ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1998) 1267–1274

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Radioimmunoassay for TA-0910, a new stable thyrotropin releasing hormone analogue and its metabolite, TA-0910 acid-type, in human plasma and urine

Shigeki Morikawa [a,*], Tsutomu Sakai [a], Kazuo Murata [a], Kiyoshi Banno [a], Tadashi Sato [a], Tsutomu Takai [b], Takehiko Suzuki [b]

[a] *Analytical Research Laboratory, Tanabe Seiyaku Company Limited, 16–89 Kashima 3-chome Yodogawa-ku, Osaka 532, Japan*
[b] *Pharmaceutics Research Laboratory, Tanabe Seiyaku Company Limited, 16–89 Kashima 3-chome Yodogawa-ky, Osaka 532, Japan*

Received 25 December 1996; accepted 7 February 1997

Abstract

Radioimmunoassay (RIA) was investigated for the determination of TA-0910 and its main metabolite, TA-0910 acid-type, in human plasma and urine. TA-0910 is a new metabolically stable analogue of thyrotropin releasing hormone (TRH). Antiserum was raised in the rabbit against the 1-fluoro-2,4-dinitrophenyl derivative of TA-0910 or TA-0910 acid-type conjugated to keyhole limpet hemocyanin (KLH). The radioligand was prepared by iodination with ^{125}I of the histidine imidazole ring of TA-0910 or TA-0910 acid-type. Cross-reactivities of anti-TA-0910 or TA-0910 acid-type antiserum for TA-0910, its metabolite and related compounds were low. The calibration range was 0.02–5 ng ml^{-1} using 100 μl human plasma or urine. Inter-day variations of TA-0910 and TA-0910 acid-type assay in plasma were 3.5–15.5 and 1.8–9.4%, respectively. The variations of the assay in urine were the same as those in plasma. The recovery of TA-0910 and TA-0910 acid-type spiked in plasma or urine samples was approximately 100%. Furthermore, this method was applied to the determination of TA-0910 and TA-0910 acid-type in human plasma and urine samples, for the evaluation of the pharmacokinetics of TA-0910 in humans. From the results it was demonstrated that he developed RIA was useful for the determination of TA-0910 and TA-0910 acid-type in human plasma and urine, and was applicable to pharmacokinetic studies in humans.

Keywords: Radioimmunoassay; TA-0910; TA-0910 acid-type; Pharmacokinetics; Humans; Plasma; Urine

1. Introduction

TA-0910, (−)-*N*-[(*S*)-hexahydro-1-methyl-2,6-dioxo-4-pyrimidinyl carbonyl]-*L*-histidyl-*L*-prolinamide tetrahydrate, is a new thyrotropin releasing hormone (TRH) analogue developed by

* Corresponding author. Tel.: +81 63002637; fax: +81 63002629.

0731-7085/98/$19.00
PII S0731-7085(97)00145-3

Tanabe Seiyaku [1]. It has various central nervous system (CNS) activities 30–100-fold more potent and about 8-fold longer than TRH [2]. In contrast, its thyroid stimulating hormone (TSH) releasing activity is only 1/50th of that of TRH [1], indicating a successful dissociation between the CNS and hormonal actions. Also, TA-0910 produced dose-dependent increases in the amplitudes of mono- and poly-synaptic reflex potentials and withdrawal flexor reflexes [3].

The clinical dose of TA-0910 is as low as 5–40 mg per person per day, therefore, it is necessary to develop a sensitive assay for TA-0910 and its metabolite in plasma and urine, in order to investigate the pharmacokinetics of TA-0910 in humans. The aim of the present study was the development of a sensitive radioimmunoassay (RIA) for TA-0910 and a main metabolite, TA-0910 acid-type, *N*-[(*S*)-hexahydro-1-methyl-2,6-dioxo-4 pyrimidinylcarbonyl]-*L*-histidyl-*L*-proline, in plasma and urine, and its application to pharmacokinetic studies in humans.

