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Enzyme-linked immunosorbent assay for TA-2005-glucuronide in human plasma

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Abstract

A sensitive enzyme-linked immunosorbent assay (ELISA) for TA-2005-glucuronide, a main metabolite of new adrenergic β-receptor agonist TA-2005, has been investigated without prior deconjugation. Coupling of the hapten with bovine serum albumin (BSA) or β-D-galactosidase was carried out by the *N*-hydoxysuccinimide ester method. An anti-TA-2005-glucuronide antiserum was obtained from guinea pig immunized with the hapten–BSA conjugate. The ELISA was based upon a competitive assay in which the separation of bound from free fraction was performed by the double antibody technique using rabbit anti guinea pig immunoglobulin antibody adsorbed to microtiter plates.

A satisfactory standard curve for the ELISA of TA-2005-glucuronide was observed in the range of 30 pg–3 ng ml^{-1} using 25 μl of human plasma. Inter-day and intra-assay variations were 7.0–17.5% and 1.0–11.7% respectively. The recoveries of TA-2005-glucuronide spiked to plasma samples were 95.5–120% (inter-assay) and 96.0–123.3% (intra-assay). The cross-reactivities of the prepared antiserum with the related compound of TA-2005-glucuronide were quite low though there was a considerable cross-reaction with TA-2005. However, TA-2005-glucuronide could be easily separated from TA-2005 by a simple pretreatment of the plasma sample with a C_{18} cartridge column. This method was applied to the determination of TA-2005-glucuronide in human plasma samples for the evaluation of the pharmacokinetics of TA-2005. From the results, it was demonstrated that the ELISA developed was useful for the determination of TA-2005-glucuronide in human plasma and that the method was applicable to pharmacokinetic studies in humans.

Keywords: TA-2005-glucuronide; Metabolite; ELISA (enzyme-linked immunosorbent assay); Human plasma; Adrenergic β-receptor agonist; Cross-reactivity; Pharmacokinetic studies

1. Introduction

Glucuronidation is an important metabolic conjugation pathway, occurring in mammals during the metabolism of both xenobiotics such as

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drugs and endogenous compounds such as hormones or bile acids. The glucuronic metabolites, however, are often neglected in pharmacokinetic and pharmacodynamic studies. Glucuronides are considered to have high water solubility, low protein binding and small volumes of distribution, and thus are rapidly excreted. They are generally assumed to be pharmacologically inactive and are rarely taken into account in evaluating drug effects. However, there are an increasing number of reports that describe an enhancement of biological activity as the result of glucuronidation [1,2]. Such contributions may consist of direct effects (e.g. morphine-6-glucuronide) or results from indirect effects due to reactivity of glucuronide during enterohepatic circulation [3–5]. The reaction of glucuronidation mediates formation of ether, ester, thiolic, *N*- or *C*-glucuronide, leading in each case to an enhanced water solubility in comparison with the parent compound. Therefore, the compounds having phenolic hydroxy group are metabolized to *O*-glucuronide and excreted in plasma and urine mainly as conjugate with glucuronic acid.

TA-2005, 8-hydroxy-5-[(1*R*)-1-hydroxy-2-[*N*-[(1*R*)-2-(*p*-methoxy-phenyl)-1-methylethyl]-amino]ethyl]-carbostyril hydrochloride, was newly synthesized as a novel bronchodilator [6]. The bronchodilating action of TA-2005 was greater than that of formoterol, suggesting that TA-2005 could exert a stronger pharmacological action at a lower blood concentration than formoterol [7]. Since the clinical dose is as low as μg order, the determinations of TA-2005 are exclusively performed by an enzyme immunoassay using a highly specific antibody for unchanged TA-2005.

In a previous study, Yoshikawa et al. (Pharmaceutical Development Research Laboratory, Tanabe Seiyaku) have demonstrated a main metabolite of TA-2005 is its *O*-glucuronide derivative (unpublished data). The determination of TA-2005-glucuronide has been attempted by the conventional method of releasing the unconjugate using β-glucuronidase following the unconjugate to determine by the enzyme immunoassay for TA-2005. However, that trial was found to be unsuccessful, considering β-glucuronidase used at the deconjugation step would interfere with the determination step of the enzyme immunoassay for the unconjugated compound. There have been a few reports about the immunoassay for direct measurement of those conjugate without using a hydrolysis, solvolysis or oxidation-fission step to release the unconjugate moiety for determination [8,9]. These steps are undesirable, for not only do they destroy the identity of the conjugate, but it is impossible to determine accurately how much of the conjugate is destroyed during the fission.

