Journal of Chromatography, 266 (1983) 129–139
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 013

USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE EVALUATION OF THE SYNTHESIS AND BINDING OF FLUORESCEIN-LINKED STEROIDS TO ESTROGEN RECEPTORS

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SUMMARY

A fluorescein-linked estrogen was synthesized as a non-invasive, non-radiochemical means of detecting the levels and distribution of estrogen receptors in histological preparations of breast and endometrium. 17α-Ethynylestradiol-21-carboxylic acid was coupled via octane-l ,8-diamine to fluorescein-isothiocyanate yielding a promising ligand, N-fluoresceinyl-5,N"-[8-(3,17P-dihydroxy- 19-nor-17α-pregna-1,3,5(10)-triene-20-yne-21-carboxylic acid amide)]octylthiourea (FXDE) for an estrogen receptor. High-performance liquid chromatography on preparative reversed-phase C_{18} columns was used to purify the final product. Using cytosolic receptor preparations from bovine uterus and human uterus and breast cancer, the binding of F8DE was determined by competition analyses to have a K_d value of 10^{-8} *M*. High- and low-molecular-weight forms of estrogen receptors were separated on TSK 3000SW and 4000SW columns by high-performance size-exclusion chromatography. Specific binding of radio labeled estradiol-17β to these forms was inhibited in the presence of F8DE, indicating association with the fluorescein-linked steroid.

INTRODUCTION

The determination of estrogen receptors in biopsy specimens of breast and endometrial carcinomas plays an important role in the selection of patients most likely to respond to additive or surgical ablative hormone therapies (e.g. refs. 1–3). Current methods of assessment of estrogen receptors employ radioligand binding assays in which either [^{3}H]estradiol-17β (ref. 4) or [^{125}I]iodoestradiol-17β (ref. 5) and cytosol, prepared from target tissues, are used.

A method of determining estrogen receptors by non-radioactive means is the incubation of tissue slices with a fluorescein-linked estrogen and visualization of the "receptor-bound" steroid under a fluorescence microscope[6–10]. So far, the use of such compounds has had two major disadvantages: (1) derivatization of estradiol in the 17β-position reduces the affinity of the ligand for the specific binding sites of the receptors, and/or (2) bonds between the spacer and the fluorescent steroid ligand were labile, making assessment of the affinity constant difficult due to contaminating

0021-9673/83/$03.00

free estradiol. These and other complicating factors such as tissue autofluorescence have made the unequivocal determination of estrogen receptors in tissue preparations difficult'[1].

To circumvent these problems we synthesized N-fluoresceinyl-5,N'-[8-(3,17β-dihydroxy-19-nor-17a-pregna-1,3,5(10)-triene-20-yne-21-carboxylic acid amide)]-octylthiourea (F8DE) from ethynylestradiol linked to fluorescein-isothiocyanate via octane-1,8-diamine. This substance has an intact 17β-hydroxyl group needed for high affinity interaction with estrogen receptors and a more stabile amide bond between the spacer and the steroid ligand.

MATERIALS AND METHODS

Columns and instrumentation

The preparative C_{18} column, Synchropak RP-P (25 cm × 10 mm I.D., particle size 5 μm) was purchased from SynChrom (Linden, IN, U.S.A.). The analytical column for steroid separations (Ultrasphere-ODS, 25 cm x 4.6 mm I.D., particle size 5 μm) and the size-exclusion columns for receptor separation (Spherogel TSK 3000SW and TSK 4000SW, particle sizes 10 and 13 μm, respectively) were obtained from Altex (Beckman Instruments, Palo Alto, CA, U.S.A.). HPLC was performed with a Beckman 112 solvent delivery module, including a Model 421 system controller and injector block. Chromatograms were recorded with a Hitachi 100-40 variable-wavelength UV spectrophotometer connected to a Zanen BD42 recorder at either 280 or 495 nm. The solvent used for separations on reversed-phase columns consisted of methanol-water-acetic acid mixtures (see legend to Fig. 2 for details). All solvents were filtered (pore size < 0.45 μm) prior to use. Receptor preparations were passed through an Altex Type GSWP precolumn (7.5 cm × 7.5 mm I.D.) prior to separation on size-exclusion columns. Calibration of size-exclusion columns was performed in TEGM buffer (10 m*M* Tri–HCl, pH 7.4 containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol and 10% glycerol) with 100 m*M* potassium chloride at a flow-rate of 0.45 ml/min as described earlier[12]. Fractions were collected at 1-min intervals and counted for ^{3}H or ^{125}I. Spectrophotometric and spectrofluorometric measurements were taken on a Model 25 Beckman spectrophotometer and an Aminco-Bowman spectrofluorometer. In certain experiments TEG buffer (TEGM without monothioglycerol) was used.