2. Experimental

2.1. Materials and reagents

TA-0910, its main metabolite, TA-0910 acid-type (Fig. 1), its related compounds, demethyl TA-0910, Oro-His-COOH, His-Pro-NH_2, TRH and TRH acid-type were synthesized at Tanabe Seiyaku, (Osaka, Japan). 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) was purchased from Fluka (Buchs, Switzerland), Keyhole limpet hemocyanin (KLH) from Calbiochem (La Jolla, CA), Freund complete adjuvant (FCA) from Difco (Detroit, MI), heparin from Mochida (Tokyo, Japan), bovine-γ-globulin (Fraction II) from Miles (Kankakee, IL), phosphate buffered salts (PBS) from Takara Shuzo (Ohtsu, Japan), chloramine-T from Wako (Osaka, Japan), sodium [^{125}I]iodine from Amersham (Buckinghamshire, UK), ammonium sulfate from Katayama (Tokyo, Japan). Deionized water was prepared by the MilliQ system (Millipore, Bedford, MA). All reagents were of special grade. Anti-TA-0910 antiserum and [^{125}I]TA-0910, which was used for the assay of TA-0910 in rat plasma, were offered by S. Chishima of the Pharmaceutical Development Research Laboratory of our company [4].

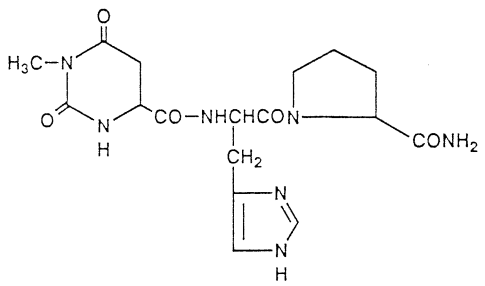
2.2. Animals

Female New Zealand white rabbits, weighing 1.0–1.3 kg, were purchased from Kitayama (Nagano, Japan).

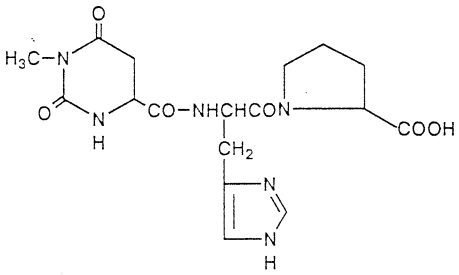
2.3. Assay method of TA-0910 in plasma and urine

2.3.1. Assay procedure

Anti-TA-0910 antiserum was diluted 5000-fold with 1/15 M PBS (pH 7.4) containing 0.1% bovine-γ-globulin, and [^{125}I]TA-0910 was diluted



TA-0910



TA-0910 acid-type

Fig. 1. Chemical structure of TA-0910 and TA-0910 acid-type.