In the present study, we describe the development of an ELISA for TA-2005-glucuronide in human plasma and its application to pharmacokinetic study in human, and also deal with the preparation of the TA-2005-glucuronide-BSA conjugate and the labeling of TA-2005-glucuronide with β-D-galactosidase. The utilization of the convenient and highly sensitive method for the measurement of glucuronide metabolite will facilitate the understanding of drug metabolism and distribution of the metabolites.

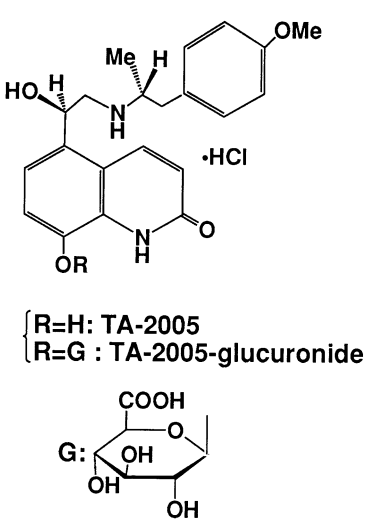

Fig. 1. Chemical structures of TA-2005 and its main metabolite, TA-2005-glucuronide.

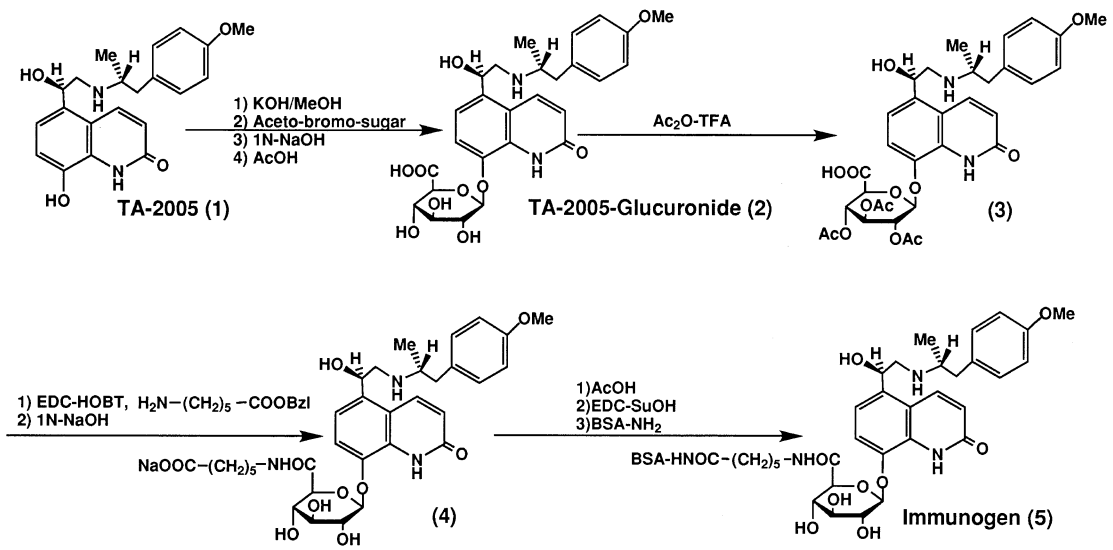

Fig. 2. Synthetic route of immunogen.

2. Experimental

2.1. Chemicals and reagents

TA-2005, 8-hydroxy-5-[(1*R*)-1-hydroxy-2-[*N*-[(1*R*)-2-(*p*-methoxy-phenyl)-1-methylethyl]-amino]-ethyl]-carbostyril hydrochloride (Fig. 1) was synthesized at Tanabe Seiyaku. ϵ-Aminohexanoic acid, 4-aminocyclohexane carboxylic acid and general purpose solvents were purchased from Katayama (Osaka, Japan) and ethyl dimethylaminopropyl-carbodiimide hydrochloride (EDC) was purchased from Aldrich (Milwaukee, WI). HiTrap™ Desalting was obtained from Pharmacia Biotech (Uppsala, Sweden) and HP-20 was obtained from Mitsubishi (Tokyo, Japan). Phosphate-buffered saline (PBS) was purchased at Life Technologies (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). β-D-Galactosidase (*Escherichia coli*) was purchased from Boehringer Mannheim (Mannheim, Germany). Freund's complete and incomplete adjuvants were purchased at DIFCO (Detroit, MI). Rabbit anti-guinea pig immunoglobulin was purchased from DAKO (Glostrup, Denmark).

