RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Radioiodinated Estrogen Derivatives

J. Krijn Mazaitis, Raymond E. Gibson, Toru Komai, William C. Eckelman, Barbara Francis, and Richard C. Reba

George Washington University, Washington, D.C. and Central Research Laboratory, Sankyo Co., Ltd., Tokyo, Japan

Monoiodohexestrol exhibits 10 to 15% specific binding to the 8S estrogen receptor while the remainder binds to nonreceptor 4S proteins. Reduction of nonreceptor binding with either thyroxine or 8-anilino-1-naphthalene sulfonic acid was not quantitative. Thus no accurate determination of the concentration of receptor sites in the radioreceptor assay was possible by graphical analysis.

Two additional estrogens—17α-[^{125}I]iodoethynylestradiol and 17α-[^{125}I]iodoethynyl-11β-methoxy estradiol—were synthesized at high specific activity. Although the iodoethynyl derivatives were stable under synthetic conditions, deiodination in the presence of proteins is too fast to allow either in vivo or in vitro use.

To make these compounds clinically useful, therefore, chemical modification to reduce nonreceptor binding and the rate of dehalogenation must be undertaken.

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In the field of nuclear medicine, a radioiodinated tracer for the hormone estradiol may provide a scanning agent to visualize estrogen-dependent tissues by binding to estrogen receptors (*1*)—especially breast tumors containing estrogen receptors, and their metastases (*2*).

An ideal tracer should have high affinity and high specificity for the receptor, and minimal binding to nonreceptor proteins. Generally speaking, iodination reduces the affinity and the specificity for the receptor (*3,4*). An example is monoiodohexestrol (I-Hex) whose specific binding of 10 to 15% is almost masked by its nonreceptor binding (*3*). This is also reflected in the in vivo distribution studies where the uterus-to-blood ratio (i.e., T/NT) was 1.87. Preinjection with thyroxine improved the ratio to 10.4, by both an increase of the percentage dose per gram in the uterus and a decrease in the blood. These results suggest a decrease of the nonreceptor binding of I-Hex in the blood and an increase in the specific binding in the uterus as a result of thyroxine. We therefore evaluated I-Hex plus thyroxine as a radioligand for the in vitro radioreceptor assay.

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For reprints contact: J. Krijn Mazaitis, Radiopharmaceutical Chemistry Sect., George Washington Univ., 2300 Eye St., N.W., Washington, DC 20037.

In our quest for a tracer we also selected 17α-ethynyl estradiol (EE_2) and 17α-ethynyl-11β-methoxy estradiol (R2858 ≡ EME_2)* as starting materials. The ethynyl group decreases metabolism of the hormone and increases affinity for the estrogen receptor; the 11β-methoxy group decreases the affinity somewhat but eliminates virtually all nonreceptor binding (*5*). As suggested by in vivo studies of halogenated 3-methoxy-ethynyl estradiols (*6*), replacement of the ethynylhydrogen by a radioiodine might give an authentic tracer for estradiol (E_2). We describe here the synthesis of these compounds (I-EE_2 and I-EME_2) with I-127 and carrier-free I-125, and the results of in vitro studies of its receptor binding.

MATERIALS AND METHODS

Synthesis of I-EE_2 via morpholine-iodine complex method (*7*). Iodine (1 mmol, 254 mg) was dissolved under stirring in 5–10 ml CH_2Cl_2/CH_3OH (1/1). First 4.5 mmol morpholine (0.4 ml) were added, followed by 1 mmol (400 mg) of the 3-benzoate ester of EE_2 (EE_2-3 OBz). After 2 to 3 days stirring at room temperature no more change occurred; the solvent was then evaporated, the residue dissolved in ethyl acetate (EtOAc), washed with water, and dried over Na_2SO_4. Purification took

place on preparative TLC (20 × 20 cm; 0.5-mm-thick silica gel F254† with CH_2Cl_2/CH_3OH (99/1) as solvent. The bands were scraped off and extracted with CH_2Cl_2. This isolated 303 mg (58%) of the iodinated benzoate ester (I-EE_2-3 OBz) and 56 mg of starting material. Purity of the product was checked by TLC on silica gel F254 glass plates† in (a) benzene/ethyl acetate (4/1) and (b) methylene chloride/methanol (99/1). The compounds were visualized under uv. The estradiol derivatives were located by the reaction of 1-nitroso-2-napthol with the phenolic ring. The following R_f values were observed: in (a), R_f for EE_2 = 0.28, for I-EE_2 = 0.36, for E_2-3 OBz = 0.41, and for I-EE_2-3 OBz = 0.53; and in (b), R_f for EE_2 = 0.15, for I-EE_2 = 0.22, and for EE_2-3 OBz = 0.38.

