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SHORT COMMUNICATION

ANALYSIS OF ESTROGEN RECEPTORS IN HUMAN BREAST CANCER BY MOLECULAR FILTRATION ON SEPHACRYL-S-200 COLUMNS

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SUMMARY

Molecular filtration on Sephacryl-S-200 columns was successfully used for the analysis of cytosol estradiol receptors, extracted from human breast cancers. This method is fast, simple to perform and readily separates the larger and smaller ER components.

Analysis of the cytosol estradiol receptors (ER) in specimens of human breast cancer is generally performed by two methods. The dextrane-coated charcoal assay gives exact information on the dissociation constant (K_D) and the number of binding sites, when a full saturation analysis is performed. The sucrose gradient sedimentation technique yields important data on the molecular size of the ER. Most human breast cancer ER sediments at 8*S* [1] but a significant proportion of tumors has ER sedimenting at around 3–4*S* [1, 2]. The application of the sucrose gradient technique is limited by its lengthy and laborious nature, usually requiring concentrated cytosols. Also, evaluation of the 4*S* complex is often difficult, because it partially overlaps with the free [^{3}H]-estradiol peak [3].

Molecular filtration on Sephacryl-S-200 columns offers several advantages. It is faster, reproducible, technically simple to perform and it readily separates the 8*S* and 3–4*S* complexes.

Sephacryl-S-200 Superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) was packed into columns in the preswollen state as supplied, followed by washing of the columns with 100 ml of a Tris–EDTA–dithiothreitol buffer (0.01 M, pH 7.4, containing 0.0015 EDTA and 0.5 mM dithiothreitol) [4]. The same buffer was used to prepare cytosols from human breast cancer specimens. Tissues (0.3–1.0 g) were homogenized in 10 ml TED buffer in a Vir-Tis homogenizer and cytosols separated by ultracentrifugation at 100,000 *g* for 1 h as described elsewhere [5]. Aliquots of cytosol (1 ml) were incubated with 0.45 pmol of [2, 4, 6, 7^{3}H]-estradiol (S.A. 110 Ci/mmol) at temperatures and lengths of time specified in the legends of figures. Incubates were treated with dextrane-coated charcoal pellets to remove unbound estradiol prior to application to the column. Charged cytosols, however, can be applied to the column without previous treatment with charcoal. Removal of excess [^{3}H]-estradiol prevents the contamination of the column with large amounts of radioactivity. The volume of cytosol applied was 0.5–1.0 ml per column, with a protein concentration between 0.3–1.5 mg/ml.

Calibration of a column (Pharmacia K9; 600 × 9 mm)

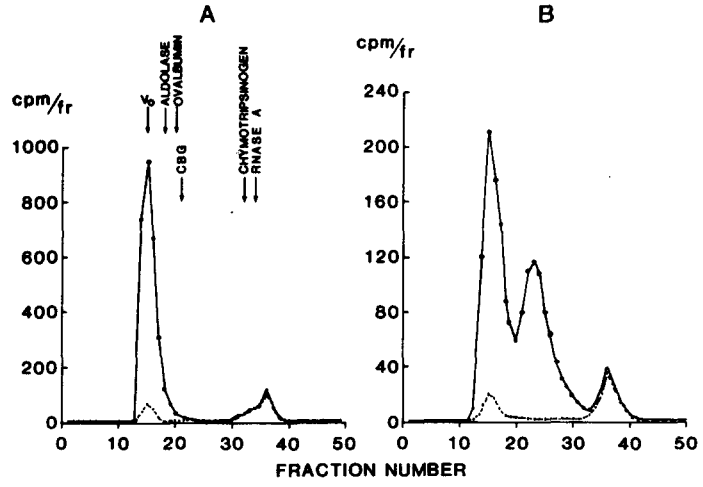

Fig. 1. A. Resolution of a single component [^{3}H]-estradiol–ER complex on a Sephacryl-S-200 column (K9). Arrows indicate elution of standard proteins. V_0 = void volume; CBG = corticosteroid binding globulin. B. Cytosol with large and small ER species (—). Broken lines indicate incubations with a 100× excess (45 pmol) of estrogen competitor (DES). Incubations were at 4° for 16 h (Column K9). Experiments were performed using low ionic strength buffer. (A & B).

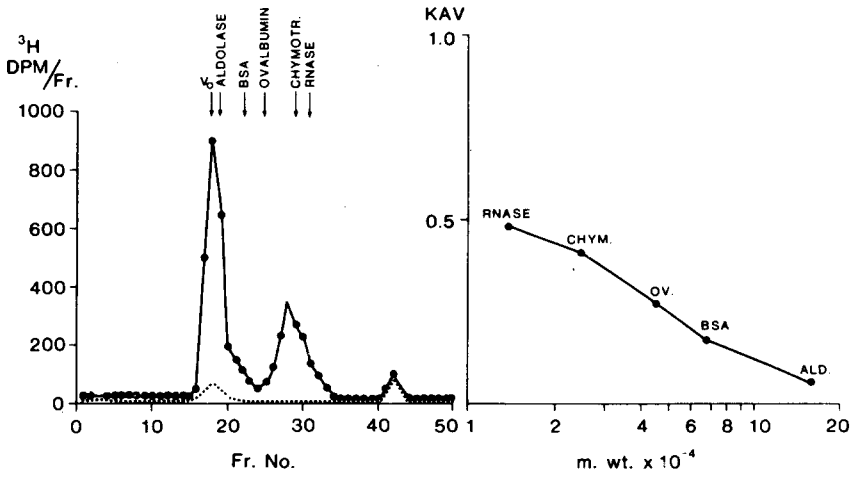

Fig. 2. The two major forms of ERC in high ionic strength buffer (0.4 M KCL in TED) as analysed on a Sephacryl column (K15) following incubations with [^{3}H]-estradiol at 4° and 16 h. Arrows indicate elution volumes of standard proteins with known mol. wt. Dotted line shows result of incubation with DES (left side). Calibration of a Sephacryl column (K15) with standard proteins. Column eluted with 0.4 M KCL in TED buffer (right side).