Synthesis of fluorescein-linked estrogen

Fig. 1 depicts the synthetic route for the preparation of the fluorescein-linked estrogen, F8DE (compound VI) following commonly accepted methods. Compound II is produced by reaction of ethynylestradiol with methyllithium in anhydrous tetrahydrofuran under a nitrogen atmosphere. The synthesis of the carboxylic acid derivative of ethynylestradiol (compound III) proceeds by the carboxylation of the anion (compound II) with carbon dioxide gas[13]. Compound V (Fig. 1) is prepared by treating octane-1,8-diamine with the N-hydroxysuccinimide-activated ester (compound IV). The coupling of compound V to fluorescein-isothiocyanate is performed in a saturated solution of potassium carbonate in ethanol overnight at room temperature to yield F8DE (compound VI). Table I provides the chemical nomenclature of F8DE and its precursors

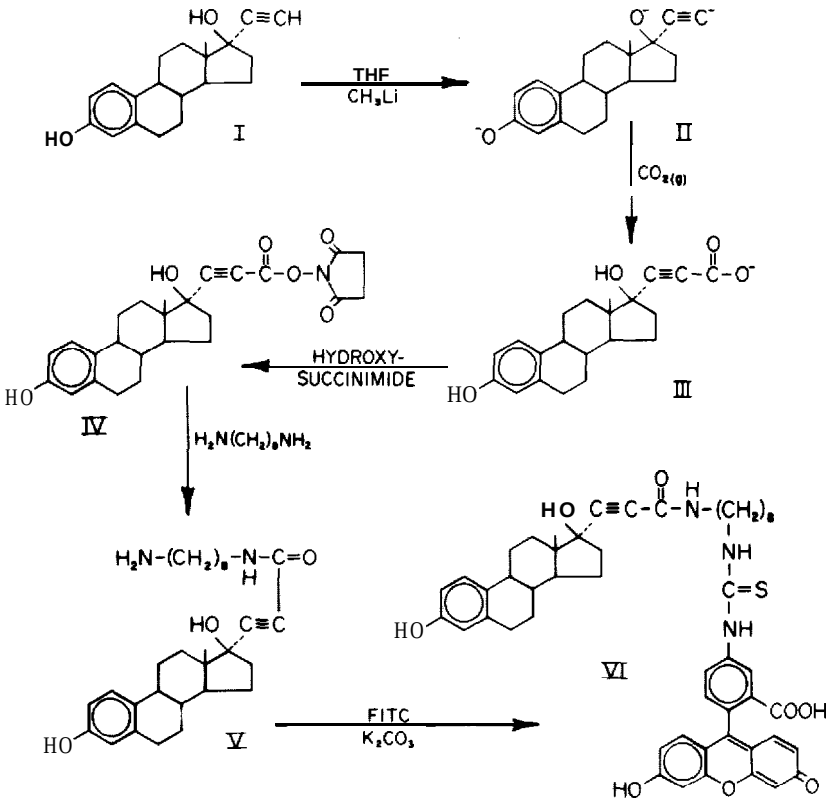

Fig. 1. Pathway for the synthesis of F8DE (compound VI). The chemical names of the fluorescein-linked steroid and its precursors are given in Table I.

TABLE I

CHEMICAL NOMENCLATURE OF F8DE AND ITS PRECURSORS

Structural formulae of F8DE (compound VI) and its precursors are given in Fig. 1

Compound No.	*Name*
I	3,17β-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-20-yne
II	Intermediate of compound I, stabilized under anhydrous conditions
III	3,17β-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-20-yne-21-carboxylic acid
IV	3,17β-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-20-yne-21-carboxylic acid N-succinimide
V	3,17β-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-20-yne-21-carboxylic acid-amide-N-(8-amino-octyl)
VI	N-Fluoresceinyl-5,N′-[8-(3,17β-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-20-yne-2l-carboxylic acid amide)]octylthiourea

