Technetium-99m Complexes with Steroid-2-aminooxyethyliminodiacetic Acid Conjugates

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Conjugates of 2-aminooxyethyliminodiacetic acid with estrone, testosterone, epiandrosterone, 17-α-hydroxyprogesterone and progesterone were synthesized and their complexes with Tc-99m were successfully prepared in good yields, which indicated the agent to be promising for metal-labeling of biomolecules and related substances containing one or more carbonyl groups. The biodistribution and scintigraphic studies in mice bearing Ehrlich tumor and mammary tumor showed that the radioactivity accumulated considerably in the tumor tissues, but the tumor images were somewhat obscured.

Key words 2-aminooxyethyliminodiacetic acid; estrone; technetium-99m; bifunctional chelating agent; Ehrlich tumor; mammary tumor

Tritium (H-3) labeled estrogen was reported to localize in breast cancer[1] in 1961, and its localization mechanism was clearly explained by means of an intracellular hormone receptor[2] in 1980. Since H-3 is a beta-emitting nuclide, a quantitative assay of the receptor levels requires removal of a portion of the tissues. A more desirable method for characterizing the tissue *in situ* would be *in vivo* imaging using the hormone labeled by a single-photon or a positron-emitting radionuclide. Such an agent would be noninvasive and permit repeated evaluations of the same site and may allow the detection of microscopic foci associated with early primary or metastatic disease. Basic studies have been made on I-125 labeled estradiol[3—7], halogenated estrogens[8] and I-125 iodovinyl estrogens, and imaging with I-123 iodovinyl estrogens was studied.[9]

Since technetium-99m (Tc-99m) has ideal physical properties for many applications in nuclear medicine, Tc-99m-labeled imaging agents for mammary tumor have been required for many years. The use of bifunctional chelating agents with steroid-binding and metal chelating sites in the molecule may enable us to meet the requirement. Katzenellenbogen and co-workers prepared several steroid conjugates with a metal chelating site containing amino and thiol groups in order to image breast or prostate cancers based on their steroid receptor content.[10—12] The Tc-99m complex of a conjugate they prepared showed high affinity for the progesterone receptor but poor target tissue uptake efficiency and selectivity *in vivo*.

In the previous paper,[13] we reported the synthesis of 2-aminooxyethyliminodiacetic acid (**1**), a possible bifunctional chelating agent with both carbonyl binding and metal chelating sites in the molecule. The agent was expected to be used for the metal-labeling of biomolecules. The agent formed oxime-type conjugates with various carbonyl compounds under mild conditions. Conjugates of **1** with estrone, testosterone, epiandrosterone, 17-α-hydroxyprogesterone and progesterone were prepared.[14] Those of testosterone and 17-α-hydroxyprogesterone were 3-oximes, in which the metal chelating site was connected to the A ring of the steroids, while the chelating site was attached to the D ring in those of estrone and epiandrosterone. The conjugate with progesterone was 3,20-dioxime. The structures of the conjugates are shown in Chart 1.

The conjugates were easily labeled with Tc-99m by the conventional stannous chloride method. The biodistribution and scintigraphic studies in mice bearing Ehrlich tumor and mammary tumor were carried out using the Tc-99m complexes. The present paper describes the results. The reason for the use of the mice bearing Ehrlich tumor is that we have studied the biodistribution of many Tc-99m complexes in the animals and many published[15] and unpublished results have been accumulated.

Methods and Materials

Synthesis of the Conjugates The agent, **1**, and its conjugates with estrone, testosterone, epiandrosterone, 17-α-hydroxyprogesterone and progesterone were synthesized according to the method described previously.[13,14]

Formation of Tc-99m Complex A solution of 20 mg of a conjugate in 1.0 ml was adjusted to pH 7.0 with 0.1 M NaOH. A 75 μl volume of freshly prepared solution (2 mg/ml in 0.1 M HCl) of stannous chloride (Nacalai