2.2. Apparatus

Spectra were obtained with the following machines: UV on Hitachi U-3210 (Hitachi, Tokyo, Japan), proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded in DMSO-d_6 using tetramethylsilane (TMS) as an internal standard on Bruker AC-200 or DRX-500 spectrometers. The abbreviations used are as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. Thin-layer-chromatography (TLC) was performed on precoated silica gel plate 0.25 mm thick or 2 mm thick (Kieselgel F254, Merck, Darmstadt, Germany), and detection was achieved by UV irradiation (254 nm) or spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 followed by heating. Fluorescence intensity was measured using CytoFluor 2350 (Millipore, Bedford, MA). The secondary ion mass spectrometry (SIMS) was measured using a Hitachi M-80A mass spectrometer equipped with a SIMS system.

2.3. Synthesis of hapten–BSA conjugate

The hapten–BSA conjugate was prepared by following four steps, as shown in Fig. 2.

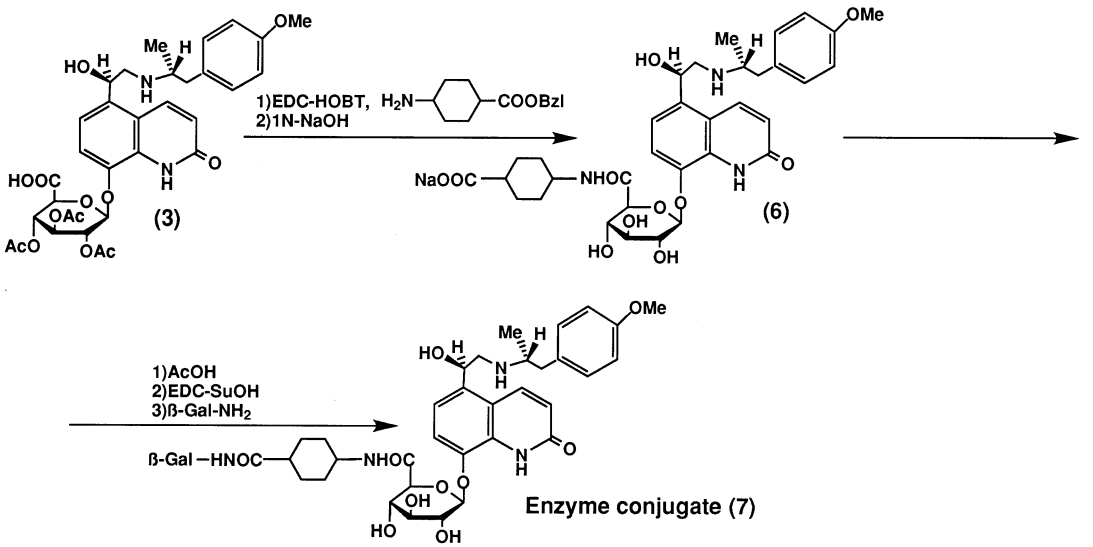

Fig. 3. Synthetic route of enzyme conjugate.

TA-2005-glucuronide (**2**): To a solution of potassium hydroxide 3.6 g (62 mmol) in methanol (250 ml) was added TA-2005 (**1**) 12.45 g (31 mmol) and the mixture was stirred at room temperature for 30 min under nitrogen atmosphere. The mixture was then cooled in an ice-water bath and then methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl)uronate-bromide [10] 21.31 g (54 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 117 h (5 days). The solvent was evaporated to dryness under reduced pressure at 40°C. The residue dissolved in a small amount of water was adsorbed on to a HP-20 column (500 ml), washed with water and eluted with 66% aq. methanol and the elution was evaporated to dryness under reduced pressure at 40°C. To a stirred solution of the above residue in methanol (200 ml) was added dropwise 1 N sodium hydroxide (50 ml) under ice-cooling. Then, the mixture was stirred at room temperature for 1 h. After concentration in vacuo, the concentrate was applied to a HP-20 column, washed with water, eluted with 33% aq. methanol and thereafter the elute was concentrated in vacuo. The concentrate was adjusted to pH 6 with acetic acid under ice-cooling. The solvent was evaporated to dryness under reduced pressure at 40°C. The residue was recrystallized from methanol–water to give compound (**2**). Yield: 1.2 g (7.0%); ^{1}H-NMR: δ 1.02 (3H, d, CH_3, J = 6.2 Hz), 2.51 (1H, m, $C\underline{H}_2$-$C_6H_5 \times 1/2$), 2.92 (3H, m, $C\underline{H}_2$-$C_6H_5 \times 1/2$, $C\underline{H}_2$-NH), 3.22 (1H, m, $CH_3C(\underline{H})$-CH_2), 3.22–3.57 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.72 (3H, s, OCH_3), 4.91 (1H, d, H-1′, J = 7.3 Hz), 5.32 (1H, m, $C(\underline{H})OH$-CH_2), 5.86 (1H, br, $C(H)O\underline{H}$-CH_2), 6.60 (1H, d, carbostyril H-3, J = 9.7 Hz), 6.84 and 7.08 (2H each, d, phenyl, J = 8.6 Hz), 7.34 and 7.43 (1H each, d, carbostyril H-6,7 J = 8.4 Hz), 8.49 (1H, d, carbostyril H-4, J = 10.1 Hz), 10.92 (1H, br, carbostyril H-1); SIMS (m/z): 545 $[M+H]^+$.