Preparation of estrogen receptors

Human tissues used in this study were obtained through pathologists at local hospitals. Specimens were either frozen at once or brought to the laboratory on dry ice and then deep-frozen in liquid nitrogen. All tissues were stored at −86°C. Tumor pathology examinations served to confirm the nature of the carcinoma specimens. Calf uteri were obtained from a local slaughterhouse. Samples of fresh or frozen tissues were homogenized at 4°C with a Brinkman Polytron (two 10-sec bursts) in TEGM buffer. The homogenate was centrifuged at 4°C at 110,000 g for 60 min to prepare the cytosol. The lower phase was separated from the supernatant lipids and used in the experiments. Protein was determined by the method of Waddell[14]; cytosols routinely contained 5510 mg cytosol protein/ml.

The ligands, [^{3}H]estradiol-17β and 16α-[^{125}I]iodoestradiol-17β, were obtained from New England Nuclear (Boston, MA, U.S.A.). Unlabeled diethylstilbestrol was purchased from Sigma (St. Louis, MO, U.S.A.). Cytosols were incubated at 4°C for 4 h with 4–5 n*M* [^{125}I]iodoestradiol-17β in the presence or absence, depending upon the experiment, of 200-fold molar excess of diethylstilbestrol. Just before the high-performance size-exclusion chromatographic (HPSEC) experiment, unbound steroid was removed as a pellet by a 10-min incubation with an equal volume of a solution of 1% dextran-coated charcoal (DCC). The cytosols were either applied immediately to Spherogel columns or used in the inhibition experiments. Radioactivity was determined with either a Micromedics 6/400 gamma counter or a Beckman LS-9000 liquid scintillation counter.

RESULTS AND DISCUSSION

Synthesis, purification and characterization

The reactions used in the synthesis of F8DE are commonly known, but new aspects of their utilization will be described in detail elsewhere[15]. The carboxylic acid (III) contained a small amount of ethynylestradiol after the conventional work-up procedures such as extraction and recrystallization[13]. The retention time of ethynylestradiol and III on C_{18} columns was short enough to separate them from either V or VI (data not shown). Initial attempts to purify V by HPLC on the preparative C_{18} column with a methanol-water eluent were unsatisfactory because the compound interacted with the column packing material. The inclusion of acetic acid (2–3%) prevented this. Higher concentrations of acetic acid made retention times too short to resolve these components.

The chromatographic profiles given in Fig. 2A and B illustrate that the successful separation of V may be accomplished by a linear gradient of methanol–water-acetic acid, ranging from a concentration ratio of 60:40:3 to 65:35:3.25 over 30 min. The purity of F8DE (peak 3, Fig. 2B) was verified by chromatographing it on the preparative reversed-phase column (Fig. 2C) and on an analytical C_{18} column (data not shown). This highly purified material was characterized further spectrophotometrically and served as the probe for interaction with estrogen receptors in both breast cancer and uterus tissues.

For further characterization, UV and visible light spectra were obtained. The spectra showed no marked differences between 3′,6′-dihydroxy-5-isothionatospiro-[isobenzofuran-1(3H),9′[9H]xanthen]-3-one (FITC) and F8DE (Fig. 3) with both