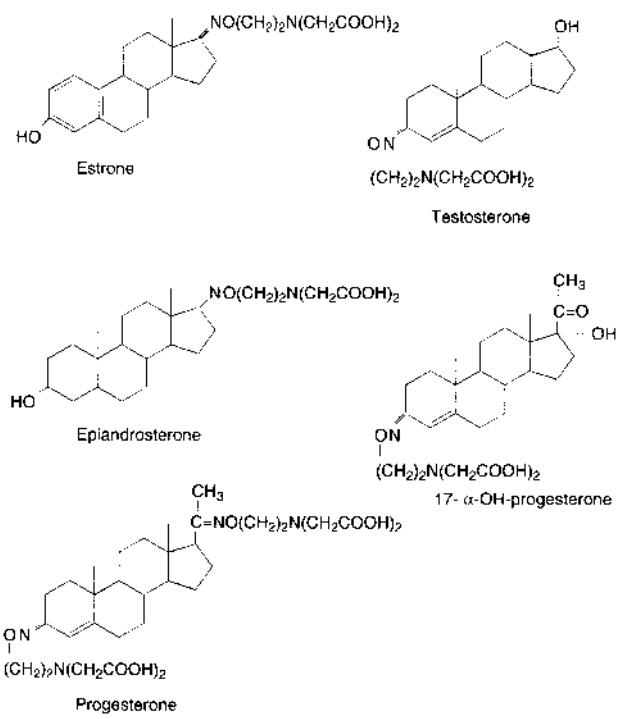

Chart 1. Steroid-2-aminooxyethyliminodiacetic Acids Conjugates

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Chemicals, Ltd., Kyoto, Japan) was then added and the pH was readjusted to 7.0. Two ml of the Tc-99m pertechnetate solution eluted from a sterile ^{99}Mo-^{99m}Tc shielded generator (Ultra-TechneKow, Dai-ichi Radioisotope Laboratories, Chiba, Japan) was added to the conjugate solution. The obtained solution was ready for injection after 10 min incubation at room temperature.

Quality Control a) Thin Layer Chromatography: The labeling efficiencies with Tc-99m were evaluated chromatographically using a 0.25 mm Silica-gel 60 F_{254} plate (E. Merck). The following three solvent systems were used to determine the radiochemical purity: Solvent A, acetonitrile : water (7 : 3), Solvent B, acetonitrile, and Solvent C, ethanol : water (7 : 3). The plates were counted by images in a gamma camera (Ohio-Nuclear Co.) equipped with a high resolution collimator with a digital computer (VP-450). The *Rf* values were determined relative to the peaks of Tc-99m pertechnetate and hydrolyzed Tc-99m colloid.

b) Paper Electrophoresis: The purities of Tc-99m-conjugates were determined by paper electrophoresis. Paper strips (Filter paper 51A, Toyo Roshi Kaisha, Ltd.) were run at a constant voltage of 600 V for 30 min using 0.1 M tris buffer, pH 7.4. The paper strips were counted by images in a gamma camera equipped with a high resolution collimator with a digital computer. Movement was determined relative to Tc-99m pertechnetate and hydrolyzed Tc-99m colloid.

Biological Studies Male ICR mice 5 weeks of age (Clea Japan, Tokyo, Japan) were inoculated subcutaneously with Ehrlich tumor cells (4×10^7 cells) into the right foreleg and left for a period of 2—3 weeks to allow tumor growth. Old aged female C3H/HeN Jcl mice (Clea Japan, Tokyo, Japan) bearing spontaneous mammary tumor were used in the scintigraphic study. Each mouse bearing a mammary tumor or Ehrlich tumor received 0.20 ml (3 MBq, 1.3 mg) of Tc-99m complex by tail vein administration. Sequential scintigrams were taken at predetermined intervals with a gamma camera equipped with a high resolution collimator with a digital computer. The ICR mice (4/group) were sacrificed with collection of blood in the heart at 1, 5, and 20 h after the injection. The organs, blood, some muscles, and the tumor were removed, weighed, and the radioactivity was counted by images in a gamma camera equipped with a high resolution collimator with a digital computer. The percentages of the injected dose per organ were determined by comparison of tissue radioactivity levels with the total radioactivity.

Results

Chemical Studies The steroids studied are practically insoluble in water and do not bind Tc-99m. Synthesized conjugates with **1** showed improved aqueous solubility of more than 40 mM at neutral and alkaline pH. The conjugates were completely labeled with Tc-99m under the conditions described above. The thin layer chromatograms (TLC) on silica gel and the paper electrophoresis did not indicate that Tc-99m pertechnetate and colloid forms of Tc-99m were present in detectable amounts in Tc-99m labeled solutions of the conjugates.

In TLC, Tc-99m pertechnetate moved to the front and hydrolyzed Tc-99m colloid remained at the origin with acetonitrile : water (7 : 3) (solvent A) and acetonitrile (solvent B) as the mobile phases. The Tc-99m complexes of the conjugates moved to the front with solvent A and remained at the origin with solvent B. With solvent C (ethanol: water (7 : 3)), the *Rf* values of Tc-99m complexes were as follows: estrone, 0.95; testosterone, 0.93; epiandrosterone, 0.91; 17-α-hydroxyprogesterone, 0.93; progesterone, 0.88; Tc-99m pertechnetate, 0.91.

