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A radioimmunoassay for TA-0910, a new metabolically stable thyrotrophin-releasing hormone analogue

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Abstract: TA-0910 [1-methyl-(*S*)-4,5-dihydroorotyl-L-histidyl-L-prolineamide] is a metabolically stable analogue of thyrotrophin-releasing hormone (TRH) and is under clinical investigation as a central nervous system function modulator. A method for determination of its plasma concentrations by radioimmunoassay (RIA) was established. TA-0910 was conjugated to bovine serum albumin and keyhole limpet haemocyanin (KLH) with bis-diazotized benzidine and 1,5-difluoro-2,4-dinitrobenzene as bridging agents. Anti-TA-0910 antisera were prepared by immunizing rabbits with the TA-0910 conjugates and Freund's complete adjuvant. The radiolabelled TA-0910 for RIA was prepared by introducing ^{125}I into the histidine imidazole ring of TA-0910 by the $Na^{125}I$/chloramine-T method, and purified by reversed phase high-performance liquid chromatography to give a specific radioactivity of 81.4 TBq mmole^{-1}. As the result of testing the cross-reactivity of the antisera with assumed TA-0910 metabolites and TRH, a TA-0910-selective antiserum was obtained from a rabbit immunized with TA-0910-dinitrophenyl-KLH. RIA using this antiserum and the radiolabelled TA-0910 afforded a determination range of 10 pg ~5 ng ml^{-1} plasma. By using this RIA, the time courses of plasma concentrations of unchanged TA-0910 after oral and intravenous administration of TA-0910 were obtained in rats.

Keywords: *Radioimmunoassay; TRH analogue; TA-0910; rat; HPLC.*

Introduction

TA-0910 [1-methyl-(*S*)-4,5-dihydroorotyl-L-histidyl-L-prolineamide] is a novel thyrotrophin-releasing hormone (TRH) analogue, which is under clinical study to treat spinocerebellar degenerative disease and dementia. TA-0910 administered either orally or intravenously exerts central nervous system (CNS) effects 100 or 30 times, respectively, as potent as TRH [1]. In contrast, its thyroid-stimulating hormone (TSH)-releasing action is only 1/50th that of TRH [1], indicating a successful dissociation between the CNS and hormonal actions. Another feature of TA-0910 is its long duration of action in comparison with TRH after oral or intravenous administration. This is due to its stability in blood plasma [1], where it is resistant to degradative enzymes such as thyroliberinase and TRH deaminase, and this may also contribute to its neuropharmacological potency. Chemical modification of TRH to more enzymatically resistant forms has been reported to yield more pharmacologically potent analogues [2, 3].

Since the clinical dosage of TA-0910 is as low as 5 ~40 mg per man a day, the method for determination of plasma concentrations has to be highly sensitive and selective. Therefore, development of a radioimmunoassay (RIA) for TA-0910 was attempted in analogy with the RIAs of DN-1417 [4], azetrirelin [5], and RX-77368 [6], which are similar synthetic analogues of TRH aimed to be more stable CNS agents.

In order to attain high sensitivity and selectivity in RIA, a radiolabel of high specific radioactivity is essential along with highly selective antibodies. Reversed phase high-performance liquid chromatography (HPLC) was utilized as a purification means to obtain a highly radioactive preparation of ^{125}I-TA-0910. The resultant RIA was highly sensitive as well as selective to unchanged TA-0910 and its lower determination limit was 10 pg ml^{-1}. Using this RIA, it was possible to establish the time courses of plasma concentrations of TA-0910 after oral and intravenous administration in rats.

Experimental

Reagents

TA-0910 (Lot No. 906030), TA-0910-acid, Oro-His-COOH, and His-Pro-NH_2 (Fig. 1) were synthesized at the Research Laboratory

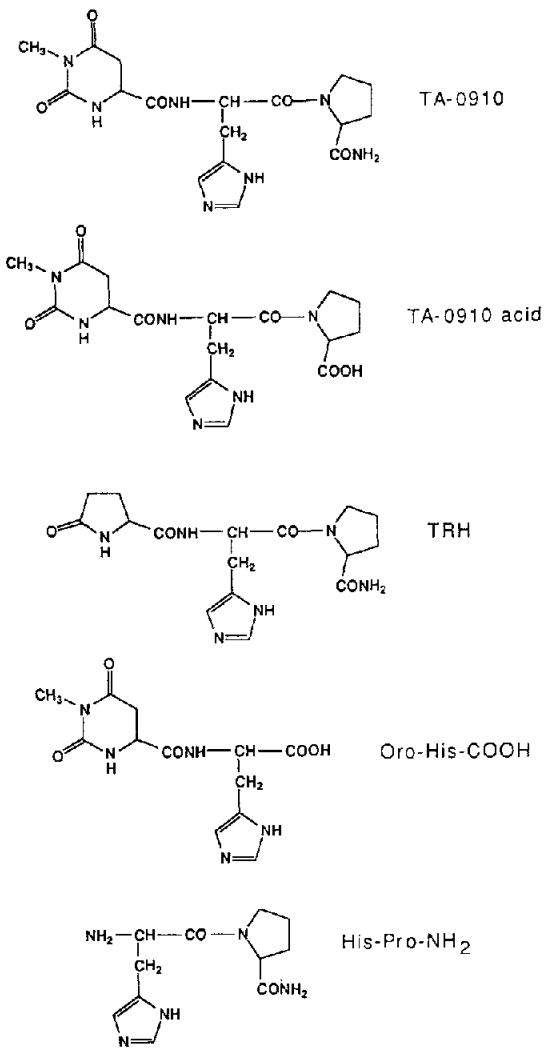


Figure 1
The chemical structures of TA-0910, TRH and metabolites used in the cross-reactivity study.