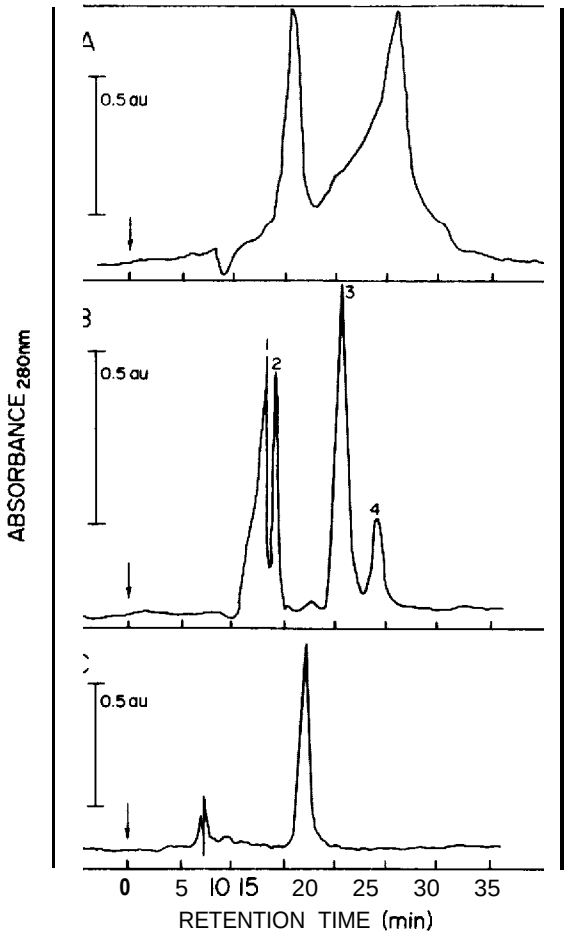

Fig. 2. Purification of F8DE by preparative C_{18} HPLC. (A) Isocratic elution with methanol-water-acetic acid (80:20:2). (B) Elution by a 30-min linear gradient of methanol-water-acetic acid, beginning at concentration ratios of 60:40:3 and ending at 65:35:3.25). (C) Isocratic elution with methanol-water-acetic acid (65:35:3.25) of peak 3 (Panel B). Fractions from several chromatograms were pooled, evaporated to dryness and redissolved in 99% ethanol. The arrows in each panel indicate the time of sample injection.

compounds exhibiting absorbance maxima at 230 and 498 nm. Fig. 4 shows the excitation and emission bands of FITC and F8DE. It is noteworthy that $\Delta(\lambda_{ex} - A_{,,})$ for F8DE decreased after the derivatization of the FITC. The extinction coefficient (ε_{498}) ofpurified F8DE (mol. wt. 857) was determined to be *ca.* 21,000 $M^{-1}cm^{-1}$. Since the spectral properties of peak 3 from the HPLC separation (Fig. 2B) were similar to FITC, it was essential to characterize further the interaction of this compound with estrogen receptors.

To test the possible use of fluorescence microscopy to detect a physiological concentration of fluorescein-labeled estradiol, the concentration dependence of the relative fluorescence intensity of FITC solutions was determined (Fig. 5). It was found that below 10^{-8} *M*, no concentration dependence of the relative fluorescence could be detected. This quantity appears to represent the upper concentration range

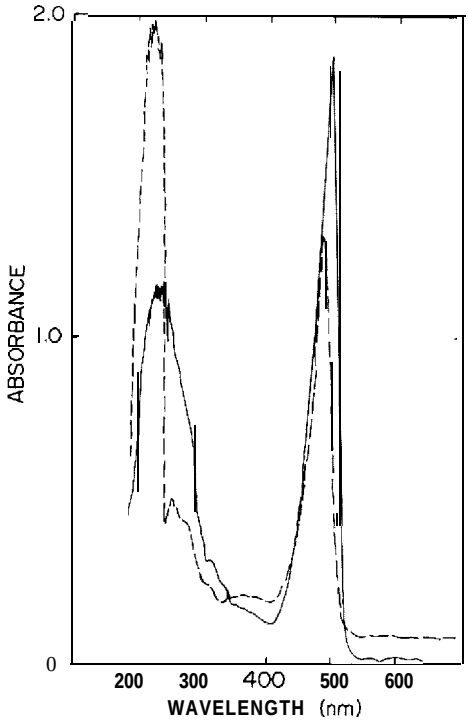

Fig. 3. Absorbance profiles of FXDE (solid line) and FITC (dashed line) in the UV and visible spectrum. Concentrations: F8DE, $1.2 10^{-4} M$: FITC, $7.5\ 10^{-5}$ A4 in TEG buffer.

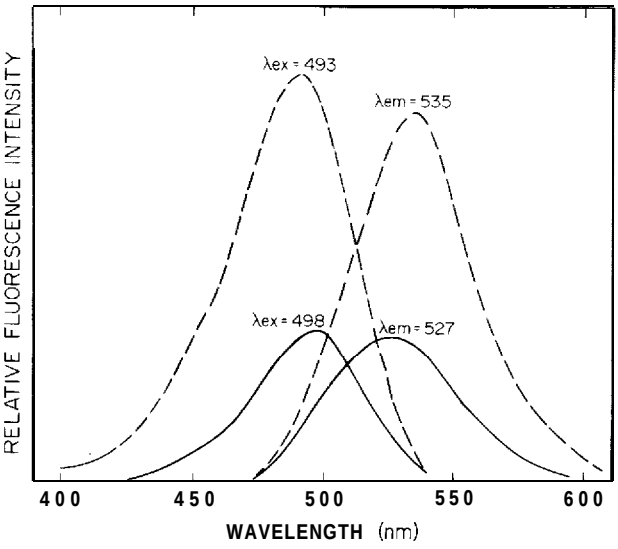

Fig. 4. Excitation (ex) and emission (em) spectra of F8DE (solid line) and FITC (dashed line). Concentrations: F8DE, $7.95\ 10^{-6} M$; FITC, $3.24\ 10^{-6} M$ in TEG buffer, pH 7.4.