1-*O*-(TA-2005)-2,3,4-tri-*O*-acetyl-D-glucuronic acid (**3**): To a solution of trifluoroacetic acid-(45 μl, 46 μmol) in acetic anhydride (100 μl) was

Table 1
Specificity of anti-TA-2005-glucuronide antiserum

Compound	Cross reaction (%)
TA-2005-glucuronide	100
TA-2005 (*R*, *R*)	80.3
6-Amino-*n*-capronic acid	<0.001
8-Hydroxy carbostyril	<0.001
D-Glucuronic acid	<0.001

added compound (**2**) (5 mg, 92 μmol) and the mixture was stirred under ice-cooling for 2 h. The solvent was evaporated to dryness under reduced pressure at 40°C. The residue dissolved in a small amount of 20% aq. methanol. The solution was adsorbed onto a HP-20 column, washed with 30% aq. methanol and eluted with 40% aq. methanol. The elution was concentrated to dryness under reduced pressure to leave an amorphous powder. Yield: 5.9 mg (96%); ^{1}H-NMR: δ 1.10 (3H, d, CH_3, J = 6.0 Hz), 2.00 (9H, s, OAc × 3), 2.63 (1H, m, C$\underline{H}_2$-NH × 1/2), 3.12 (1H, m, C$\underline{H}_2$-NH × 1/2), 3.55 (2H, m, C$\underline{H}_2$-C_6H_5), 3.73 (3H, s, OCH_3), 4.57 (1H, d, H-1′, J = 7.2 Hz), 5.18 (1H, t, H-2′), 5.33 (1H, m, H-4′), 5.39–5.46 (2H, m, C($\underline{H}$)OH-CH_2, H-3′), 5.79 (1H, d, H-5′), 6.35 (1H, brs, C(H)O$\underline{H}$-CH_2), 6.68 (1H, d, carbostyril H-3, J = 10.2 Hz), 6.91 and 7.16 (2H each, d, phenyl, J = 8.4 Hz), 7.19 and 7.36 (1H each, d, carbostyril H-6,7, J = 8.4 Hz), 8.20 (1H, d, carbostyril H-4, J = 10.1 Hz), 8.65 (1H, brs, CH_2-N$\underline{H}$), 10.49 (1H, brs, carbostyril H-1); SIMS (*m*/*z*): 671 $[M + H]^+$.

N-(5-carboxypentyl)-1-*O*-(TA-2005)-D-glucuronamide sodium salt (**4**): EDC (1.7 mg, 89 μmol) was added to a solution of compound (**3**) (5 mg, 7.4 μmol), HOBT (1.2 mg, 8.9 μmol) and benzyl-ϵ-aminohexanoate-*p*-toluenesulfonate [11] (5.8 mg, 7.4 μmol) in tetrahydrofuran (1 ml) under ice-cooling. The mixture was stirred at room temperature for 2 h. The solvent was evaporated to dryness under reduced pressure at 40°C. The residue was purified by PTLC using 17% methanol–chloroform as a developing solvent. The elution was evaporated to dryness under reduced pressure to give the residue. The residue was dissolved in methanol (600 μl), then to this was added 1 N sodium hydroxide dropwise under ice-cooling. The reaction mixture was stirred at the same temperature for 50 min. The solvent was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of 10% aq. methanol. The solution was adsorbed on to a HP-20 column, washed with 5% aq. methanol and eluted with 30% aq. methanol. The elution was evaporated to dryness under reduced pressure to leave an amorphous powder. Yield: 3.1 mg (62%); ^{1}H-NMR: δ 1.07 (3H, d, CH_3, J = 6.3 Hz), 1.28–1.38 (8H, m, NH-C$\underline{H}_2$-C$\underline{H}_2$-C$\underline{H}_2$-C$\underline{H}_2$), 1.54 (2H, m, C$\underline{H}_2$-COOH), 2.56 (2H, d, C$\underline{H}_2$-C_6H_5, J = 5.1 Hz), 2.76 and 3.00 (1H each, m, CH(OH)-C$\underline{H}_2$), 2.81 (1H, m, CH_3C($\underline{H}$)-CH_2-C_6H_5), 3.54–3.96 (4H, m, H-2′,H-3′,H-4′,H-5′), 3.75 (3H, s, OCH_3), 4.98 (1H, d, H-1′, J = 7.9 Hz), 5.15 (1H, m, C($\underline{H}$)OH-CH_2), 6.65 (1H, d, carbostyril H-3, J = 10.4 Hz), 6.83 and 6.95 (2H each, d, phenyl, J = 8.5 Hz), 7.36 and 7.41 (1H each, d, carbostyril H-6,7, J = 8.4 Hz), 8.32 (1H, d, carbostyril H-4, J = 9.9 Hz); SIMS (*m*/*z*): 680 $[M + H]^+$.