of Applied Biochemisty, and 1-$^{14}CH_3$-labelled TA-0910 (specific radioactivity: 1.3 MBq mg^{-1}) by S. Furuuchi at this Laboratory. 1,5-Difluoro-2,4-dinitrobenzene (DFDNB), chloramine-T, and inorganic reagents are commercial products of the special grade, and trifluoroacetic acid (TFA) and methanol are those for protein research and HPLC, respectively, all from Nacalai tesque, Inc (Kyoto, Japan). Also purchased were TRH (Protein Research Institute, Osaka, Japan), $Na^{125}I$ (specific activity: 81.4 TBq $mmol^{-1}$, Dupont/NEN, Boston, MA, USA), bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA), bovine serum albumin (BSA, Sigma, St Louis, MO, USA), keyhole limpet haemocyanine (KLH, Calbiochem, La Jolla, CA, USA), Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, MI, USA), dialysis membrane (spectra/POR, spectrum, Houston, TX, USA) and heparin (Mochida, 5000 units $ampoule^{-1}$, Tokyo, Japan). Other reagents used were all of the special grade. Deionized water was prepared by the MilliQ system (Millipore, Woburn, MA, USA).

Animals

Male New Zealand white rabbits, weighing 2.5–2.8 kg, were purchased from Kitayama labes (Nagano, Japan) and maintained on 120 g pellets (LRC4, Oriental Yeast, Tokyo, Japan) once a day per animal and tap water *ad libitum*.

Male Sprague–Dawley rats, weighing 400–475 g, were purchased from Japan SLC (Shizuoka, Japan) and maintained on pellets (CE-2, CLEA Japan, Tokyo, Japan) and tap water *ad libitum*. All animals were kept at a room temperature of 23 ± 1°C and a humidity of 55 ± 5%, and used after the acclimatization period of 1 week after purchase.

Instruments

The HPLC system consisted of a Waters M45J pump, a U6K sample injector (Waters, Milford, MA, USA), a Shimadzu SPD-2A detector (Shimadzu, Kyoto, Japan), and a Shodex F411A column (ODS, 4.6 mm × 150 mm, 5 μm, Showa Denko, Tokyo, Japan) and was used with 0.05% TFA–methanol (6:1, v/v) as the mobile phase at a flow rate of 1.0 ml min^{-1}.

Synthesis of TA-0910-dinitrophenyl (DNP)–KLH conjugate and immunization

TA-0910 (3.2 mg) dissolved in 0.2 ml of 0.1 M phosphate buffer (pH 7.2) was mixed with 30 mg DFDNB dissolved in 1 ml methanol, and reaction was allowed to proceed for 15 min at room temperature. Deionized water (0.5 ml) was added to the reaction mixture, which was then washed with 4 ml ether four times. KLH (2.5 mg) was dissolved in 0.8 ml 0.1 M borate buffer (pH 10.0) [7]. The above aqueous layer containing the TA-0910-1-fluoro-2,4-dinitrophenyl (DNPF) derivative was added to the KLH solution. The reaction mixture was protected from light and stirred for 24 h at room temperature. The mixture was transferred to a sack of dialysis membrane and dialysed against 2 l of deionized water for 2

days with changing of dialysis water once a day. After dialysis, the solution was adjusted by dilution with deionized water to make the protein concentration to 1 mg ml^{-1} and kept frozen at −80°C until use. The protein concentration was determined by the BCA method against BSA as the standard. The amount of TA-0910 bound to KLH was determined as follows: ^{14}C-TA-0190 was used to prepare the conjugate in the same manner as above, the crude conjugate was dialysed until the radioactivity of dialysis water was lowered to the background level (about 30 dpm ml^{-1}), and then the amount of TA-0910 bound to KLH was calculated from the radioactivity and the amount of protein inside the dialysis bag.

Five rabbits were immunized with TA-0910-DNP–KLH (0.5 mg KLH $animal^{-1}$) in an emulsified 1:2 (v/v) mixture with FCA by intradermal injection at several sites on the back and intramuscular injection into the hind limb femoral muscle. Subsequent booster injection was performed in the same manner as above at 4–6 week intervals. The antibody production was followed by withdrawing blood from the ear vein 2–3 weeks after each booster injection and measuring the amount of ^{14}C-TA-0910 bound to the serum protein precipitate at 50% ammonium sulphate saturation. The whole blood was withdrawn when the antibody production leveled off 15–23 weeks after the first immunizing injection.

