (DES). Elution of the AX-1000 column was performed on 200 μl of cytosol (7.4 mg/ml) cleared of unbound ligand at 1.0 ml/min using a gradient of potassium phosphate at pH 7.4 (– – –). A, 1-ml fractions were collected and radioactivity measured manually with a gamma counter; B, radioactivity recorded continuously using on-line Model 170 radioisotope detector with conductivity flow cell. Total binding is indicated by ● in A and by ——— in B, and non-specific binding is indicated by O in A and by —·— in B. Recovery of radioactivity from the column was 97% for the total bound curve determined by counting a 10-μl aliquot before sample injection. Specific binding was 167 fmol receptor/mg cytosol protein determined by multi-point titration analysis.

was performed by the Model 170 radioisotope detector and compared within one area percent to calculations performed manually on the data from the plot shown in Fig. 1A. Clearly, greater than two-thirds of the population of estrogen receptors for this patient existed in a high salt-eluting isoform. Retention times corresponding to peak maxima for all peaks detected are provided by the Model 170. Either method distinguished the isoform profile clearly but the information presented in Fig. 1B was gathered more quickly.

Additionally, the on-line method of analysis permitted the detection of very low levels of receptor isoforms in small quantities of cytosol (10–20 μl). Fig. 2 compares the manual and on-line profiles of 6.9 fmol of total receptor-bound radioactivity from human uterine tissue separated by HPIEC. Measurements obtained manually are shown in Fig. 2A, with the continuous tracings presented in Fig. 2B. Conditions for separation were identical to those in Fig. 1 except that 10 μl cytosol

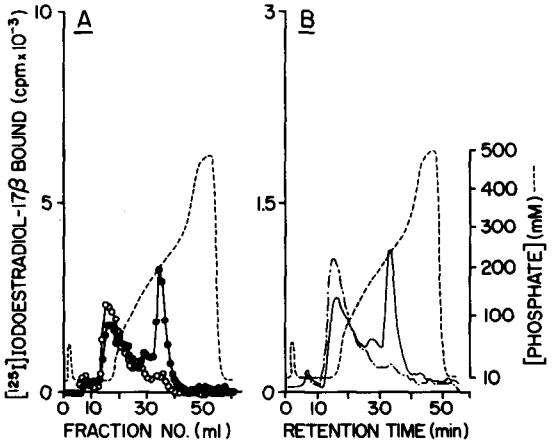

Fig. 2. HPIEC separation of micro quantities of ionic isoforms of the estrogen receptor from human uterus. The conditions used in this experiment were the same as described in the legend to Fig. 1 but human uterine tissue was used. The curves shown are the results of receptors separated in the presence or absence of 200-fold excess diethylstilbestrol. Elution was performed on 10 μl of cytosol (10 mg/ml) equivalent to 6.9 fmol receptor at 1.0 ml/min using a gradient of potassium phosphate (– – –). A, 1-ml fractions were collected and radioactivity measured manually; B, radioactivity recorded continuously using on-line Model 170 radioisotope detector with conductivity flow cell. Total binding is indicated by ● in A and by ——— in B, and non-specific binding is indicated by ○ in A and by —·—· in B. Recovery of radioactivity from the column was 93% for the total bound curve determined by counting a 10-μl aliquot before sample injection. Specific binding was 30 fmol receptor/mg cytosol protein determined by multipoint titration analysis.

containing 100 μg of protein were applied to the column. An elution pattern similar to that for estrogen receptors in breast tissue emerged. Two non-specific binding components that did not interact with the column were also present in uterine cytosol. However, one receptor isoform was shown to have slightly different surface charge properties as characterized by an altered elution from the phosphate gradient. The first species of receptors eluted at a phosphate concentration of 150–180 m*M* (retention time, 27.9 min) and was equivalent to 1.4 fmol of bound steroid (22% of total). The second isoform eluted between 225–255 m*M* phosphate (retention time, 33.5 min) and was equal to 5.0 fmol of receptor (78% of total). A 93% recovery was observed in this representative experiment. The presence of different ionic isoforms of the receptor in breast and uterine tissues revealed by the rapid format with HPIEC allows the comparison required of clinical studies.