N-(TA-2005-glucuronyl)-ϵ-aminohexanoic acid–BSA conjugate (**5**): Compound (**4**) (15 mg, 22 μmol) was dissolved in water (1 ml) and the solution was adjusted to pH 6 with acetic acid under ice-cooling. The solvent was lyophilized. The residue suspended in tetrahydrofuran (1.5 ml), pyridine (0.5 ml), EDC (6.3 mg, 33 μmol) and *N*-hydroxysuccinimide (3.8 mg, 33 μmol) was added to the neutralized solution of compound (**4**). The mixture was stirred at room temperature for 2 h. A solution of the crude activated ester was added to PBS (4 ml) solution of BSA (101 mg, 2 μmol) and the mixture was stirred at 4°C overnight. The resulting turbid solution was purified by a HiTrap™ Desalting column. This conjugate was stored at −20°C until use. The UV spectrometric analysis was performed by comparing the absorbance at 254 nm of the conjugate (**5**) with those of BSA and compound (**4**) as controls in PBS. The protein content of the conjugate (**5**) was determined by the method of Lowry et al. [12].

2.4. Preparation of anti-TA-2005-glucuronide antiserum

The hapten–BSA conjugate (1 mg) was dissolved in PBS and emulsified with the same amount of complete Freund's adjuvant. The emulsion was injected i.p. and s.c. at multiple sites on the back into Hartley female guinea pig (Japan SLC Sizuoka, Japan). Booster injections with half the initial amount of immunogen were administered three times every 3 weeks during 2 months. The blood was collected from postcava after the last booster injection. The serum was separated by centrifugation and was stored at −80°C until use.

Table 2
Inhibition of bound enzymatic activity of enzyme-conjugate by 1 ng/ml of TA-2005-glucuronide in the assays

Enzyme conjugate	Inhibition (%)
Homologous	44
Heterologous	95

2.5. Preparation of TA-2005-glucuronide-β-D-galactosidase conjugate

TA-2005-glucuronide-β-D-galactosidase conjugate was prepared by following two steps from compound (**3**) as shown in Fig. 3.

N-(4-carboxycyclohexyl)-1-*O*-(TA-2005)-D-glucuronamide sodium salt (**6**): EDC (11 mg, 0.11 mmol) was added to a suspension of compound (**3**) (31 mg, 46 μmol), HOBT (1 mg, 11 μmol), and benzyl-4-aminocyclohexane-carboxylate-*p*-toluenesulfonate [11] (37 mg, 92 μmol) in tetrahydrofuran (2 ml) under ice-cooling. The mixture was stirred at room temperature for 2 h. The solvent was evaporated to dryness under reduced pressure at 40°C. The residue was purified by PTLC using 17% methanol–chloroform as a developing solvent. The elution was evaporated to dryness under reduced pressure to leave an amorphous powder. The residue was dissolved in methanol (600 μl), then to this was added 1 N sodium hydroxide dropwise under ice-cooling. The reaction mixture was stirred at the same temperature for 50 min and was adjusted to pH 6 with acetic acid under ice-cooling. The solvent was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of 10% aq. methanol. The solution was adsorbed on to a HP-20 column, washed with 5% aq. methanol, and eluted with 30% aq. methanol. The elution was evaporated to dryness under reduced pressure to leave an amorphous powder. Yield: 3.1 mg (62%); ^{1}H-NMR: δ 1.08 (3H, d, CH_3, J = 6.3 Hz), 1.28–1.67 (10H, m, NH-$C_6\underline{H}_{10}$-COOH), 2.55 (2H, d, $C\underline{H}_2$-C_6H_5), 2.76 and 3.00 (1H each, m, C(H)OH-$C\underline{H}_2$), 2.85 (1H, m, $CH_3C(\underline{H})$-CH_2), 3.44–3.96 (4H, m, H-2′,H-3′,H-4′,H-5′), 3.77 (3H, s, OCH_3), 4.99 (1H, d, H-1′, J = 7.3 Hz), 5.14 (1H, m, $C\underline{H}$(OH)-CH_2), 6.64 (1H, d, carbostyril H-3, J = 9.9 Hz), 6.81 and 6.96 (2H each, d, phenyl, J = 8.4 Hz), 7.29 and 7.36 (1H each, d, carbostyril H-6,7, J = 8.3 Hz), 8.32 (1H, d, carbostyril H-4, J = 9.9 Hz); SIMS (*m*/*z*): 693 $[M + H]^+$.