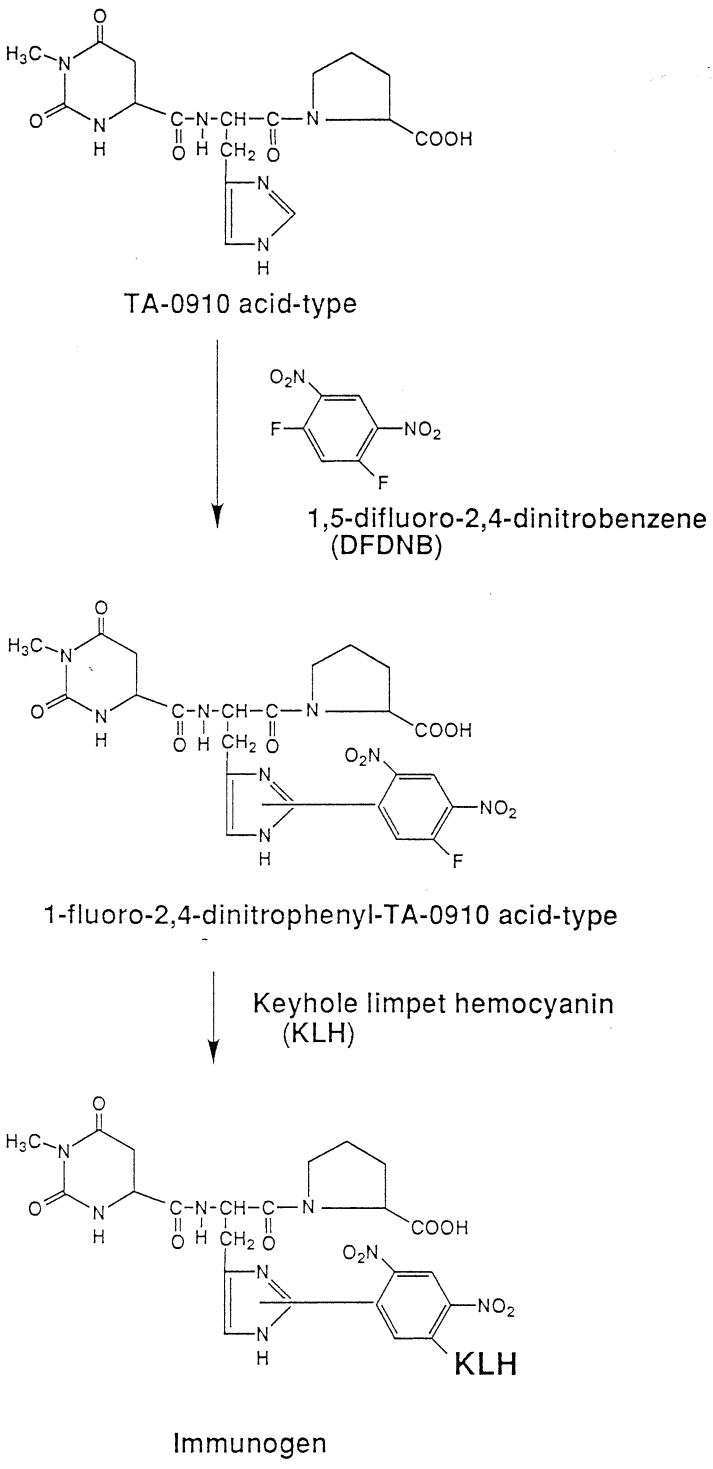


Fig. 2. Synthesis of the immunogen.

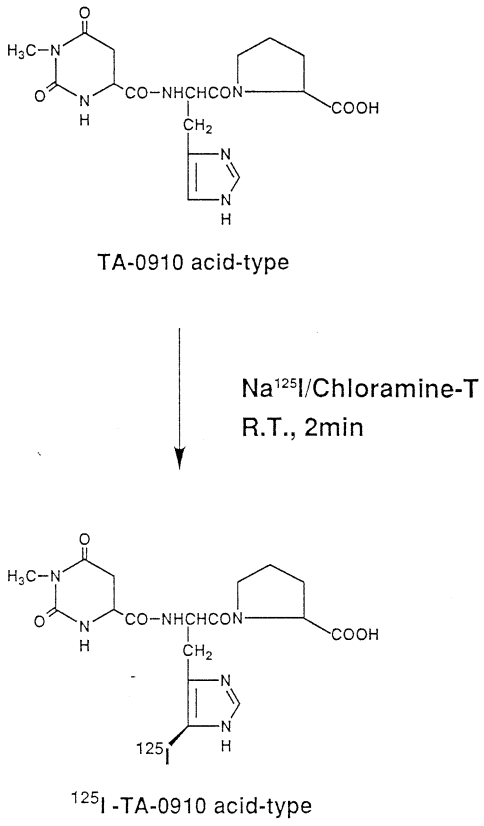


Fig. 3. Synthesis of the radioligand.