Hydrolysis of I-EE_2-3 OBz. To hydrolyze I-EE_2-3 OBz, 303 mg were dissolved in 10 ml MeOH and 0.5 ml H_2O. The reaction mixture was gently refluxed with an excess of $NaHCO_3$ until the hydrolysis was complete (≃ 2 hr) as checked by TLC. Less than 10% deiodination took place to form EE_2.

The purification was done on preparative TLC with CH_2Cl_2/CH_3OH (99/1) as solvent. The I-EE_2 band was scaped off, extracted with CH_2Cl_2/0.5% CH_3OH, and the solvent evaporated to yield 174 mg I-EE_2 (71%). Purity was checked by TLC. The uv spectrum in CH_3OH had a maximum at 281 nm with a shoulder at 287 nm. The product has the same spectrum as EE_2, indicating an unchanged phenolic ring. IR spectrum‡ in KBr pellet showed absorption for −C ≡ CI at 2160, 620, and 630 cm^{-1}, whereas EE_2 had absorption bands at 2100, 640, 660, and 3300 cm^{-1} for −C ≡ CH. Elemental analysis (EA) for $C_{20}H_{23}O_2I \cdot H_2O$: calculated C = 54.60, H = 5.67; found C = 54.40, H = 5.66.

Synthesis of EE_2-3 OBz. EE_2 (1 mmol, 300 mg), benzoic anhydride (10 mmol, 2.5 g), and dry pyridine (0.5 mmol, 0.048 ml) were dissolved in 5 ml dry benzene under stirring. The solution was refluxed under a drying tube for 2 to 3 days. The benzene was evaporated and the residue dissolved in a small amount of CH_2Cl_2/0.2% CH_3OH. The benzoate ester was purified over a column (25 cm long, 2.5 cm in diameter) packed with silica gel 60, eluted with CH_2Cl_2/0.2% CH_3OH, and monitored at 280 nm. The product was checked for purity by TLC: m.p. 199–201°C; IR 1060 cm^{-1} 17β-OH, 1740 cm^{-1} C = 0 ester; yield 90%.

Synthesis of I-EE_2 via chloramine T and NaI iodination. EE_2-3OBz (133 mg, 0.33 mmol) and NaI (50 mg, 0.33 mmol) were dissolved in 8 ml tetrahydrofuran (THF) and 2 ml H_2O. To this 94 mg (0.33 mmoles) of chloramine-T were added and the reaction mixture was stirred until a reasonable yield was detected using TLC. About 15 mg of I-EE_2-3 OBz were isolated and purified. Elemental analysis for $C_{27}H_{27}O_3I$: calculated C = 61.60, H = 5.17; found C = 61.53, H = 5.26.

Hydrolysis and subsequent isolation of this I-EE_2 proceeded as in the morpholine-iodine procedure. The two products showed the same chromotographic behavior, spectral properties, and elemental analysis.

Synthesis of EME_2. EME_2 was iodinated by the morpholine-iodine procedure. Because of the small amounts of material available, the identification of product was done via R_f value on TLC (uv visualization and color reaction indicated an E_2 derivative). In benzene/ethyl acetate (4/1), R_f for EME_2 = 0.21, for I-EME_2 = 0.27, for EME_2-3 OBz = 0.57, and for I-EME_2-3 OBz = 0.62. The uv spectrum of I-EME_2 showed an unchanged A ring.

Synthesis of carrier-free [^{125}I]I-EE_2. Two microliters of 0.5 *N* HCl and 300 μg (0.75 μmoles) of EE_2-3 OBz dissolved in 50 μl tetrahydrofuran were added to a vial containing 3 mCi of high-specific-activity Na^{125}I. Chloramine-T (211 μg, 0.75 μmole) dissolved in 5 μl of water was then added to the vial and the reaction mixture was monitored for the production of [^{125}I]I-EE_2-3 OBz by TLC as described above. [^{125}I]I-EE_2-3 OBz was isolated and purified on a 10-μ column, 250 mm in length and 3.2 mm internal diameter.‖ The solvent system was CH_3OH/H_2O (75/25) at 2 ml/min; EE_2-3 OBz appeared at 15.5 min; I-EE_2-3 OBz at 20.5 min.