Synthesis of TA-0910-DNP–BSA conjugate and immunization

TA-0910 (6.5 mg) dissolved in 0.2 ml 0.1 M phosphate buffer (pH 7.2) was mixed with 30 mg DFDNB dissolved in 1 ml methanol, and reaction was allowed to proceed for 20 min at room temperature. Phosphate buffer (0.5 ml) was added to the reaction mixture, which was then washed with 4 ml ether three times. BSA (50 mg) was dissolved in 0.8 ml 0.1 M borate buffer (pH 10.0) [8], and to this was added the above aqueous layer containing the TA-0910-DNPF derivative. The reaction mixture was protected from light and stirred for 24 h at room temperature. The reaction mixture was dialysed against 2 l deionized water for 140 h and dialysis water was changed once a day. The solution was adjusted by dilution with deionized water to give the protein concentration of 1 mg ml^{-1}. The amount of TA-0910 bound to BSA was determined as described above.

Seven rabbits were immunized with TA-0910-DNP–BSA (1 mg BSA $animal^{-1}$) in an emulsified 1:1 mixture with FCA and the antibody production was followed as described above.

Synthesis of TA-0910-bis-diazotized benzidine–BSA conjugate and immunization

Bis-diazotized benzidine (BDB) was synthesized by adding 173 mg sodium nitrite to a solution of 230 mg benzidine in 45 ml 0.2 N HCl at 4°C and stirring the mixture for 60 min. The reaction mixture (25 μmol ml^{-1}) was subdivided into 3 ml aliquots and kept frozen at −80°C until use [9].

TA-0910 (6.5 mg) and BSA 50 mg were dissolved in 5 ml 0.16 M borate buffer (pH 9.0) containing 0.13 M NaCl. To this solution was added 12.5 μmol BDB in a solution diluted 10 times with the same buffer at room temperature. Immediately, the reaction mixture was cooled in ice-water and allowed to stand for 2 h. Then, the reaction mixture was dialysed against 2 l deionized water for 106 h with changing of dialysis water once a day, and finally dialysed against 2 l of 0.15 M NaCl. The solution was adjusted with 0.15 M NaCl to give the protein concentration of 1 mg ml^{-1}.

Five rabbits were immunized with TA-0910-BDB–BSA (1 mg BSA $animal^{-1}$) in an emulsified 1:1 mixture with FCA and the antibody production was followed as described above.

Synthesis of ^{125}I-(imidazole-5-^{125}I-histidine)-TA-0910

(1) Synthesis of I-TA-0910. Non-radioactive I-TA-0910 was first synthesized by the NaI/chloramine-T method [10] to obtain a standard for purification of ^{125}I-TA-0910 by HPLC. A 10 mg mass TA-0910 (0.0213 mmol), 3.1 mg NaI, 6.0 mg chloramine-T, and 4.1 mg sodium metabisulphite were individually dissolved in 0.5 ml each of 0.5 M phosphate buffer (pH 7.4). TA-0910 was mixed with NaI, and then with chloramine-T. Reaction was allowed to proceed for 2 min and terminated by adding sodium metabisulfite. The iodinated TA-0910 was purified by HPLC (UV absorbance at 220 nm) and its structure was confirmed by fast atom bombardment (FAB) mass spectrometry and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy.

(2) Synthesis of ^{125}I-TA-0910. Synthesis of

^{125}I-TA-0910 was performed in a NENSURE vial (DuPont/NEN) from 37 MBq Na^{125}I, 2.5 μg/15 μl TA-0910, 1.5 μg/10 μl chloramine-T, and 1.1 μg/10 μl sodium metabisulphite dissolved in 0.5 M phosphate buffer as described above. The ^{125}I-labelled TA-0910 was purified by HPLC with the non-radioactive I-TA-0910 as the standard. The fraction corresponding to the retention time (*ca* 5.5–6.5 min) of I-TA-0910 was taken, diluted with deionized water to give the radioactivity concentration of about 10,000 dpm/100 μl, and stored in a refrigerator at 4°C. In order to confirm the validity of the diluted ^{125}I-TA-0910 as the radiolabel for RIA, binding inhibition by TA-0910 was examined at 0, 7, 15, 23, 28, 42 and 63 days.

