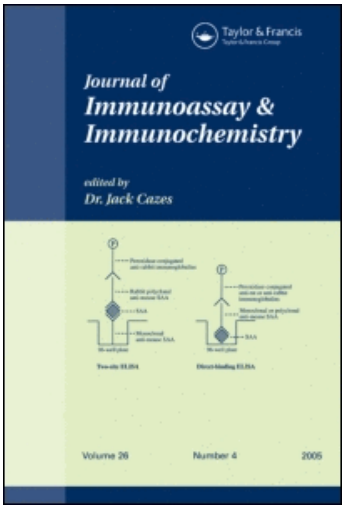

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Development of Radioimmunoassay for TG-51, a New Anti-Ulcer Drug, and Its Application

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DEVELOPMENT OF RADIOIMMUNOASSAY FOR TG-51, A NEW ANTI-ULCER DRUG, AND ITS APPLICATION

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KEY WORDS: radioimmunoassay, TG-51, anti-ulcer drug, high performance liquid chromatography, plasma levels of TG-51.

ABSTRACT

A sensitive and specific radioimmunoassay for a new anti-ulcer drug TG-51 has been developed and applied to the evaluation of its pharmacokinetics in humans. The antiserum was raised in rabbits against an immunogen of *N*-Acetyl-TG-51 coupled to human serum albumin. The radioactive compound was prepared by acetylating TG-51 with ^{3}H-Acetic anhydride. The separation of free and antibody-bound *N*-Acetyl-TG-51 was performed by the dextran coated charcoal technique. TG-51 in biological fluids could be quantitatively converted to *N*-Acetyl-TG-51 prior to radioimmunoassay. This assay system made it possible to ascertain values of 3 ng/ml of TG-51 in plasma using 100 µl of samples. Results were in good agreement with a high performance liquid chromatography method (HPLC), and the detection limit was raised 25 fold. The accuracy and reproducibility were also satisfactory.

By use of this assay method, plasma levels of TG-51 could be determined after a single oral administration of clinical doses of human volunteers.

INTRODUCTION

TG-51; 3-[*p*-(*trans*-4-aminomethylcyclohexylcarbonyl)-phenyl]propionic acid hydrochloride is a new anti-ulcer drug that exerts pharmacological effects mainly by directly acting on gastric mucosa from the lumen after oral administrations (1–4). The metabolic fate of TG-51 has been studied in rats and dogs with ^{14}C-TG-51, and its metabolism defined (5–6) [Fig. 1].

The plasma levels of unchanged TG-51 in humans are quite low despite high clinical doses (200 mg), because of low bioavailability after oral administration and rapid elimination. Until now, plasma levels of TG-51 had been measured by reversed phase and ion-paired HPLC methods. However, the detection limit of HPLC (80 ng/ml) is not sensitive enough for evaluating pharmacokinetics in humans. It has been applied only to animal studies at high oral doses.

For this reason, a much more sensitive method is required. This paper describes a sensitive and specific radioimmunoassay for determination of TG-51 in biological fluids.

MATERIALS AND METHODS

Materials

TG-51, *N*-Acetyl-TG-51, metabolite M-1, M-2 and the related compounds were synthesized at Teijin Institute for Bio-Medical Research. The chemical structures are illustrated in Fig. 1. ^{3}H-Acetic anhydride (Lot No. batch 97, specific activity 6.3 Ci/mmol)