to give radioactivity of 10 000–12 000 cpm 100 μl^{-1} with water. In the determination of TA-0910 in plasma, 100 μl water, 100 μl sample, 100 μl antiserum and 100 μl radioligand were added to 200 μl 1/15 M PBS (pH 7.4) containing 0.1% bovine-γ-globulin in a tube. After mixing on a vortex mixer, the tubes were incubated for 40 h at 4°C. 600 μl Saturated ammonium sulfate solution (pH 7.4) was added to the tubes. After mixing on a vortex mixer, the tubes were stood for 25 min in ice water. After centrifugation at $2000 \times g$ for 30 min at 4°C the supernatant was removed by aspiration. 500 μl 50% Ammonium sulfate solution was added to the tubes. After mixing on a vortex mixer, the tubes were centrifuged at $2000 \times g$ for 30 min at 4°C. The supernatant was removed by aspiration. The radioactivity of the pellets was counted for 3 min using a γ-scintillation counter. Concentration of samples were calculated from the standard curve by Immuno Fit™-EIA/RIA (Beckman). TA-0910 in urine was determined according to the same assay procedure as for plasma.

2.4. Assay method of TA-0910 acid-type in plasma and urine

2.4.1. Preparation of immunogen

As shown in Fig. 2, immunogen was prepared by coupling hapten to KLH using DFDNB. For this, 150 mg DFDNB in 5 ml methanol was added to a solution of 14.5 mg TA-0910 acid-type in 1 ml 0.1 M phosphate buffer (pH 7.2). After reaction for 20 min at room temperature, 2.5 ml 0.1 M phosphate buffer (pH 7.2) was added to the mixture. It was washed three times with 20 ml ethyl acetate under ice cooling. 125 mg KLH in 4 ml 0.1 M borate buffer (pH 10.0) was added to the water layer containing the 1-fluoro-2,4-dinitrophenyl-TA-0910 acid-type derivative. After stirring for 24 h at room temperature in the dark, the reaction mixture was dialyzed against 5 l water three times a day for 2 days. The dialyzed solution was adjusted with water to bring the protein concentration to 2 mg ml^{-1} and was stored in a deep freeze at -80°C.

2.4.2. Immunization

The immunogen solution was an emulsified 1:2 (v/v) mixture with FCA. Five rabbits were injected intracutaneously at several sites on the back and intramuscularly at the hind limb femoral with 750 μl emulsion. A booster injection was given with 375 μl emulsion at 3–4 week intervals for 20 weeks. At 1–2 week intervals after each injection, blood samples were taken from the marginal ear vein for determination of the antibody titre. After the final injection, the whole blood was bled by heart puncture under anesthesia. The serum was stored in small aliquots at -80°C. Each serum sample was diluted (1:5000) and its B/T value was measured. The serum taken 20 weeks after immunization was used as an antiserum, since the B/T value of the serum was about 50%.

2.4.3. Preparation of the radioligand

The radioligand was prepared by introducing ^{125}I into the histidine imidazole ring of TA-0910 acid-type by the Na[^{125}I]chloramine-T method [5] as shown in Fig. 3. 15 μl 0.5 M phosphate buffer (pH 7.4), 15 μl of a 167 μl ml^{-1} solution TA-0910 acid-type dissolved in 0.5 M phosphate buffer (pH 7.4) and 10 μl of a 0.5 mg ml^{-1} solution of chloramine-T dissolved in 0.5 M phosphate buffer (pH 7.4) were added to 3.7 MBq of sodium [^{125}I]iodide. After 2 min at room temperature, the reaction was stopped by addition of 10 μl of a 1 mg ml^{-1} solution of sodium metabisulfite dissolved in 0.5 M phosphate buffer (pH 7.4). 20 μl 0.5 M phosphate buffer (pH 7.4) was added to the solution. The reaction mixture was purified by reversed-phase HPLC using a column (4.6 mm $\phi \times 150$ mm) packed with Shodex gel F-411 A (5 μm) (Showa Denko, Tokyo, Japan). The mobile phase consisted of a mixture of 0.05 M TFA and methanol (6.5:1). The flow rate was 1.0 ml min^{-1} and the column was operated at room temperature. The eluate was collected every 0.5 ml and the radioactivity of each fraction was counted by a γ-scintillation counter (COBRA; Packard, Meriden, CT). Two peaks (F4, F18) were observed, and F18 was adopted as a radioligand since the peak of F18 was reached with antiserum. The radioligand was stored at −20°C and used for two months.