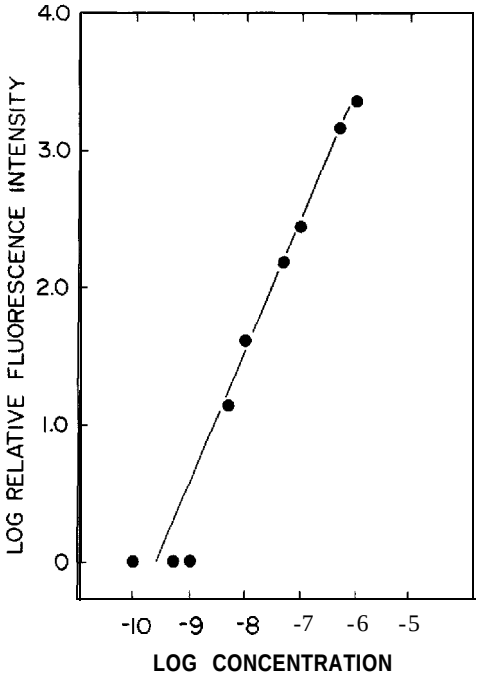

Fig. 5. The concentration-dependent fluorescence of FITC in TEG buffer, pH 7.4.

of a physiologically occurring receptor of about 20,000 sites per cell[16]. It appears that special fluorescence microscopy techniques, such as single photon counting, will have to be employed for the detection of lower levels of estrogen receptors in tissue slices. Incubation with very high concentrations of fluorescein-labeled steroid may result in the labeling of type II and III estrogen binding sites[17].

The structure of F8DE confers a potential advantage relative to N-fluoresceino-N'-[17β-(estradiol-hemisuccinamido)ethyl]-thiourea[8], another fluorescein-labeled estrogen previously analyzed for receptor-binding character in this laboratory. This compound has been criticized' as being susceptible to cleavage by cytosolic enzymes, particularly at the ester bond formed with the oxygen function of the 17β-hydroxyl group. F8DE contains no ester bonds. However, prolonged incubation of F8DE with cytosol or cells would be necessary to dismiss any possibility of enzymatic or time-dependent hydrolysis postulated by Chamness et *al.*[7] as the phenomenon responsible for the appearance of unlabeled estradiol-17β in a sample of the compound synthesized by Barrows ***et al.***[8].

Receptor interaction

To ascertain the interaction of F8DE and its precursors with estrogen receptors, cytosol was prepared from both calf and human uterus as well as from human breast carcinomas. Using a competition assay with either [^{3}H]estradiol-17β or [^{125}I]iodoestradiol-17β as ligand, increasing concentrations of F8DE, FITC, estradiol-17β and diethylstilbestrol were added to cytosol to evaluate binding (Fig. 6). As expected, both estradiol-17β and diethylstilbestrol were potent competitors, exhibit-