Tests for anti-TA-0910 antibody titres and cross-reactivities

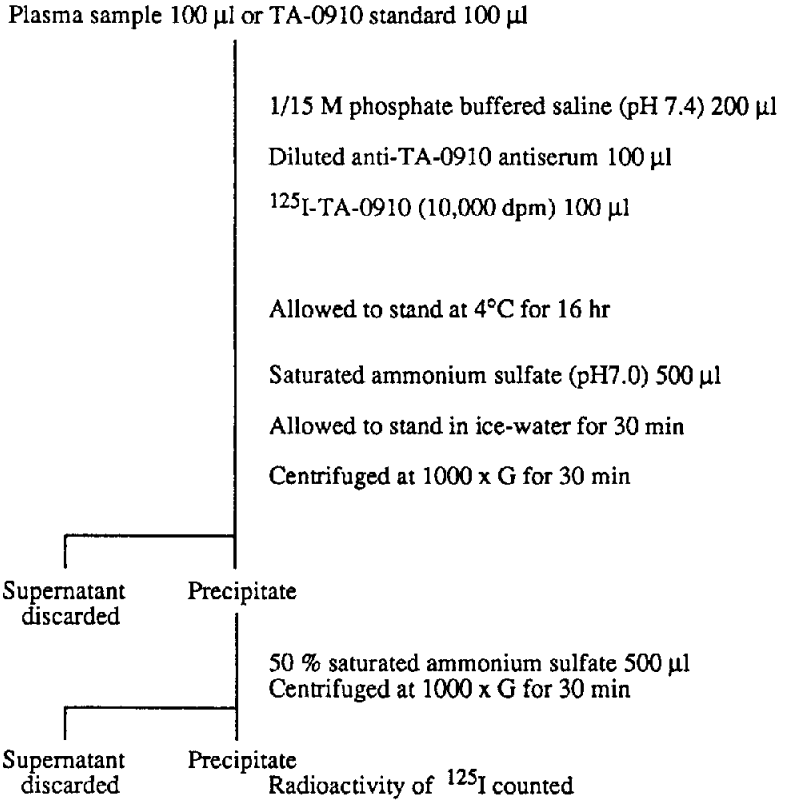
Serum samples were diluted 100–20,000-fold with 1/15 M phosphate-buffered saline (PBS) (pH 7.4) containing 0.1% bovine-γ-globulin. ^{125}I-TA-0910 (about 10,000 dpm/100 μl) was added to 100 μl each of the serum dilutions, and the reaction was allowed to proceed for 16 h at 4°C. The bound and free forms of ^{125}I-TA-0910 were separated by the ammonium sulphate precipitation method, and the dilution fold giving the bound/total ratio of 0.5 was determined as the antibody titre.

Cross-reactivity was examined by using 100 μl each of the serum dilutions which gave the bound/total ratio of 0.5. To each tube was added an aqueous solution (100 μl) of TA-0910 in the range of 5–500 pg and TA-0910 metabolites and TRH in the range of 50 pg–2 ng, and the antigen–antibody reaction was carried out in the presence of about 10,000 dpm ^{125}I-TA-0910 per tube. The chemical structures of TA-0910, its metabolites, and TRH are shown in Fig. 1.

The procedure for the antigen–antibody reaction to measure antibody titres and cross-reactivities is outlined in Scheme I.

RIA sensitivity

Standard solutions of TA-0910 (1–500 pg 100 μl^{-1}) were prepared with deionized water. Anti-TA-0910 antiserum No. 10 was diluted 8,000-fold with 1/15 M PBS containing 0.1% bovine-γ-globulin and 0.1% NaN_3, and 100 μl of this dilution was allowed to react with 100 μl



Scheme I
Radioimmunoassay procedure for TA-0910

of the TA-0910 standards in the presence of about 10,000 dpm (100 μl) of ^{125}I-TA-0910 according to the RIA procedure outlined in Scheme I. Interference by biological materials was examined by replacing PBS with 100 μl rat plasma in Scheme I. The working concentration range of the RIA was determined by applying the four-parameter logistic function to the inhibition curves [11].

Time-courses of plasma concentrations of TA-0910 in rats

TA-0910 in a saline solution was administered to rats orally at the dose of 1 mg ml^{-1} animal^{-1} (n = 4, body weight 400–425 g) with a stomach tube or intravenously at the dose of 100 μg 0.5 ml^{-1} animal^{-1} (n = 3, body weight 430–475 g) from the tail vein.

Blood samples were taken from the tail artery through a polyethylene cannula (0.5 mm i.d. × 0.9 mm o.d.) under arrest in a Bollman cage immediately before, 15, 30, 60, 90, 120, 180, 240 and 360 min after oral administration, and immediately before 3, 5, 10, 30, 60, 90, 120, 180, 240 and 360 min after intravenous administration. The volume of blood samples was about 0.3 ml. After addition of a small drop of heparin, the blood was centrifuged and the plasma was kept at −80°C until analysis.

Results

Identification of I-TA-0910 and stability of ^{125}I-TA-0910

Figure 2(A) shows the high-performance liquid chromatogram of the purified fraction of I-TA-0910, which gave a single peak at *ca* 6 min. The FAB mass-spectrum and ^{13}C-NMR chart of I-TA-0910 are shown in Fig. 3. The molecular ion at MH$^+$ 532 indicates that one iodine atom was introduced to the molecule. The peak at 85.3 ppm in the NMR chart proves that one iodine atom has been incorporated at position 5 of the imidazole ring. Other NMR peaks were compatible with the structure of I-TA-0910.

Using HPLC as the means of purification, it was possible to obtain a pure, highly radioactive preparation of ^{125}I-TA-0910 (81.4 TBq mmole^{-1}) free of the starting materials and degradation products. As the high-performance liquid chromatogram of the reaction mixture shows (Fig. 2B), the peak for TA-0910 was clearly separated from the radioactive fraction corresponding to the retention time of

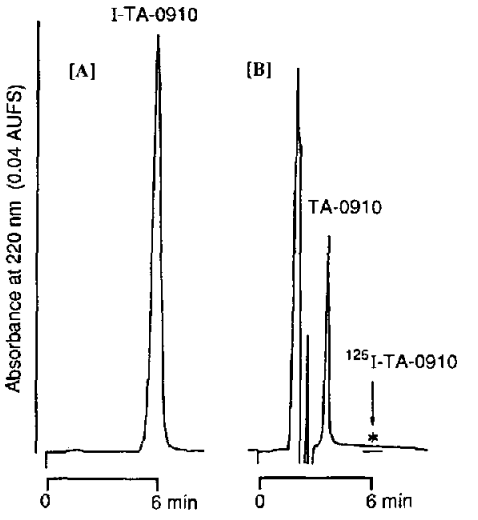


Figure 2
High-performance liquid chromatograms of I-TA-0910 (A) and iodination reaction mixture for preparation of ^{125}I-TA-0910 (B). See the text for HPLC conditions. The bar indicates the interval effluent collection.