The iodinated benzoate was hydrolyzed with 10 μl of 0.1 *N* NaOH while most of the solvent was being evaporated. The hydrolysis was monitored by TLC. The [^{125}I]I-EE_2 was also isolated and purified by HPLC using ethanol/H_2O (35/65) at 0.7 ml/min (EE_2 at 10 min: I-EE_2 at 16.5 min). Approximately 200 μCi of the [^{125}I]I-EE_2 were prepared at a specific activity of 2200 Ci/mmol based on complete removal of unreacted EE_2 by the two preparative HPLC chromatographic procedures.

Synthesis of carrier-free [^{125}I]I-EME_2-3 OBz. The procedure was the same as for carrier-free iodination of [^{125}I]I-EE_2.

Synthesis of Carrier-Free [^{125}I]I-Hex. To a U-shaped vial containing approximately 3 mCi of carrier-free Na^{125}I in less than 10 μl was added 10 μl of hexestrol (225 μg) dissolved in ethanol/0.03 *M* phosphate buffer (55/45, pH ~7.5) and 5 μl chloramine-T (0.15 μmol) dissolved in ethanol/phosphate buffer (1/1). After the reaction proceeded for 5 min, the reaction mixture was injected onto an Altex reverse-phase column‖ and eluted with ethanol/water (42/58) at 1 ml/min (Hex at 7.5 min; Cl-Hex at 13 min; I-Hex at 17 min). [^{125}I]I-Hex of specific activity 2200 Ci/mmol was isolated.

Synthesis of 3-Iodo-4-0-methyl hexestrol and 3-Iodo-4-0-hydroxyethyl hexestrol. Both compounds were prepared using the procedure for ether formation as described in (*8*).

Competitive binding assay using immature rat-uterus cytosol. This assay has been described in our previous report (*3*).

TABLE 1. COMPETITIVE BINDING ASSAY USING IMMATURE RAT-UTERUS CYTOSOL

pmol added	% [^{3}H]E_2 bound to receptor*		
	E_2	EE_2	I-EE_2
0	100	100	100
1	64.72	60.78	62.93
2	50.31	49.55	49.28
5	26.02	30.43	27.63
10	—	—	16.19

* Mean of two experiments.

Sucrose-gradient centrifugation. See ref. *3*.

In vitro stability of [^{125}I]I-EE_2 and [^{125}I]I-EME_2-3 OBz in rat plasma and uterine cytosol. [^{125}I]I-EE_2 was stored in 33% ethanol at room temperature. The radiochemical was chromatographed in three TLC systems: on System 1 (Whatman paper with 85% CH_3OH), R_f for I-EE_2 = 0.9, for I^- = 0.7; on System 2 (silica gel with $CHCl_3$/5% CH_3OH), R_f for I-EE_2 = 0.55, for I-EME_2-3 OBz = 0.4, for I^- = zero; on System 3 (silica gel with toluene/ethyl acetate [8:2]), R_f for I-EE_2 = 0.5, for I-EME_2-3 OBz = 0.26, for I^- = zero. Five microliters (20 μCi) [^{125}I]I-EE_2 or [^{125}I]I-EME_2-3 OBz were mixed with 1 ml of rat plasma or uterus cytosol and chromatographed every half hour up to 2 hr in two systems with nonradioactive EE_2 and I-EE_2 as references. The amounts of radioactivity corresponding to I-EME_2-3 OBz, I-EE_2, and I^- were estimated from integration of the radioactive peaks obtained by TLC scanning.

RESULTS AND DISCUSSION

Synthesis. It is clear that we can iodinate the ethynyl group of EE_2 without iodinating the phenolic ring (see Materials and Methods), and that this can be accomplished via the chloramine-T/NaI route (*10*). We thus have a protocol for the production of carrier-free [^{125}I]I-EE_2 with specific activity as high as 2200 Ci/mmol.

Competitive binding assay. Table 1 shows that the percentage inhibitions of [^{3}H]E_2 binding to rat-uterus cytosol receptor by E_2, EE_2, and I-EE_2 are virtually the same. Korenman, using rabbit-uterus cytosol, found that EE_2 is a more potent inhibitor (*11*).