The migration distances toward the anode in the electrophoresis on paper strips obtained by the Tc-99m complexes were as follows: estrone, 2.4 cm; testosterone, 2.4 cm; epiandrosterone, 2.1 cm; 17-α-hydroxyprogesterone, 2.2 cm; progesterone, 2.2 cm; Tc-99m pertechnetate, 7.8 cm. Under the same experimental conditions, hydrolyzed Tc-99m colloid showed no migration.

Biological Studies The organ distribution of the Tc-99m complexes in mice at 30 min, 1 h, and 3 h after injection are shown in Tables 1—5. Reduced urinary excretion of Tc-99m estrone conjugate compared with that of epiandrosterone should be ascribed to the aromatic nature of the steroid moiety. Similar biodistributions were observed in Tc-99m complexes of the conjugates of testosterone and epiandrosterone. The higher urinary excretion shown by that of progesterone should be due to the increased quantity of introduced iminodiacetic acid moiety. Lower tumor-to-blood activity ratios were observed with the Tc-99m complexes. The conjugates did not contribute largely to the accumulation of mammary tumors at an early stage.

Typical scintigrams of mice bearing mammary tumor, shown in Fig. 1, indicated that the Tc-99m complexes were excreted by the hepatobiliary route. The images of the mammary tumor and Ehrlich tumor were rather obscured. It

Table 1. Biodistribution of Tc-99m Estrone-1 Conjugate[a)]

Organ	30 min	1 h	3 h
Salivary glands	0.26±0.01	0.15±0.03	0.11±0.01
Spleen	0.12±0.02	0.09±0.01	0.08±0.02
Stomach	0.30±0.10	0.19±0.03	0.14±0.05
Intestine	11.04±2.42	13.66±0.85	15.52±0.49
Liver	29.89±4.26	28.96±1.36	22.84±0.33
Kidneys	9.74±2.41	11.54±0.61	8.58±1.68
Urine	23.13±2.89	29.10±0.31	38.97±5.34
Muscle 1 g	0.47±0.12	0.38±0.05	0.30±0.08
Tumor 1 g	1.05±0.22	0.81±0.14	0.68±0.17
Blood 1 g	2.34±0.35	1.65±0.18	1.44±0.22

a) Expressed as percent injected dose per organ. Each value is mean±S.D. for three mice.

Table 2. Biodistribution of Tc-99m Testosterone-1 Conjugate[a)]

Organ	30 min	1 h	3 h
Salivary glands	0.21±0.01	0.12±0.04	0.07±0.02
Spleen	0.19±0.01	0.09±0.01	0.07±0.01
Stomach	0.23±0.02	0.20±0.07	0.12±0.01
Intestine	6.65±0.06	9.41±0.47	9.77±1.06
Liver	12.71±0.98	11.11±0.19	9.51±0.02
Kidneys	11.81±1.53	12.02±0.92	6.57±0.61
Urine	38.83±1.88	52.80±0.42	65.33±1.19
Muscle 1 g	0.71±0.22	0.28±0.02	0.22±0.03
Tumor 1 g	0.93±0.21	0.77±0.01	0.39±0.04
Blood 1 g	2.87±0.30	1.80±0.16	0.87±0.05

a) Expressed as percent injected dose per organ. Each value is mean±S.D. for three mice.

Table 3. Biodistribution of Tc-99m Epiandrosterone-1 Conjugate[a)]

Organ	30 min	1 h	3 h
Salivary glands	0.21±0.01	0.15±0.02	0.09±0.02
Spleen	0.20±0.03	0.13±0.02	0.08±0.02
Stomach	0.21±0.01	0.17±0.01	0.12±0.01
Intestine	1.80±0.10	1.85±0.32	1.58±0.21
Liver	25.56±0.42	23.08±0.54	18.52±1.85
Kidneys	9.92±0.42	12.85±2.51	9.70±2.67
Urine	41.79±2.16	47.58±3.62	62.16±4.22
Muscle 1 g	0.72±0.07	0.40±0.06	0.16±0.02
Tumor 1 g	1.53±0.79	0.63±0.04	0.40±0.04
Blood 1 g	2.31±0.09	1.72±0.18	0.97±0.08

a) Expressed as percent injected dose per organ. Each value is mean±S.D. for three mice.