I-TA-0910. The preparation of ^{125}I-TA-0910 stored in the refrigerator for 63 days gave the same binding inhibition curve as the fresh preparation, indicating that no interfering degradation products were generated during storage.

Antibody production and cross-reactivity

The amounts of bound TA-0910 in the preparations of TA-0910-DNP–KLH, TA-0910-DNP–BSA, and TA-0910-BDB–BSA were 400, 11.0 and 6.1 molecules, respectively, per molecule of protein, corresponding to 1–2 molecules per 10,000 molecular weight.

The time-courses of anti-TA-0910 antibody production in individual rabbits were determined by measuring the amount of ^{14}C-TA-0910 bound per 0.1 ml serum and are shown in Fig. 4. Antibody production sharply increased with time after the first booster immunization and plateaued around the 15th to 20th week. Among the antigens used, those obtained with DNP as the bridging agent tended to give higher antibody titres than TA-0910-BDB–BSA.

Four antisera (Nos 7, 9, 10 and 14) were chosen so as to represent each antigen group and their cross-reactivities with TRH and TA-0910 metabolites were examined as shown in Table 1. The antisera Nos 7 and 10 showed no cross-reactivity with TRH and the TA-0910

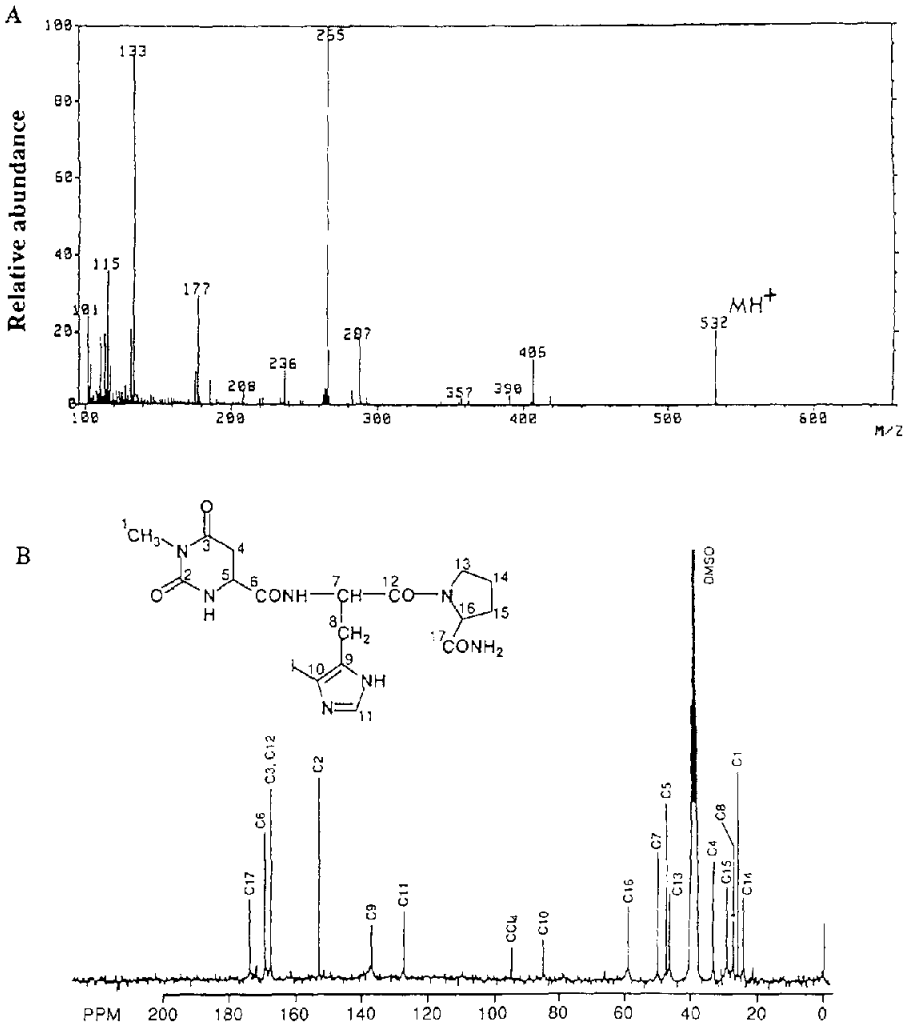


Figure 3
FAB mass spectrum (A) and ^{13}C-NMR spectrum (B) of the prepared cold-I-TA-0910. The FAB mass spectrum was taken on a JEOL HX100 mass spectrometer. Xenon was used the FAB gas. The FAB gun was operated at 3 kV. The sample was dissolved in methanol. The sample and liquid matrix (glycerol-DMSO-18-crown-8 mixture) were mixed on the FAB target. ^{13}C-NMR was recorded on Hitachi FX200 (200 MHz) spectrometer using DMSO-d-6 as solvent and trimethylsilane as an internal standard.