In a competitive binding assay, [^{125}I]I-EE_2 could not be displaced by E_2, EE_2, or I-EE_2, but 50% of the radioactivity stayed in the protein fraction after 20 min, and 26% could not be absorbed by dextran coated charcoal (DCC) even at 24 hr. A sucrose-gradient centrifugation pattern, however, did not show radioactivity in the 8S or 4S estrogen receptor peak, indicating that I-EE_2 is not receptor-bound. This finding, and the displacement of [^{3}H]E_2 by cold I-EE_2, are both explainable by deiodination of I-EE_2 to EE_2.

TABLE 2. STABILITY OF IODINATED ESTROGENS IN RAT PLASMA
(% Radioactivity)

Time (hr)	Chromatographic System 2			
	I-EE_2	I^-	I-R2858 benzoate ester	I^-
0	70	30	40	60
0.5	60	40	0	100
1	30	70	0	100
1.5	15	85	0	100
2	5	95	0	100
Control 0–2 hr			85	15

In vitro stability of the iodoethynyl group. In spite of reports suggesting the contrary (*7,12,13*), we find that the iodoethynyl moiety is quite stable, both under the conditions of synthesis (pH 10, NaOH in CH_3OH, under reflux) and in methanol and water at neutral pH. The stability may depend on the presence of sulfhydryl groups.

Table 2 shows the results of the in vitro stability studies of I-EE_2 and I-EME_2-3 OBz in rat plasma. The results in System 3 in rat plasma and in Systems 1 and 3 in rat-uterus cytosol are similar. The bulk of the deiodination occurs within the first half hour and is complete within 1 hr. Since accumulation of estrogens occurs in 30–60 min in estrogen-responsive tissues, clearly deiodination is too fast for I-EE_2 or I-EME_2 to be effective tracers.

In-vitro binding of [^{125}I]I-Hex to estrogen receptors. In the present experiments we have used [^{125}I]I-Hex that is carrier free, of high specific activity, and free from Cl-Hex and Cl-I-Hex. As in our earlier in vivo work (*3*), cytosol was incubated with [^{125}I]I-Hex and a large amount of thyroxine (10^{-5} *M*) or the more soluble 8-anilino-1-naphthalene sulfonic acid (ANS) (10^{-4} *M*). ANS should have the same effect as thyroxine (*15*), since it is used in triiodothyronine radioimmunoassay to displace prealbumin binding. The [^{125}I]I-Hex binding was analyzed by sucrose-gradient centrifugation.

The patterns in Fig. 1 show that the specific binding of I-Hex to the estrogen receptor (8S peak, competable by E_2) increased from 13 to 30% when either thyroxine (10^{-5} *M*) or ANS (10^{-4} *M*) was added. The amount of [^{125}I]I-Hex that is nonreceptor-bound (4S peak, noncompetable by E_2) decreased.

We observed that the amount of E_2 bound to the receptor is twice that for I-Hex in the presence of thyroxine or ANS (Table 3). The ratio of the concentrations of receptor-bound E_2 and I-Hex ($B_{E_2}/B_{I\text{-}Hex}$) can be calculated from the ratio of the mass-action equations describing binding for each ligand:

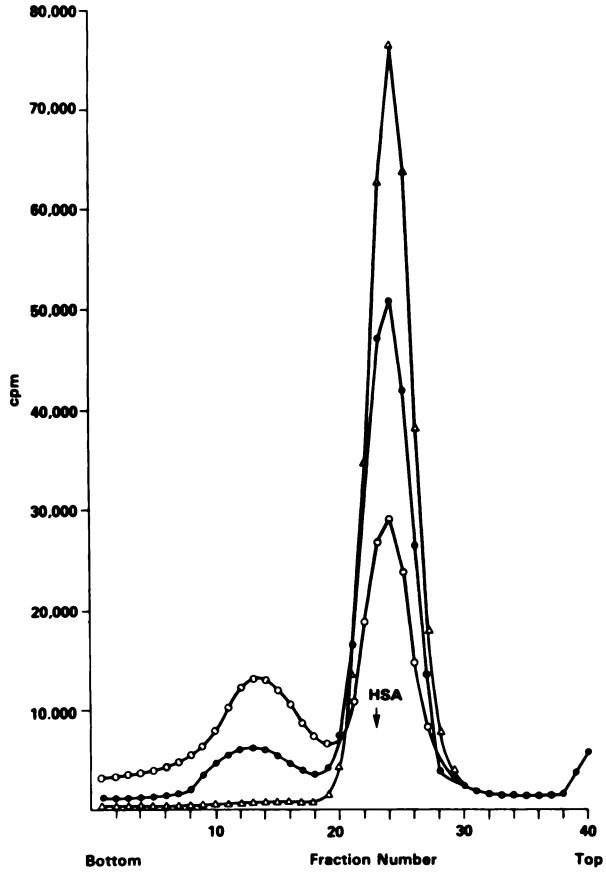

FIG. 1. Binding of [^{125}I]I-Hex to cytosol from immature rat uterus. Sucrose-gradient centrifugation patterns, (●) [^{125}I]I-Hex, (O) with 10^{-5} *M* ANS or T_4 and (△) with 10^{-5} *M* ANS or T_4 and 1 μmole of E_2.

$$\frac{B_{E_2}}{B_{I\text{-}Hex}} = \frac{(K_{d_{I\text{-}Hex}} + [I\text{-}Hex])[E_2]}{(K_{d_{E_2}} + [E_2])[I\text{-}Hex]}$$

Assuming that: (a) the dissociation constants, K_d, for the estrogen receptors of E_2 and I-Hex are the same (1.4×10^{-10}) (*14*); and (b) $[E_2]$ and [I-Hex], the free concentrations for E_2 or I-Hex, are the same as the initial concentrations. The calculated value of $B_{E_2}/B_{I\text{-}Hex}$ is about 1.1 but our observed value is 2.

This means that the dissociation constant of I-Hex would be five times that of E_2, and/or that the free concentrations of E_2 and I-Hex are much different from the initial concentrations. The affinity of I-Hex for the estrogen receptor is reported as 40% of that of E_2 (*14*), so it seems that the major difference is in the free concentrations. In the case of E_2, this is 70% of the initial concentration (Table 3), while for I-Hex it must be very small, since it cannot be measured by sucrose-gradient analysis (Fig. 1). This indicates that the 4S proteins compete effectively with the estrogen receptor for the I-Hex, either by affinity or more likely by capacity (*14*).

A Scatchard analysis to obtain the affinity constant of I-Hex for the estrogen receptor and the number of receptors per uterus as compared with E_2 will not be practicable until conditions can be found under which there is an accurately measurable amount of free I-Hex. In an attempt to achieve this by reduction of the nonreceptor binding, two derivatives of I-Hex less similar to thyroxine were synthesized: 3-iodo-4-O-methyl hexestrol, and the more polar 3-iodo-4-O-hydroxyethyl hexestrol. However, both compounds failed to displace [^{3}H]E_2 effectively from the receptor, most likely because of reduced affinity (*16*). Other methods to minimize the nonreceptor binding are purification of the estrogen receptor or addition of a more efficient competitor for the 4S-protein binding.

CONCLUSION

I-Hex binds too much to nonreceptor proteins—even in the presence of thyroxine or ANS—to be useful in the radioreceptor assay. Chemical modification of I-Hex, even a slightly more polar derivative, did not reduce the problems of nonreceptor binding.

I-EE_2 and I-EME_2 deiodinate at a rate that does not allow their use in vitro or in vivo. The bromoderivatives of these compounds should be more stable and consequently be better tracers for the parent compound.

FOOTNOTE

* Kindly furnished by J.-P. Raynaud, Centre de Recherches Roussel, Uclaf, France.

† EM Laboratories

‡ Beckman IR-20Z, Beckman Instruments

‖ Altex Reverse Phase Lichrosorb RP 18

TABLE 3. ANALYSIS OF RECEPTOR BINDING CAPACITY BY SUCROSE-GRADIENT CENTRIFUGATION

	[^{3}H]E_2	[^{125}I]I-Hex	[^{125}I]I-Hex + ANS	[^{125}I]I-Hex + T_4
Total concentration	1.06 n*M*	0.5 n*M*	0.5 n*M*	0.5 n*M*
% receptor bound	31.3	13.7	29.0	33.7
pmole bound	0.0663	0.0146	0.0273	0.0338
$\frac{\text{pmole bound}}{\text{g wet uterus}}$	1.84	0.406	0.758	0.939

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