Table 4. Biodistribution of Tc-99m 17-α-Hydroxyprogesterone-1 Conjugate[a)]

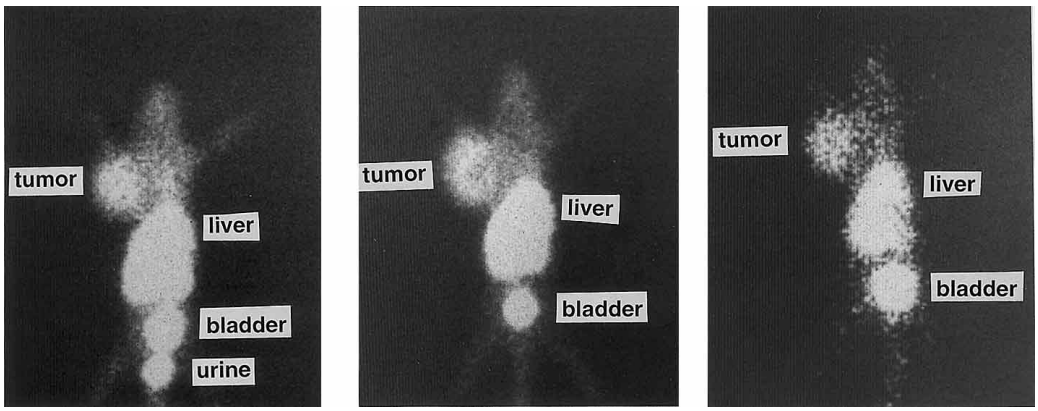
Organ	30 min	1 h	3 h
Salivary glands	0.18±0.01	0.12±0.01	0.08±0.02
Spleen	0.14±0.03	0.13±0.03	0.13±0.03
Stomach	0.21±0.07	0.17±0.02	0.14±0.02
Intestine	5.26±1.58	7.13±0.47	10.85±2.01
Liver	45.89±1.58	40.07±2.72	32.42±1.79
Kidneys	9.54±1.23	11.50±2.46	6.99±0.68
Urine	21.57±2.46	27.18±0.41	46.12±2.48
Muscle 1 g	0.47±0.05	0.33±0.08	0.17±0.02
Tumor 1 g	0.58±0.02	0.46±0.07	0.35±0.02
Blood 1 g	2.94±0.33	1.69±0.02	0.97±0.16

a) Expressed as percent injected dose per organ. Each value is mean±S.D. for three mice.

Table 5. Biodistribution of Tc-99m Progesterone-1 Conjugate[a)]

Organ	30 min	1 h	3 h
Salivary glands	0.23±0.01	0.20±0.01	0.09±0.01
Spleen	0.17±0.01	0.09±0.02	0.07±0.01
Stomach	0.21±0.01	0.20±0.01	0.16±0.02
Intestine	6.89±0.23	12.11±0.62	16.27±0.99
Liver	18.58±0.12	14.92±0.40	8.51±0.17
Kidneys	12.68±0.79	9.76±1.98	5.13±0.97
Urine	42.16±0.97	46.83±2.31	63.37±0.12
Muscle 1 g	0.59±0.03	0.41±0.01	0.19±0.05
Tumor 1 g	0.99±0.09	0.82±0.12	0.37±0.05
Blood 1 g	2.76±0.05	2.15±0.06	0.87±0.02

a) Expressed as percent injected dose per organ. Each value is mean±S.D. for three mice.



a) b) c)

Fig. 1. Scintigram of Mice Bearing Mammary Tumor

Scintigrams were taken 1 h after the administration of the Tc-99m complexes of 1-conjugates with a) estrone, b) progesterone, and c) testosterone.

seems likely that the tumor image is dependent on blood pool and not on specificity to the receptor of the steroid moiety. The thyroid gland and stomach were not visualized at any time.

Discussion

The results showed that 2-aminooxyethyliminodiacetic acid is an excellent bifunctional chelating agent, with both carbonyl binding and metal chelating sites in the molecule. The agent formed conjugates with the steroids in good yields. The conjugates were stable and were readily labeled with Tc-99m by the ordinary stannous chloride-method, whereas the steroids do not bind Tc-99m. The agent proved promising for metal-labeling of biomolecules and related substances containing one or more carbonyl groups.

The Tc-99m complexes of the conjugates studied did not show high affinity or selectivity in mammary tumor and Ehrlich tumor. They were initially distributed to all tissues, including nontarget sites, and then reached equilibria between those tissues and plasma. The Tc-99m estrone conjugate was excreted in 3 h, about 38% by the hepatobiliary system and 48% by the urinary system. The urinary excretion increased in the epiandrosterone and testosterone conjugates and increased further in progesterone, possibly due to the increased hydrophilic nature of the conjugates. The molecular sizes of the steroids in the present study are not so large compared to that of the bifunctional agent that we may not expect their original bioactivity to be fully restored in the conjugates and their metal chelates.

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