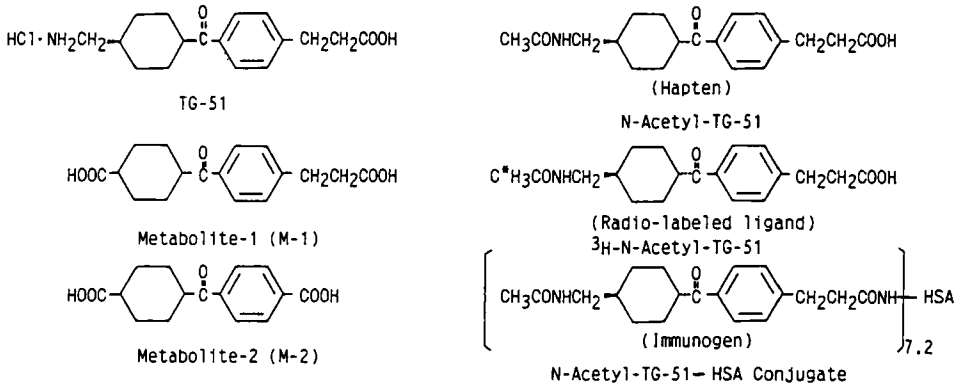

FIGURE 1. Chemical structure of TG-51, metabolites, hapten, radio-labeled ligand and immunogen.

was obtained from Radiochemical Centre, Amersham (London); Human serum albumin (HSA, lyophilized powder), from Kabi Diagnostica (Stockholm, Sweden); Bovine serum albumin (BSA, fraction V) from Sigma Chemical Co. (St. Louis, MO.); Human control serum (lyophilized powder) from Ortho Diagnostic Systems Inc. (Raritan, NJ.); Freund's complete and incomplete adjuvants, from Difco Lab. (Detroit, Mich.); Omniflour® (pre-mixed powder), from New England Nuclear (Boston, MA.); Norit extra, dextran (M.W. 100,0000 - 200,000), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and triethylamine, from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals and solvents from commercial sources were of analytical quality.

Animals and Immunization

Male New Zealand White rabbits weighing approximately 2 kg were used for immunization.

Synthesis of Radioactive Ligand

^{3}H-Acetic anhydride (12 mCi) sealed in a breakable ample was added to 4 ml of anhydrous pyridine containing 16 mg of TG-51. After 24 hr at room temperature, 2.5 ml of 6 N HCl was added to the reaction mixture followed by extraction with 3 ml of ethyl acetate three times. The ethyl acetate layer was washed with 2 ml of 1 N HCl and evaporated to dryness. ^{3}H-*N*-Acetyl-TG-51 was separated and purified by HPLC using a Shimadzu Liquid Chromatograph Model LC-3A, with a μ-Porasil column, (3.8 × 300 mm) Waters Associates, eluent: $CH_2Cl_2/CH_3OH/CH_3COOH$ (100/3/0.5), flow rate: 1.2 ml/min, and detection: ultraviolet spectrophotometer, wave length at 254 nm. The labelled product had a specific activity of 3.1 Ci/mmol, and the final yield was 16.7%. Radiochemical purity exceeded 95% and practically no degradation was observed for 1 year at −20°C in ethanol solution.

Preparation of Immunogen

N-Acetyl-TG-51 was conjugated to human serum albumin (HSA) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and the resultant conjugate purified by dialysis. 100 mg HSA was dissolved in 10 ml of phosphate buffer 100 mmol/L, pH 5.5, followed by the addition of 50 mg EDC. 30 mg *N*-Acetyl-TG-51 in 0.8 ml of dimethylformamide was then added dropwise with constant stirring. After the pH was readjusted to 5.5 by adding 0.4 ml of Na_2CO_3 solution, the mixture was allowed to react at room temperature for 18 hr. Dialysis was performed against phosphate buffer 10 mmol/L, pH 7.2, containing 0.15 M NaCl at 4°C for 72 hr with frequent changes of dialyzing solution.

Protein was assayed by the Lowry-Folin method and spectrophotometric determination of hapten indicated a conjugate of about 7.2 *N*-Acetyl-TG-51 residues per HSA molecules. This conjugate was stored at −20°C without further purification.