Table 1
Cross reactivity of anti-TA-0910 antisera

Compounds	Concentrations for 50% binding (pg tube $^{-1}$) Antisera no. 7	9	10	14
TA-0910	50	100	20	40
TA-0910 acid (main metabolite)	>2000	1000	>2000	100
TRH	>2000	>2000	>2000	>2000
Oro-His-COOH	>2000	>2000	>2000	>2000
His-pro-NH_2	>2000	>2000	>2000	>2000

TA-0910 metabolites and TRH were assayed by the radioimmunoassay over the range 5–2000 pg tube^{-1}. All compounds were dissolved in water. TA-0910 or an analogue, diluted antiserum and ^{125}I-TA-0910 (about 10000 dpm) were incubated for 16 h at 4°C.

No. 7 (1:5000 dilution) was immunized with TA-0910-BDB–BSA.

No. 9 (1:5000 dilution) and no. 10 (1:8000 dilution) were immunized with TA-0910-DNP-KLH.

No. 14 (1:20000 dilution) was immunized with TA-0910-DNP–BSA.

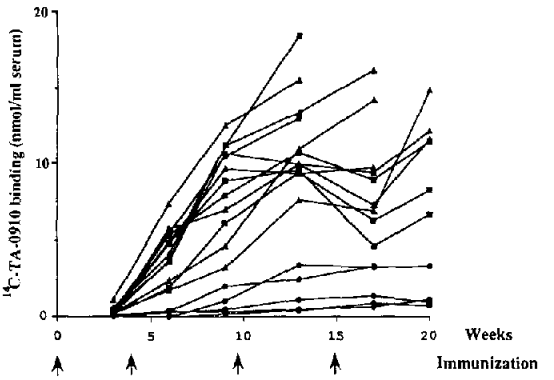


Figure 4
Serum levels of anti-TA-0910 antibody after immunization with TA-0910-protein conjugates. Blood samples were taken from the ear veins at 2 ~ 3 weeks intervals after each booster injection. Anti-TA-0910 antibody was determined by the method of 50% saturated ammonium sulphate using ^{14}C-TA-0910 (about 10000 dpm) (see text for further details). —●—, TA-0910-BDB–BSA conjugate; —■—, TA-0910-DNP–KLH conjugate; —▲—, TA-0910-DNP–BSA conjugate.

metabolites although they were raised by different antigens. The antisera Nos 9 and 14, which had high anti-TA-0910 activities, showed cross-reactivities toward the main metabolite TA-0910-acid with an equal or somewhat less affinity compared to their anti-TA-0910 activities.

The antisera Nos 7 and 10, both selective enough to be useful for RIA, had the antibody titres of 2000 and 8000, respectively. Therefore, the serum No. 10 was selected as the standard antiserum for RIA in subsequent studies.

Binding inhibition curves and assay sensitivity

The curves of inhibition of ^{125}I-TA-0910 binding to anti-TA-0910 antibody (the diluted antiserum of No. 10) by spiked TA-0910 (1–500 pg tube^{-1}) in the presence and absence of 100 μl normal rat plasma are shown in Fig. 5.

An appreciable inhibition was observed by 1 pg of TA-0910, and the working range of quantitation was calculated to be between 10 and 5000 pg ml^{-1} test sample. The presence of 100 μl rat plasma showed no significant interference with the assay as shown by the two nearly superimposable curves. The intra-assay variation for a standard curve of 1–500 pg/100 μl rat plasma showed relative standard deviations (RSD) in the range of ±0.5 to ±4.5%. The RSD for the slope factor in the four-parameter logistic function after four experiments was ±5.9%.

Time-courses of plasma concentrations of TA-0910 after oral and intravenous administration in rats