TA-2005-glucuronide-β-D-galactosidase conjugate (**7**): Compound (**6**) (4 mg, 5.8 μmol) was dissolved in water. The solution was adjusted to pH 6 with acetic acid under ice-cooling. The solvent was lyophilized. The residue suspended in tetrahydrofuran (0.5 ml) and pyridine (0.5 ml), EDC (1.8 mg, 8.7 μmol) and *N*-hydroxysuccinimide (1.0 mg, 8.7 μmol) was added to the solution. The mixture was stirred at room temperature for 2 h. This active ester (0.2 μmol; 35 μl of the solution was pipetted off) was added to a solution of β-D-galactosidase (1 mg, 2 nmol) in PBS (0.5 ml) and the mixture was stirred at 4°C overnight. The mixture was directly chromatographed on a HiTrap™ Desalting column. The peak fractions were pooled at 4°C until use.

2.6. Pretreatment of plasma samples

The human plasma sample (0.2 ml) was diluted with PBS (1.8 ml). A 1.0-ml aliquot of the solution was applied to a Bond Elut C_{18} column (Varian, Harbor City, CA) which had been serially pre-washed with 3 ml each of methanol and water. The TA-2005-glucuronide fraction was eluted with 1 ml each of 10% aq. methanol and 20% aq. methanol. After removal of the solvent under N_2 gas stream at 50°C, the residue was dissolved in PBS (300 μl).

2.7. Assay procedure

The wells in ELISA plates (Corning, NY) were coated with 50 μl of rabbit anti-guinea pig immunoglobulin in PBS (10 μg ml^{-1}). After incubation overnight at 4°C, the plates were washed six times with PBS containing 0.05% Tween 20 (PBST). The plates were then coated with 250 μl of blocking reagent (Blockace, Snow Brand Milk Products, Tokyo, Japan) overnight at 4°C to prevent nonspecific adsorption. After washing the plates with PBST, the anti-guinea pig IgG coated wells were filled with 25 μl of plasma samples pretreated as already described, and 25 μl of

water or standard solution (5 pg–10 ng ml^{-1}). Then 25 μl of anti-TA-2005-glucuronide antiserum (1:10000) and 100 μl of TA-2005-glucuronide-β-D-galactosidase conjugate were added to the wells immediately. The wells were incubated overnight at 4°C and washed with PBST. A 100-μl aliquot of 0.1 mM 7-β-D-galactopyranosyloxy-4-methylcoumarin (substrate solution) was added to the wells. After incubation at 37°C for 4 h, the enzymatic reaction was stopped by addition of 100 μl of 0.2 M glycine–NaOH (pH 10.4). The fluorescence intensity of each well was measured (360 and 460 nm for excitation and emission, respectively) using a fluorescence plate reader. The concentrations of TA-2005 glucuronide in human plasma samples were determined by computer interpolation from a four-parameter logistic function plot of the standard data using a computer program [13].

2.8. Human study

A tablet of TA-2005 (40 μg) was orally administered to three healthy volunteers. Blood samples were taken from a forearm vein before drug administration and at 0.5, 1, 2, 3, 4, 6, 8, 10 and 12 h after treatment. Each blood sample was collected into tubes containing heparin as an anticoagulant. After centrifuging, the plasma samples were separated and frozen at −20°C until analysis. The plasma concentrations of unchanged TA-2005 were determined by a specific enzyme immunoassay developed at Pharmaceutical Development Research Laboratory of Tanabe Seiyaku.

2.9. Pharmacokinetic analysis

The time to reach the maximum plasma concentration was determined from the observed plasma concentrations of TA-2005 and TA-2005-glucuronide. The elimination half-life was calculated by log-linear regression for the declining plasma concentrations against time after administration.