Table 1
Cross-reactivity of anti-TA-0910 and TA-0910 acid-type antiserum

Compound	Cross-reactivity (%)	
	Anti-TA-0910 antiserum	Anti-TA-0910 acid-type antiserum
TA-0910	100	0.12
TA-0910 acid-type	<0.01	100
Demethyl TA-0910	0.89	<0.01
Oro-His-COOH	<0.01	<0.01
His-Pro-NH_2	<0.01	<0.01
TRH	<0.01	<0.01
TRH acid-type	<0.01	<0.01

2.4.4. Assay procedure

Anti-TA-0910 acid-type antiserum was diluted 5000-fold with 1/15 M PBS (pH 7.4) containing 0.1% bovine-γ-globulin, and [^{125}I]TA-0910 acid-type was diluted to give radioactivity of 10 000–12 000 cpm 100 μl^{-1} with water. In the determination of TA-0910 acid-type in plasma, 100 μl water, 100 μl sample, 100 μl antiserum and 100 μl radioligand were added to 200 μl 1/15 M PBS (pH 7.4) containing 0.1% bovine-γ-globulin in a tube. The following assay procedure was performed according to the same assay procedure as for TA-0910.

2.5. Applications

2.5.1. Human study

A tablet of TA-0910 (5 mg) was administered orally to six healthy male volunteers. Blood samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h. Urine samples were collected for 0–2, 2–4, 4–6, 6–8, 8–12 and 12–24 h. The plasma and urine samples were frozen at −20°C until used.

2.5.2. Pharmacokinetic analysis

The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined from the observed plasma concentrations of TA-0910 and TA-0910 acid-type. The area under the plasma concentration–time curve (AUC_∞) from zero to infinity was calculated as the sum of $AUC_t + AUC_{t-\infty}$. AUC_t from zero to the last measurable time was calculated by trapezoidal rule and $AUC_{t-\infty}$ was calculated according to C_n/K_{el}. C_n was the last observed plasma concentration and K_{el} the elimination rate constant. Elimination half-life ($t_{1/2}$) was calculated by log-linear regression for the declining plasma concentrations against time after administration.

3. Results and discussion

3.1. Specificity of antiserum

Cross-reactivity of anti-TA-0910 or anti-TA-0910 acid-type antiserum were examined for TA-0910, its metabolite and its related compounds. It

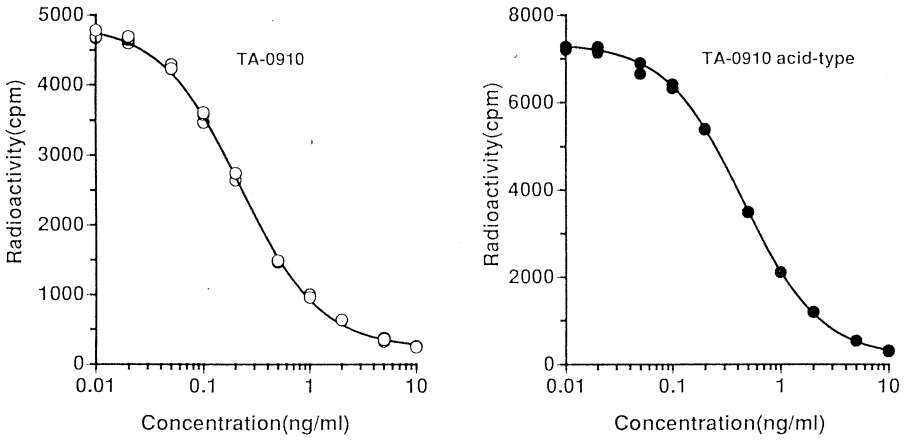


Fig. 4. Typical standard curves for TA-0910 (○) and TA-0910 acid-type (●) in plasma obtained by RIA.