Immunization

Antibody to *N*-Acetyl-TG-51 was produced in rabbits by repeating intradermal injections. The first immunization was performed with 0.5 mg of conjugate emulsified in Freund's complete adjuvant and the booster doses of 0.2 mg of the immunogen emulsified in Freund's incomplete adjuvant were given at one month intervals. The titre of the antibody was frequently inspected employing ligand binding affinity test as described by Yamamoto *et al.* (7–10). On the 7th day after the second booster, blood was taken from the carotid artery. The antiserum obtained was lyophilized and stored at −20°C until use.

Ligand Binding Affinity Test

^{3}H-*N*-Acetyl-TG-51 (25,000 dpm, 3.5 pmol) in 100 µl of sodium acetate buffer 50 mmol/L, containing 0.1% BSA, pH 6.5, and diluted antisera (1:200) in 100 µl of same buffer were incubated at 4°C for 18 hr. 500 µl of dextran coated charcoal suspension (0.075% dextran, 0.5% Norit extra and 0.5% BSA) was added to the mixture, which was then cooled in an ice bath for 20 min. After centrifugation at 3000 rpm and 4°C, 500 µl of the supernatant was removed to measure the antibody-bound radioactivity. A blank tube contained buffer instead of antiserum. The (B/T)% reflected the *N*–Acetyl-TG-51 titre in rabbit sera.

Radioimmunoassay

This radioimmunoassay is based on the principle of competitive binding reaction against N-Acetyl-TG-51 antiserum instead of TG-51. Therefore, TG-51 in biological samples has to be quantitatively acetylated prior to radioimmunoassay. A preliminary study revealed that TG-51 in plasma or urine was rapidly converted to *N*-acetyl-TG-51 by the addition of acetylating reagent which contained a small quantity of acetic anhydride and triethylamine in dioxane or acetone.

The determination of TG-51 in plasma was carried out as follows. Briefly, 50 µl of acetylating reagent (4 mg of acetic anhydride, 5 µl of triethylamine and 45 µl of acetone) was added to 100 µl of samples. After vortexing, the mixture was allowed to stand for 30 min at room temperature and then diluted with 850 µl of assay buffer (sodium acetate buffer 50 mmol/L, containing 0.1% BSA, pH 6.5). For the competitive reaction, glass culture tubes containing 100 ml of ^{3}H-*N*-Acetyl-TG-51 (15,000 dpm, 2.5 pmol) in buffer, 500 µl of the acetylated sample or standard and 200 µl of antiserum (diluted 1:600 with buffer) were incubated at 4°C for 18 hr. 500 µl of dextran coated charcoal suspension was added to the mixture, which was immediately cooled in an ice bath for 20 min. The charcoal was precipitated at 3000 rpm and 4°C for 10 min, and 1.0 ml of supernatant was transferred to counting vials at room temperature. Scintillation fluid was then added (4 g of Omniflour®, 700 ml toluene and 300 ml of Triton X-100) and the samples were measured in a Packard liquid scintillation counter, Model 3255.

Standard curves were made out in the assay buffer and human control serum which contained known amounts of TG-51. The concentrations of TG-51 were read from the standard curve plotted as a semilogarithmic function. All determinations were carried out in duplicate.

High Performance Liquid Chromatography Method

The apparatus consisted of a Model LC-3A Chromatograph, a UV detector (Shimadzu, SPD-2A), and high pressure sample valves (Rheodyne, Model 7125 with 500 μl sample loop). The analytical conditions for TG-51, M-1 and M-2 were as follows; column: μ-Bondapak C_{18} (4.6 × 250 mm, Waters Associates), eluent; methanol/H_2O/PIC-B-7 reagent (Waters Associates), (600 ml/400 ml/1 vial), wave length: UV-254 nm, flow rate: 1.3 ml/min.

A plasma sample (0.5 ml) was deproteinized by 2 ml of ethanol which contained 1.0 μg of *N*-Propyl-TG-51 as an internal standard. The precipitates were removed by centrifugation at 3000 rpm for 10 min, and the supernatant solution was evaporated to dryness. The residue was dissolved in 500 μl of the mobile phase and an aliquot was introduced into the HPLC systems. The plasma sample for the calibration was prepared by adding known amounts of TG-51 and its motabolites in control plasma.