3. Results and discussion

3.1. Preparation of immunogen

The BSA conjugate of TA-2005-glucuronide was obtained in good yield from TA-2005 glucuronide by selectively protecting the hydroxy groups of 2, 3, 4 position of glucuronic acid moiety with acetate. It is generally supposed that selective *O*-acetylation of glucuronic acid moiety is difficult, because the simultaneous procession of dehydration and degradation of the molecule has occurred. Provided a general acetylation method using pyridine-Ac_2O is carried out, a *N*-acetyl protecting group, that is not deprotected easily, is formed at the same time. Hence, we have managed *O*-acetylation using Ac_2O-trifluoroacetic acid (TFA), by that we have been able to protect selectively the hydroxy groups of glucuronic acid moiety without *N*-acetylation. We considered that the deprotonation of the amino group was depressed due to the addition of acid-catalyst. Consequently, it was considered that the method described above was a very useful derivatization method. Thereafter, we have selected ε-aminohexanoic acid as a chemical bridge between hapten and carrier protein, and the immunogen was prepared by condensation of a hapten having an activated ester with a carrier protein of BSA. The resultant immunogen was found to have a hapten/carrier molar ratio of 26.4:1.

3.2. Specificity of the antiserum

The specificity of the antiserum was defined as the ratio (%) of the concentration of TA-2005-glucuronide to the related compounds for 50% displacement of the enzyme conjugate. As shown in Table 1, the cross-reactivities of the antiserum to the related compounds of TA-2005-glucuronide were low, indicating the antiserum reacted neither with the part of 8-hydroxycarbostyryl moiety, nor with that of glucuronic acid moiety of TA-2005-glucuronide. However, there was a substantial cross-reaction with TA-2005. The reactivity toward TA-2005 was inconvenient for the precise determination of TA-2005-glucuronide in the plasma samples of humans orally administered

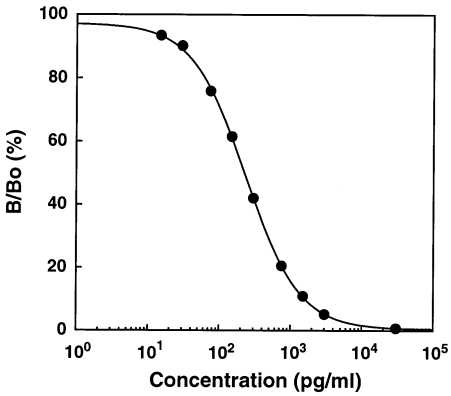

Fig. 4. A typical standard curve for TA-2005-glucuronide in human plasma.

TA-2005. Considering the apparent difference of the hydrophilicity between TA-2005 and TA-2005-glucuronide, we have examined a favorable procedure for the separation of the two compounds in human plasma. Thus, the pretreatment of the plasma samples by solid-phase extraction using Bond Elut C_{18} was found to be an easy and selective method for the separation of TA-2005-glucuronide from TA-2005 in human plasma.

For the refinement of the ELISA system, it was considered that the enzyme conjugate structure was an important factor prescribing the assay sensitivity. We have constructed two distinct enzyme conjugates, one has a hexanoate (homologous to the TA-2005-glucuronide-BSA conjugate spacer structure) and the other has cyclohexanoate (heterologous to the TA-2005-glucuronide-BSA conjugate spacer structure) spacer arm. Using two enzyme conjugates, we have examined the inhibition of enzymatic activity caused by the addition of 1 ng ml^{-1} of TA-2005-glucuronide. From the result, it was clearly demonstrated that the heterologous enzyme conjugate (cyclohexanoate type) was more effective than the homologous hexanoate spaced enzyme conjugate for increasing the sensitivity (Table 2). It was supposed that the anti-TA-2005-glucuronide antiserum contained various antibodies showing different specificity and at least a portion of the antibody population recognized not only the TA-2005-glucuronide molecule but also the bridge structure. It might be assumed that the low inhibition by TA-2005-glucuronide observed with homologous enzyme conjugate showed that this enzyme conjugate had a stronger affinity with the antiserum. As the antiserum did not have a cross-reaction with the bridge structure of 6-amino-*n*-capronic acid (Table 1), we thought that a part of the antiserum recognized both the bridge structure and glucuronic acid moiety together. Further studies are being conducted to determine the effect of the bridge length of the enzyme conjugate upon the assay sensitivity.

3.3. Sensitivity of the assay

A typical standard curve for enzyme-labeled TA-2005-glucuronide binding to the antiserum by addition of TA-2005-glucuronide ranging from 15

Table 3
Precision and recovery of TA-2005-glucuronide in human plasma (inter-assay)

Rep.	Nominal concentration (pg/ml)					
	30	75	150	300	1500	3000
1	34	85	174	310	1340	2345
2	44	71	143	326	1243	2679
3	32	89	148	288	1559	2999
4	41	61	128	323	1489	3398
5	29	89	141	278	1530	3258
Mean	36	79	147	305	1432	2936
S.D.	6	12	17	21	135	429
R.S.D. (%)	17.5	15.8	11.5	7.0	9.4	14.6
Recovery (%)	120.0	105.3	97.9	101.7	95.5	97.9