The time-courses of plasma concentrations

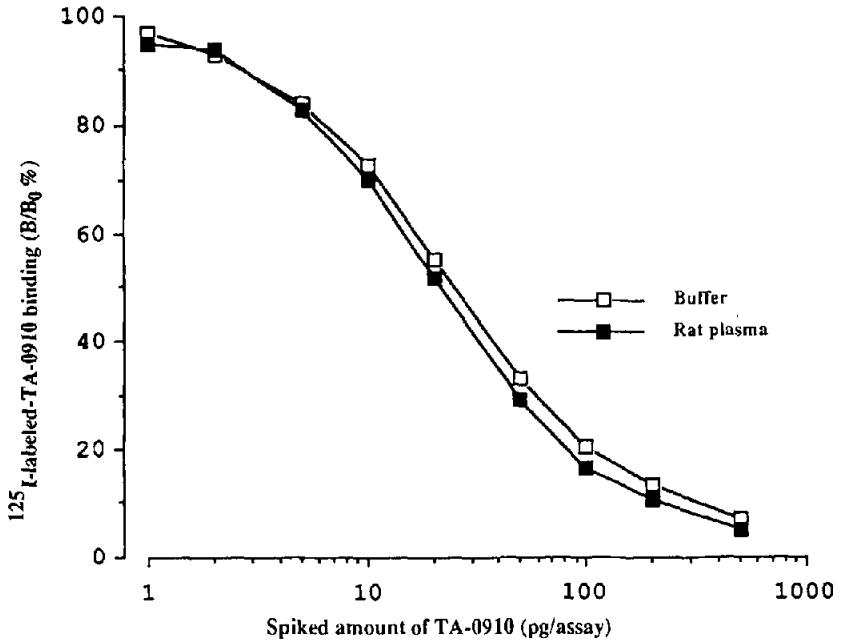


Figure 5
Radioimmunoassay standard curves for TA-0910. TA-0910 was spiked to rat plasma and phosphate buffer. TA-0910, diluted antiserum (no. 10,1:8000) and ^{125}I-TA-0910 (about 10000 dpm) were incubated for 16 h at 4°C (see text for further details).

of unchanged TA-0910 after oral administration at the dose of 1 mg animal^{-1} (about 2.4 mg kg^{-1} body weight) and after intravenous administration at the dose of 100 μg animal^{-1} (about 0.22 mg kg^{-1}) are shown in Figs 6 and 7, respectively. After oral administration, the plasma concentration reached the maximum (21.8 ng ml^{-1}) at 44.4 min and declined with a half-life of 51 min. The half-life of plasma concentration decay after intravenous administration was 29 min. The mean plasma concentration at 360 min was 0.9 ng ml^{-1} after oral administration, but it was under the lower detection limit after the intravenous dosage. The absolute bioavailability by the oral route was calculated to be 1.66% from the areas under the plasma concentration (AUC) for the two routes of administration.

Discussion

Performance of a RIA is determined by its

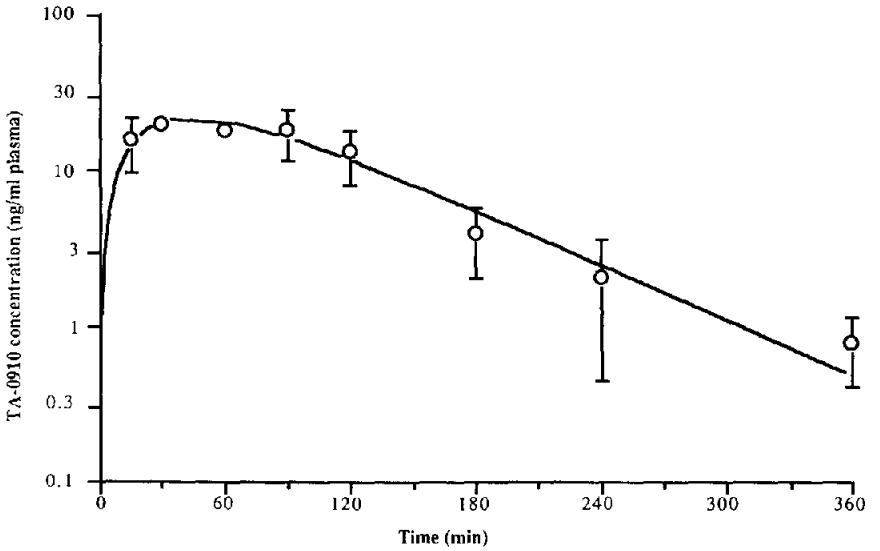


Figure 6
Plasma levels of unchanged TA-0910 after oral administration of TA-0910 (1 mg rat^{-1}). Unchanged TA-0910 was estimated by the radioimmunoassay. Each point represents the mean ±SD for three rats.

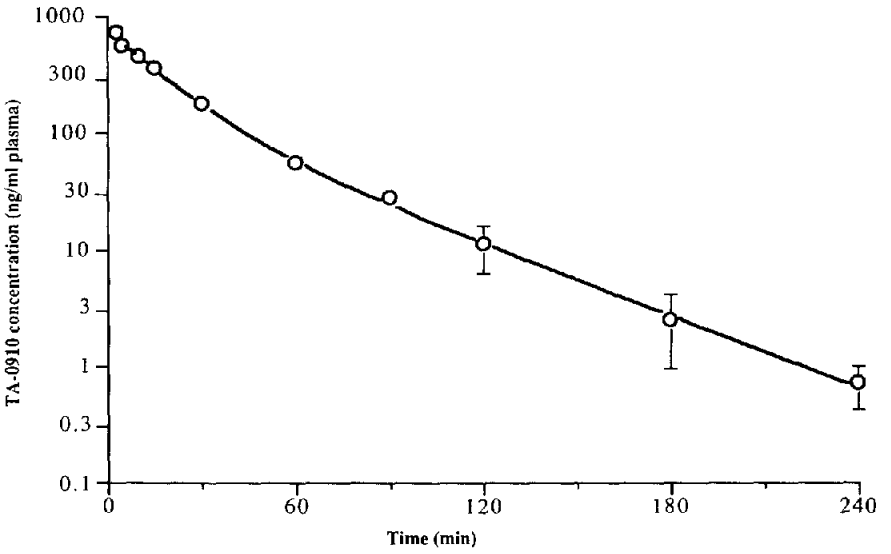


Figure 7
Plasma levels of unchanged TA-0910 after intravenous administration of TA-0910 (0.1 mg rat^{-1}). Unchanged TA-0910 was estimated by the radioimmunoassay. Each point represents the mean ±SD for four rats.