Plama Levels of TG-51 in Rats

Male Wistar rats (160–175 g) were used. TG-51 was administered orally (200 mg/kg) as an aqueous solution. Blood was withdrawn at 0.5, 1, 2, 6, 12 and 24 hr after administration

and centrifuged for 10 min at 3000 rpm. Plasma obtained was kept at −20°C until use.

Plasma Levels of TG-51 in human

The preliminary pharmacokinetic studies of TG-51 in humans were carried out in male healthy volunteers. The capsules each containing 200–1600 mg TG-51 were taken postprandially. Blood was taken from the antecubital vein before and 2, 4, 8, 24 hr after administration. Plasma was stored at −20°C until measurement.

RESULTS

Immunization and Ligand Binding Affinity Test

TG-51 is a bi-functional aminocarboxylic acid. Therefore, by acetylating the amino group of TG-51, it became possible to prepare a uniform conjugate, through the amide linkage between the carboxylic group of hapten and the lysine residue of HSA.

Calibration Curve and Cross Reactivity for Radioimmunoassay of *N*-Acetyl-TG-51

Typical calibration curves for the radioimmunoassay of *N*-Acetyl-TG-51 are shown in Fig. 2, which reveal linear displacement of tritiated labelled hapten by unlabelled *N*-Acetyl-TG-51, when plotted as a semilogarithmic function from 100 pg to 10 ng/tube. The specificity of the antiserum was assessed with TG-51 and its two metabolites. The cross reactivity of these compounds with antiserum was found to be less than 0.1%. These results suggest that the present radioimmunoassay system using *N*-Acetyl-TG-51 as a hapten is specific to TG-51 after a quantitative conversion to *N*-Acetyl-TG-51.

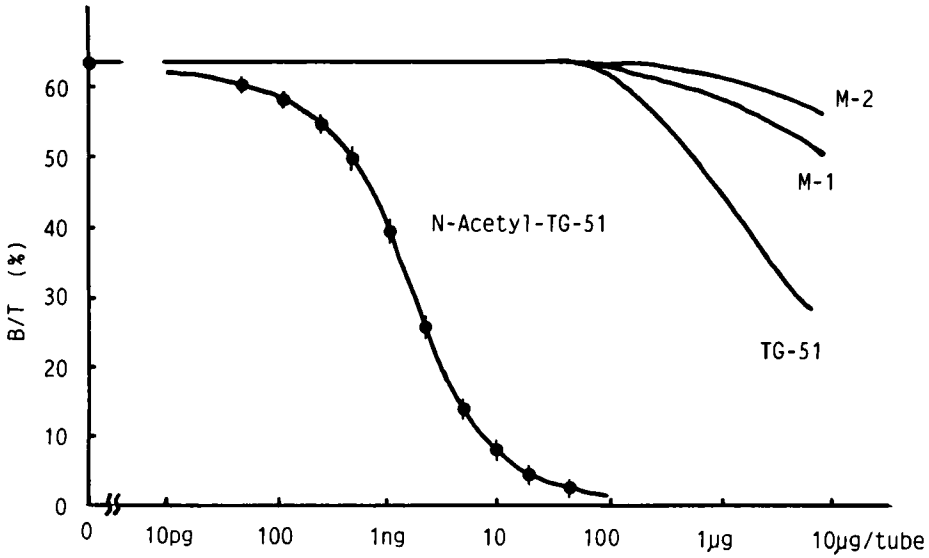

FIGURE 2. Typical calibration curve for N-Acetyl-TG-51 and cross reactivity by some TG-51 related compounds in the radioimmunoassay.