was defined as the ratio (%) of the concentration of TA-0910 or TA-0910 acid-type to that of the related compounds which is necessary to cause 50% displacement of the radioligand. The cross-reactivity of anti-TA-0910 antiserum and anti-TA-0910 acid-type are summarized in Table 1. The cross-reactivity of anti-TA-0910 antiserum for demethyl TA-0910 was only 0.89% and was below 0.01% for other compounds. Also, anti-TA-0910 acid-type antiserum for TA-0910 was only 0.12% and it was below 0.01% for other compounds. Therefore, it was concluded that the anti-TA-0910 and anti-TA-0910 acid-type antiserum were specific for TA-0910 and TA-0910 acid-type, respectively.

3.2. The standard curve

Plasma concentrations of TA-0910 and TA-0910 acid-type were at the ng ml^{-1} level following oral administration of TA-0910 (dose 0.5–40 mg) to humans, and therefore a range of 0.02–5 ng ml^{-1} for the standard curve was considered to be suitable for the determination of TA-0910 and TA-0910 acid-type in the samples. Typical standard curves for [^{125}I]TA-0910 binding to anti-TA-0910 antiserum by adding TA-0910 (0.01–10 ng ml^{-1}) and [^{125}I]TA-0910 acid-type binding to anti-TA-0910 acid-type antiserum by adding TA-0910 acid-type (0.01–10 ng ml^{-1}) in the presence of 100 μl human plasma are shown in Fig. 4.

The inter-assay variations of the standard curves for TA-0910 and TA-0910 acid-type in plasma were 0.2–10.8 and 0.6–13.0% (0.02–10 ng ml^{-1}), respectively as R.S.D., and the accuracy of the standard curves were very high (data not shown). Also, inter-assay variation and accuracy for urine were the same as those for plasma.

3.3. Precision and recovery

The inter-assay variations of RIA for TA-0910 and TA-0910 acid-type using plasma spiked with different levels of TA-0910 and TA-0910 acid-type are shown in Table 2. The R.S.D. values of TA-0910 and TA-0910 acid-type in plasma were 3.5–15.5 and 1.8–9.4%, respectively. The R.S.D. values for urine were the same as those for plasma (data not shown). The recovery was 95.2–98.9 and 92.1–95.0%, respectively, for plasma and 87.4–96.5 and 92.4–103.0%, respectively, for urine. Also, the inter-assay variations for TA-0910 and TA-0910 acid-type in plasma (0.02 ng ml^{-1}) showed approximately 20% as R.S.D. values, though the data is not shown in Table 2. Thus, it was considered that the quantitative limit of both compounds was 0.02 ng ml^{-1}.

Table 2
Precision and recovery of TA-0910 and TA-0910 acid-type in human plasma

Repetition	TA-0910			TA-0910 acid-type		
	Nominal concentration (ng ml^{-1})			Nominal concentration (ng ml^{-1})		
	0.040	0.40	4.00	0.050	0.50	5.00
1	0.040	0.39	3.89	0.051	0.48	4.54
2	0.037	0.39	3.36	0.051	0.49	4.89
3	0.027	0.42	3.77	0.046	0.47	4.70
4	0.040	0.40	3.46	0.045	0.48	4.61
5	0.043	0.38	3.79	0.043	0.47	4.79
6	0.043	0.40	4.57	0.041	0.47	4.88
Mean (ng ml^{-1})	0.038	0.40	3.81	0.046	0.48	4.74
R.S.D. (%)	15.5	3.5	11.2	9.4	1.8	3.0
Recovery (%)	95.4	98.9	95.2	92.1	95.0	94.7

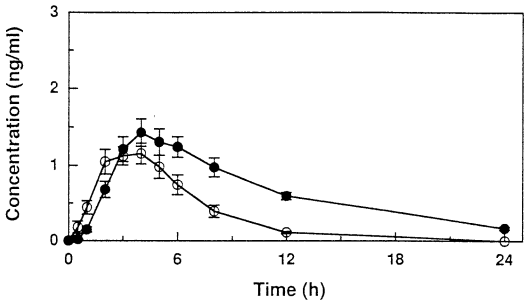


Fig. 5. The mean plasma concentration of TA-0910 (○) and TA-0910 acid-type (●) in six healthy volunteers after a single oral administration of TA-0910 at a dose of 5 mg (mean ± S.E.).