sensitivity and selectivity. The sensitivity in turn depends on the specific radioactivity of the radiolabelled ligand which competes with the compound to be determined for the antigen-binding site. In the present method, the ligand ^{125}I-TA-0910 of a high specific radioactivity free of interfering TA-0910 was obtained by the use of analytical HPLC. In the previously published methods of RIA for TRH and its derivatives, purification of the radiolabelled ligand was performed by gel-filtration [4, 7, 9] or on a reversed-phase mini-cartridge column [6]. In these methods, resolution between the radiolabelled ligand and the parent compound was not complete to give a pure preparation of the labelled ligand. The capacity of analytical HPLC is large enough to handle the microquantity of the carrier-free labelled ligand and once the retention time is defined with non-labelled I-TA-0910, collection of the specified fraction gives pure ^{125}I-TA-0910 reproducibly. This has largely contributed to the high sensitivity of the present method, the lower limit of detection (10 pg ml^{-1} plasma) being 5–10 times below those of the published RIAs for TRH and its analogues [4, 6, 7, 9]. In a clinical phase 1 study, the plasma concentration of TA-0910 has been successfully followed over the period of 8 h after single oral administration by using the present RIA (T. Suzuki, personal communication, Pharmaceutics Research Laboratory, Tanabe Seiyaku Co., Ltd).

Another essential feature of good RIA for TRH analogues is a high selectivity; i.e. the antibody should not cross-react either with their metabolites or endogenous TRH. This was achieved by using as many as 17 rabbits (the more the animals used, the higher the probability of encountering an animal which produces highly selective antibodies) and three different types of antigen; i.e. TA-0910-DNP-KLH, TA-0910-DNP-BSA, and TA-0910-BDB-BSA, all of which carry the hapten molecules through the imidazole moiety. The last point is important, because the antigen linked through a carbodiimide group at the carboxyl terminal of TRH is known to produce antibodies that react with both TRH and TRH-acid [12]. However, as shown in Table 1, the choice of the imidazole nucleus as the linking position did not prevent the production of an antibody which also reacted with TA-0910-acid, the main metabolite of TA-0910.

The individual variability of rabbits in response to the identical antigenic stimulus and the variation of the structure of immunogens resulted in the various degrees of antibody production and antigenic selectivity as shown in Fig. 4 and Table 1, respectively. The level of antibody production as measured in ^{14}C-TA-0910 binding did not differ greatly between the two carrier protein species, but the use of BDB as the bridging moiety made the immunogen TA-0910-BDB–BSA less efficient than TA-0910-DNP–BSA (Fig. 4). The reason for this is not clear, but it seems possible that the preparation of TA-0910-BDB–BSA was less homogeneous than TA-0910-DNP–BSA because the reactivity of BDB as a diazotizing agent in the one-step reaction could have resulted in impurities such as protein–protein and/or hapten–hapten conjugates [12] that could compete for antigenic recognition with TA-0910-BDB–BSA. TA-0910-DNP–BSA, on the other hand, was prepared in two steps; i.e. the monofluorodinitrophenyl hapten was first prepared and purified, and then made to react with the carrier protein so that a purer immunogen was prepared under more controlled reaction conditions than the case for BDB bridging.

Yamamura *et al.* [1] showed that the CNS effects of TA-0910 were not only much more potent but also longer-acting than those of TRH in mice, and similar activity differences were also observed in rats [13]. They ascribed these ratios to differences in pharmacokinetics between the two drugs based on the unpublished observation by Furuuchi *et al.* (S. Furuuchi, personal communication) that TA-0910 was metabolically more stable than TRH owing to its resistance to catabolic enzymes in plasma. This point was confirmed by the present study which showed that the plasma decay ($t_{1/2}$ = *ca* 29 min) of intravenously administered TA-0910 (about 0.22 mg kg^{-1}) was markedly slower than the published value ($t_{1/2}$ = *ca* 7 min) for TRH (0.2 mg kg^{-1}, i.v.) in rats [14]. The absolute bioavailability of TA-0910 (1.66%) calculated from the two AUC values for the p.o. and i.v routes was not substantially higher than the published value (1.48%) for TRH [14], although these values may not be directly comparable to each other because of the differences in dosage (TRH 29.2 mg kg^{-1}, p.o. vs 0.5 mg, i.v.). The gastrointestinal absorption rate of orally administered ^{14}C-TA-0910 (3 mg kg^{-1}) in rats has been determined to be 11.5% by Furuuchi *et*

al. (S. Furuuchi, personal communication of this Laboratory).

Conclusions

A RIA method was established for the long-acting TRH analogue TA-0910 and the resulting method was sensitive enough to measure the drug concentration in clinical plasma samples. The metabolic stability of TA-0910 as the evidence for its long-lasting pharmacological effect was demonstrated by measuring the decay of drug concentrations in plasma after oral and intravenous administration in rats.

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