with standard proteins of various molecular weights illustrates the separating ability of this particular column (Fig. 1). The resolution of a typical ER-[^{3}H]-estradiol complex obtained following a 16-h incubation at 4°C is illustrated in Fig. 1.

The running time of the K9-column is approximately 7 h to collect 50 1-ml fractions. The columns were run at 4°C and fractions were collected by an LKB 7000 automatic fraction collector.

The ER-[^{3}H]-estradiol complex is eluted in fraction No. 15 that correspond to the exclusion volume (V_0). This indicates a mol. wt over 250,000. A certain amount of nonspecific binding is always associated with this fraction. (Fig. 1).

Most human breast cancer cytosols show a single peak at this location, if prepared in low ionic strength buffer, following a 16-h incubation at 4°C. Some cytosols however, have an additional peak at the 30–40,000 mol. wt. range. This smaller molecule corresponds to the 4*S* estradiol binder described [1, 2] in human breast cancer cytosols. An additional, 3rd peak of nonspecific binding was present in all the cytosols in fractions 36 (K9) and 43 (K15), representing a mol. wt of 5–10,000 (Figs 1 and 2).

Prolonged incubation (i.e. 16 h) at 4°C appears to increase the amount of the larger mol. wt ER complex in relation to the smaller binder (8*S* vs 4*S*), suggestive of an aggregation. This phenomenon, however, can be inhibited by high ionic strength media, i.e. 0.4 M KCL in the buffer used to homogenize the tumor and to run the column.

Free [^{3}H]-estradiol is eluted in around fraction 150 from this column. Therefore it is suggested that the column be washed with 200 ml of buffer after each use to eliminate free estradiol. It is also possible to use fresh columns for each run and to wash Sephacryl in batches after use.

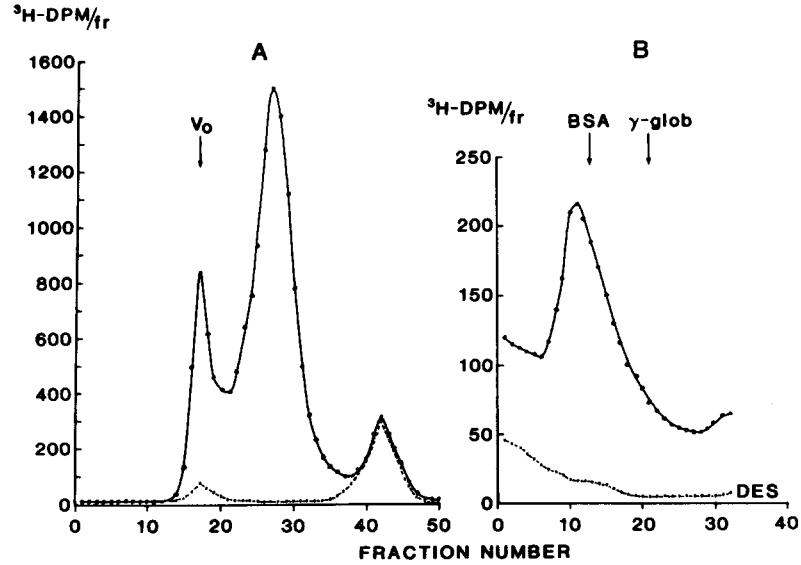

Fig. 3. Analysis of ER with dominating small molecular species in TED buffer (no KCL) on a K15 Sephacryl column (A) and on a 5–20% sucrose gradient (B). Incubations were at 4°C for 2 h only with 0.45 pmol of [^{3}H]-estradiol. Broken lines: results of incubations in the presence of a 100× excess of DES.

Excellent resolution can be obtained with much shorter separation times using a shorter but wider column (30 × 1.5 cm) by Pharmacia (K15). Running time is reduced to 2.5 h and peaks are slightly shifted to higher fraction numbers (Fig. 2). The collection of 50 1-ml fractions still covers the entire receptor spectrum.

Analysis of cytosol specimens from the same tumor by Sephacryl column and the sucrose gradient technique (Fig. 3) reveals a certain amount of aggregation of a purely 4*S* ER molecular species on the Sephacryl column. This phenomenon can be observed even if the cytosol is incubated and run in buffer containing 0.4 M KCL. ER complexes that sediment at 8*S* on sucrose gradients are eluted in the exclusion volume of these Sephacryl columns (not illustrated).

It appears that chromatography on Sephacryl-S-200 columns can be used with advantage to supplement the sucrose gradient technique, when information is sought on the molecular size(s) of ER in human breast cancer cytosols. It could also be used as an extension or addition to the charcoal assay, especially if the available material is scanty and a single dose assay is performed.

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