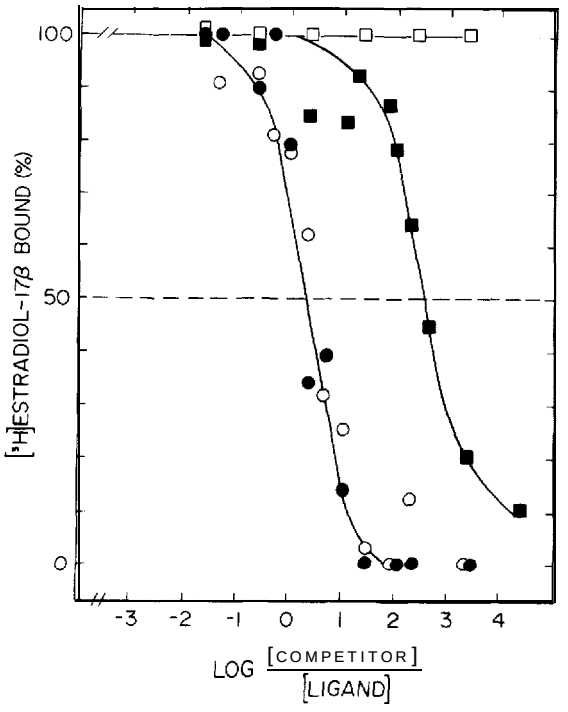

Fig. 6. Inhibition of [³H]estradiol-17β binding to bovine uterine estrogen receptors by F8DE (■) was compared to inhibition by estradiol-17β (●), diethylstilbestrol (○) and FITC (□). Cytosol prepared from powdered tissue in TEG buffer, pH 7.4 (0°C) incubated for 16 h at 0–3°C with 6 n*M* [³H]estradiol and assayed for specific binding in the presence of inhibitors, using the DCC technique as described in Materials and methods.

ing apparent dissociation constant (K_d) values of 10^{-10}–10^{-11} A4 (ref. 18). In the type of analysis suggested by Rodbard[19], F8DE gave an apparent dissociation constant k_d value of *ca.* 10^{-8} *M*, as might be expected upon the derivatization of an estrogenic ligand[13]. Similar results for the K_d value of F8DE were obtained with human breast carcinomas. Furthermore, no inhibition of estradiol binding was observed with FITC alone at concentrations in up to 10,000-fold excess of the labeled ligand (Fig. 6).

Other investigators have reported the synthesis of fluorescein-labeled derivatives that apparently interact with estrogen receptors in cell-free preparations and in tissue slices. For example, Barrows et *al.*[8] prepared a fluorescein derivative of estradiol linked through the 17β position which was purported to have an apparent K_d value of 10^{-6}–10^{-7} *M*. This compound later proved to be contaminated with estradiol-17β. (ref. 7). Another attempt was reported at the synthesis of estradiol derivatized at the 6-position[6], a compound which reportedly showed spontaneous decomposition[10]. Two additional attempts to synthesize fluorescein-linked estrogen, an estrone 17 derivative[20] and an estradiol- 17β derivative[9], yielded compounds which gave apparent K_d values of *ca.* 10^{-9} *M* when cell-free preparations of estrogen receptors were used in a competition assay similar to that reported in this paper. A crucial problem in the study of Daxenbichler *et al.*[9] was that a saturating concentration of [³H]estradiol-17β was not used and that the apparent K_d value of the ³H-ligand–

receptor complexes was reported as 0.8 10^{-9} M -clearly higher than expected of estrogen receptors (e.g. refs. 4 and 18). Furthermore, in unpublished experiments conducted in our laboratory that compound exhibited the same instability problems in solution as the substance reported by Barrows *et al.*[8].

A more promising fluorescein-linked estrogen was reported by Danliker *et al.*[20]. That estrone derivative was evaluated in human and mouse breast cancer cells with some success[21]. Dandliker et *al.*[20] were first to demonstrate by a direct binding assay, *i.e.* fluorescence polarization, that the estrone-fluorescein derivative was interacting with specific estrogen binding sites. The interaction of fluorescein-estrogen