Table 4
Precision and recovery of TA-2005-glucuronide in human plasma (intra-assay)

Rep.	Nominal concentration (pg/ml)					
	30	75	150	300	1500	3000
1	42	76	138	304	1321	2985
2	36	66	136	309	1560	3250
3	34	74	166	305	1530	2890
Mean	37	72	145	306	1470	3042
S.D.	4	5	17	3	130	187
R.S.D. (%)	10.8	6.9	11.7	1.0	8.8	6.1
Recovery (%)	123.3	96.0	96.7	102.0	98.0	101.4

pg to 30 ng ml^{-1} in the presence of 25 μl of human plasma is shown in Fig. 4. Satisfactory curves were always obtainable in that range of TA-2005-glucuronide concentration. Consequently, it was defined that the quantitation limit of TA-2005-glucuronide with the ELISA was 30 pg ml^{-1}.

3.4. Precision and recovery

The inter-assay and intra-assay variations of ELISA for TA-2005-glucuronide were examined using human plasma spiked with different levels of TA-2005-glucuronide of 30, 75, 150, 300, 1500 and 3000 pg ml^{-1}. Inter-assay variation was calculated from the data on five different days. The relative standard deviations for inter-assay (Table 3) and intra-assay (Table 4) were 7.0–17.5% and 1.0–11.7% in the range of 30 pg–3 ng ml^{-1} of TA-2005-glucuronide addition. The recovery of TA-2005-glucuronide was 95.5–123.3% for human plasma.

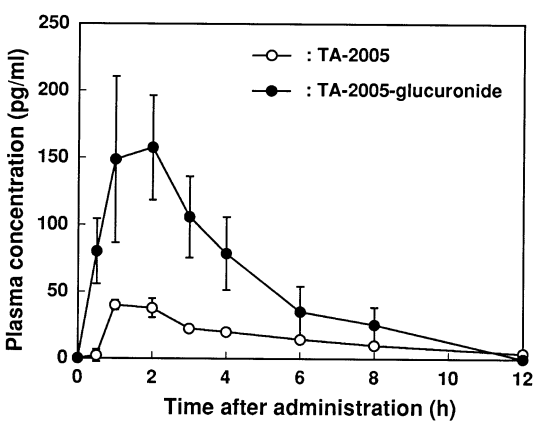

Fig. 5. Plasma concentrations of TA-2005 and TA-2005-glucuronide after oral administration of TA-2005 to healthy volunteers.

3.5. Application to pharmacokinetics of TA-2005-glucuronide in humans

The constructed ELISA was applied to the determination of TA-2005-glucuronide in human plasma samples for the evaluation of the pharmacokinetics in humans (Fig. 5). Plasma concentrations of TA-2005 are shown together in the same figure. The mean value of $AUC_{0\text{-}12}$ calculated by the linear trapezoidal rule for three volunteers following a single 40-μg dose of the drug was 651.2 pg·h·ml^{-1} for TA-2005-glucuronide, that was 2.3-fold molar excess of TA-2005. The time required to reach the peak concentration (Tmax) was 1.3 h. The Cmax value for TA-2005-glucuronide was 176.1 pg ml^{-1}, that was 2.7-fold molar excess of TA-2005. The half-lives of TA-2005-glucuronide and TA-2005 for the elimination phase ($T_{1/2}$) were estimated to be 2.7 and 4.1 h, respectively (Table 5).

Most drugs are taken orally because of the convenience offered by this route of administration. The bioavailability of orally administered drugs may be reduced due to presystemic elimination. The first-pass effect can occur in the gastrointestinal tract, the liver and lung, where both phase I (preconjugation) and phase II (conjuga-

tion) reactions are taking place. Phenolic xenobiotics such as TA-2005 are typical substrates for the first-pass metabolism of glucuronidation, the resulting products being ether glucuronides. From the result of human study, the elimination of plasma TA-2005-glucuronide showed a tendency to be faster than that of TA-2005, as the glucuronide was in general supposed to be excreted rapidly. Orally administered TA-2005 was prevailing substrate for the first-pass metabolism of glucuronidation and the plasma concentrations of TA-2005-glucuronide were much higher than those of unchanged TA-2005 throughout the study. Besides, the Tmax value for TA-2005-glucuronide was almost the same as that of TA-2005, suggesting that the first-pass effect on this drug was more significant than the metabolism during systemic circulation in human.

4. Conclusions

It was found that the developed ELISA was useful for the determination of TA-2005-glucuronide in human plasma. The methods for the preparation of hapten molecules and the construction of the enzyme immunoassay system described here will supply many practical suggestions for the measurement of the metabolic derivatives of drugs such as glucuronides in humans.

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