Table 3
Pharmacokinetic parameters in humans for TA-0910 and TA-0910 acid-type

Parameters	Compound	
	TA-0910	TA-0910 acid-type
C_{max} (ng ml^{-1})	1.20 ± 0.14	1.45 ± 0.17
AUC_{∞} (ng·h ml^{-1})	7.58 ± 1.07	16.81 ± 1.67
T_{max} (h)	3.5 ± 0.2	4.3 ± 0.3
$t_{1/2}$ (h)	2.19 ± 0.12	6.36 ± 0.29

Each value represents the mean ± S.E. for six subjects.

3.4. Application to pharmacokinetics of TA-0910 in humans

The RIA method was applied to the determination of TA-0910 and TA-0910 acid-type in human plasma and urine samples for the evaluation of the pharmacokinetics in humans. Plasma and urine concentrations of TA-0910 and TA-0910 acid-type were determined up to 24 h after oral administration of one tablet containing 5 mg TA-0910 to six healthy volunteers.

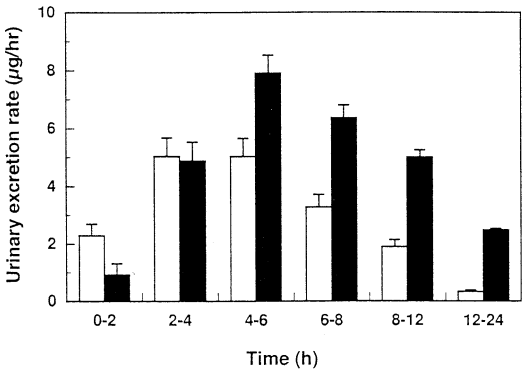


Fig. 6. Urinary excretion of TA-0910 (□) and TA-0910 acid-type (■) in six healthy volunteers after a single oral administration of TA-0910 at a dose of 5 mg (mean + S.E.).

The mean plasma concentrations of TA-0910 and TA-0910 acid-type are shown in Fig. 5. Pharmacokinetic parameters for TA-0910 and TA-0910 acid-type are shown in Table 3. The plasma concentration of TA-0910 reached a maximum 3.5 h after administration, and thereafter decreased with an elimination half-life ($t_{1/2}$) of 2.19 h. On the other hand, the plasma concentration of TA-0910 acid-type reached a maximum 4.3 h after administration, and thereafter decreased with $t_{1/2}$ of 6.36 h. The ratio of C_{max} for TA-0910 acid-type to TA-0910 was 1.21, and the ratio of AUC_{∞} was 2.22.

The urinary excretion of TA-0910 and TA-0910 acid-type are shown in Fig. 6. The urinary excretion rates of TA-0910 and TA-0910 acid-type show a similar profile to the plasma concentration curves, and the urinary excretion of TA-0910 and TA-0910 acid-type within 24 h after dosing were 1 and 2% of the dose, respectively.

In conclusion, it was found that RIA is useful for the determination of TA-0910 and TA-0910 acid-type in human plasma and urine, and that it is applicable to pharmacokinetic studies in humans.

References

[1] M. Suzuki, H. Sugano, K. Matsumoto, M. Yamamura, R. Ishida, J. Med. Chem. 33 (1990) 2130–2137.
[2] M. Yamamura, K. Kinoshita, H. Nakagawa, T. Tanaka, K. Maeda, R. Ishida, Jap. J. Pharmacol. 53 (1990) 451–461.
[3] K. Kinoshita, T. Nagano, H. Ono, Neuropharmacology. 33 (1994) 1183–1188.
[4] S. Chishima, J. Pharm. Biomed. Anal. 12 (1994) 795–804.
[5] F.C. Greenwood, W.M. Hunter, J.S. Glover, Biochem. J. 89 (1963) 114–123.