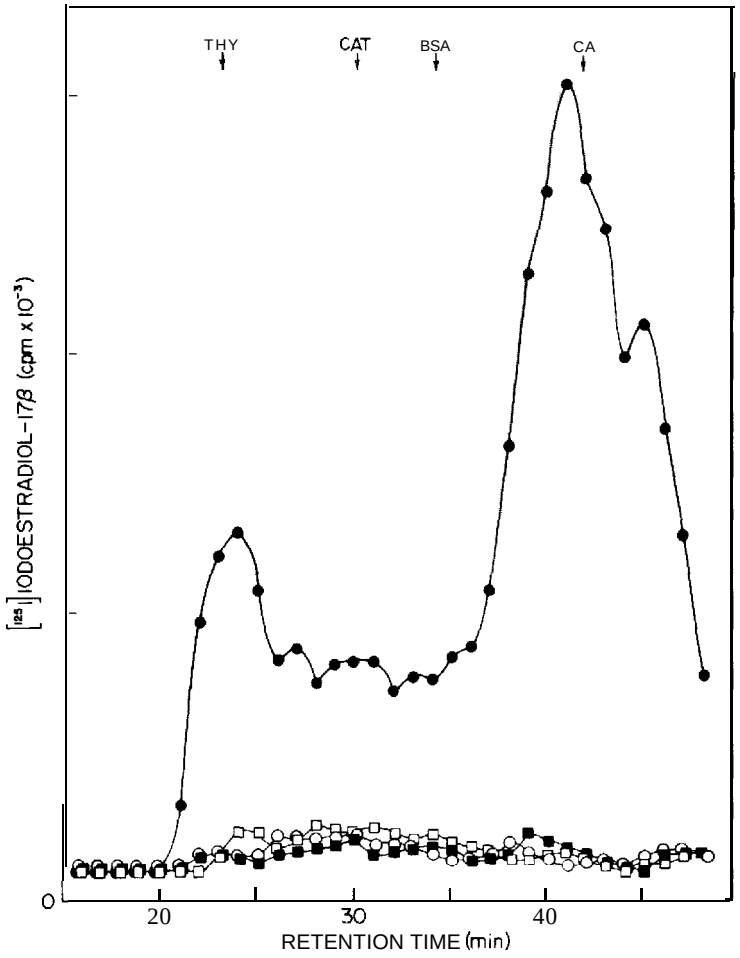

Fig. 7. Estrogen receptors from cytosol of human breast carcinomas were chromatographed on a TSK 3000SW column at a rate of 0.45 ml/min with 15 mM Tris–HCl buffer, pH 7.4 (0°C) containing 1.5 mM EDTA, 10 mM monothioglycerol, 100 mM potassium chloride and 10% glycerol. Marker proteins were separated in a similar fashion: THY = thyroglobulin; CAT = catalase; BSA = bovine serum albumin; CA = carbonic anhydrase. Cytosol was prepared as described under Materials and methods and incubated for 16 h with 4 nM [^{125}I]iodoestradiol-17β alone (●) or in the presence of 0.8 . 10^{-6} M DES (0), 4 · 10^{-5} M FIDE (■) or 4 10^{-5} M compound V (□). Free steroid was removed by DCC pellet prior to application of sample to column.

derivatives with the different species (isoforms) of the estrogen receptors in target organs, which we have demonstrated[12,22] has not been evaluated.

This important question cannot be addressed readily in a conventional competition assay. However, we have demonstrated that the various isoforms of estrogen receptors in cytosol may be separated by HPSEC[12,22] and by HPLC chromatofocusing[23]. Employing HPSEC on TSK 3000SW and TSK 4000SW columns, we have demonstrated that F8DE associates with a wide variety of estrogen receptor isoforms (Fig. 7). When receptor preparations from both human breast carcinomas and uteri were used, F8DE and its precursor (compound V) inhibited both the lower- (Stokes radius, 29-32 Å) and higher- (Stokes radius, 61-63 Å) molecular-weight forms[12] at a large excess of competitor as shown by analysis on TSK 3000SW columns. Furthermore, this inhibition of the isoforms by both compounds was concentration-dependent and showed no discernable difference between the species under the conditions analyzed. Both receptor forms, isolated by TSK 4000SW chromatography, showed inhibition by F8DE (data not presented).

In conclusion, we have synthesized a promising fluorescein-linked estrogen, F8DE, which may be readily purified in large amounts (1 mg/chromatogram) by HPLC on preparative C_{18} reversed-phase columns. Competition analysis with cell-free preparations from calf and human uteri and breast cancer indicated that F8DE exhibited a high affinity for estrogen receptors. Finally, it has been demonstrated that F8DE inhibited the binding of radiolabeled estradiol-17β to both the high- and low-molecular-weight forms of estrogen receptors, separated by HPSEC. These data suggest that HPLC separations of both the lower-molecular-weight ligands and the high-molecular-weight receptor proteins will play an important role in the characterization of these entities in the future.

ACKNOWLEDGEMENTS

We wish to acknowledge the technical assistance of Ms. Rhonda Huotari and Ms. Judith Siegel and the preparation of the typescript by Ms. Debbie Keidel. We appreciate the critical review of the manuscript by Drs. T. W. Hutchens and R. D. Wiehle. The assistance of Drs. James G. Kuhns, George Barrows and Joseph Sanfilippo in the collection of human tissues is greatly appreciated. This research was supported in part by the Marie Overbey Memorial Grant from the American Cancer Society and by the Phi Beta Psi Sorority. M. L. is the recipient of fellowships from the University of Louisville Cancer Center and the Deutsche Forschungsgemeinschaft. N. C. C., Jr., is the recipient of a Homberger Fellowship.

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