Additional information on the surface charge heterogeneity of receptor molecules can be gained through HPLC methods that exploit differences in the isoelectric points of these proteins as the basis for selective adsorption to ion-exchange columns. Previously we showed that a chemically defined, internal pH gradient generated with appropriately diluted mixtures of polyampholyte buffers on AX-500 columns will separate as many as ten different [^{125}I]iodoestradiol-labeled binding proteins in cytosols prepared from breast tissue and uterus[9,13]. The elution profiles shown in Fig. 3 demonstrate three isoforms in cytosol from a human breast cancer biopsy by the same type of analysis. These isoforms eluted at pH values of 7.3, 6.7, and 5.9–5.6

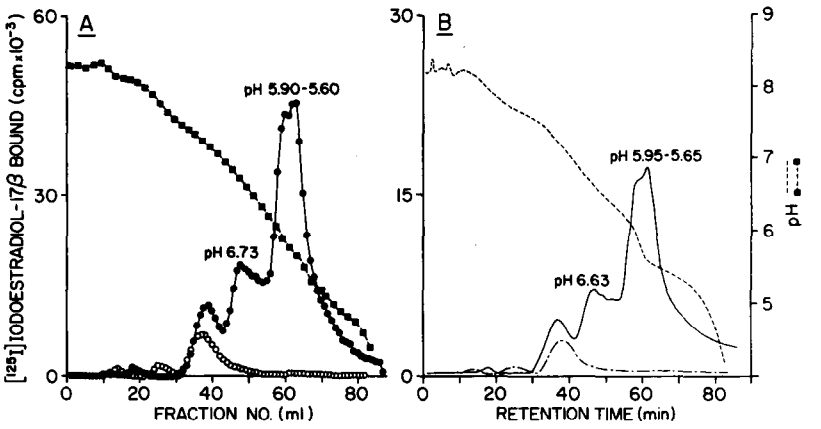

Fig. 3. HCPF separation of ionic isoforms of estrogen receptors from human breast cancer. The sample of breast tissue and conditions used in this experiment were the same as described in the legend to Fig. 1 but were performed on separate days. The curves of [^{125}I]iodoestradiol-17β bound shown are the results of receptors separated in the presence or absence of 200-fold excess diethylstilbestrol. Elution of the AX-500 column by a pH gradient was performed isocratically on 200 μl of cleared cytosol (7.0 mg/ml) at 1.0 ml/min. A 30:70 mixture of Polybuffers 96 and 74 diluted 1:10 with water–glycerol (80:20, v/v) adjusted to pH 3.0 was employed. The pH gradient is indicated as ■ in A and as — — — in B. The column was initially equilibrated with water, containing 25 m*M* Tris–HCl, 1 m*M* DTT, 20% glycerol (v/v), pH 8.3. (A) 1 ml fractions were collected and radioactivity or pH measured manually, or (B) radioactivity and pH recorded continuously using on-line Model radioisotope detector with flow-through electrode. Total binding is indicated by ● in A and by ——— in B, and non-specific binding indicated by ○ in A and by —·—·— in B. Recovery of radioactivity from the column was 97% for the total bound curve determined by counting a 10-μl aliquot before sample injection. Specific binding was 167 fmol receptor/mg cytosol protein determined by multipoint titration analysis.

and contained 23, 59, or 203 fmol of receptor, respectively. The larger peak of radioactivity between pH 5.9–5.6 in Fig. 3B appears to contain more than a single isoform. Peak retention times during chromatofocusing were not as useful since the slope of the pH gradient (column titration capacity) differed slightly from column to column. Compared to the same data plotted in Fig. 3A, it appears that the on-line tracing more clearly describes the true microheterogeneity of receptor-bound radioactivity uninfluenced by the counting of individual fractions. The recovery of radiolabeled receptor was 96% in this experiment which was completed in 3 h. Thus, on-line methods accurately depict estrogen receptor heterogeneity separated by HPCF with high resolution and short analysis times.

The necessity of conducting these analyses at 4°C is due to the lability of the ligand-binding properties of steroid receptor proteins. It is likely that the resolution of receptor proteins from ion-exchange columns in this case was hindered at lower temperatures leading to peak broadening. Peak broadening (increased peak to flow cell volume) decreases the sensitivity of peak detection which can be somewhat overcome by lower flow-rates and proper choice of flow cell volume.

In this report, we compared conventional manual methods with on-line techniques for the detection of radioactivity in chromatograms from HPIEC and HPCF that describe different ionic species of estrogen receptors. Utilizing the ligand

[^{125}I]iodoestradiol-17β with a high specific radioactivity has enabled the measurement of femtomole quantities of receptor isoforms. It is clear that the automated, on-line system for estrogen receptor analysis is superior in many ways. The time required for the complete analysis of isoforms in a single biopsy is dictated only by the length of time to perform the separation since all quantification is performed by the Beckman Model 170 flow-through radioisotope detector. Sensitivity is extended to amounts of receptor of less than 1 fmol. Separations are performed automatically using HPLC methodologies which take advantage of the size, shape, and surface charge properties of receptors. Reproducibility of chromatographic conditions is consistent from run to run (see Experimental) using microprocessor-controlled equipment. In summary, the use of flow-through equipment to monitor radioactivity, conductivity, and pH in the assessment of estrogen receptor heterogeneity in the clinical laboratory can now be a routine procedure.

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