Conversion of TG-51 to *N*-Acetyl-TG-51 in Biological Fluids

Conversion of TG-51 to *N*-Acetyl-TG-51 was studied in plasma, urine and buffer using ^{14}C-TG-51, followed by reversed phase HPLC analysis. Figure 3 shows that the conversion is achieved within 15 min. by adding the acetylating reagent at room temperature.

Radioimmunoassay of TG-51 in Plasma

Typical calibration curves for the radioimmunoassay of TG-51 in assay buffer and plasma also showed a linear displacement of labelled ligand (Fig. 4). As little as 3 ng/ml of TG-51 could be distinguished from zero using a 100 μl sample. Fig. 5 shows the

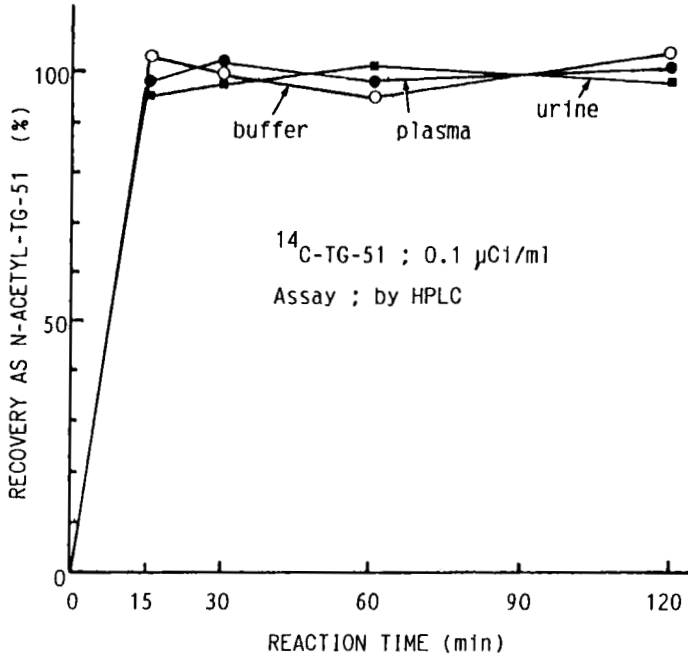

FIGURE 3. N-Acetylation of TG-51 in biological fluids by adding acetic anhydride-triethylamine-acetone (acetylating reagent).

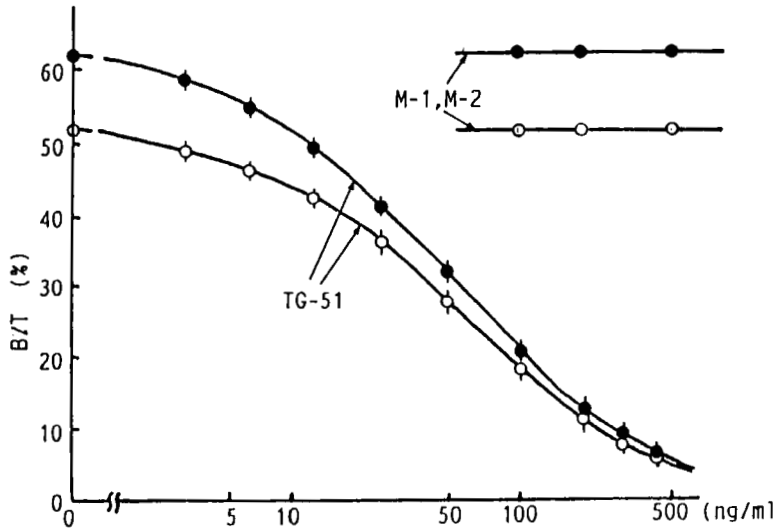

FIGURE 4. Calibration curves for the determination of TG-51 in plasma or buffer. Known amounts of TG-51 or the metabolite M-1,M-2 were added to control plasma and buffer. Radioimmunoassay was performed after the acetylation of the samples as described under "MATERIALS AND METHODS".
(—○— plasma , —●— buffer)

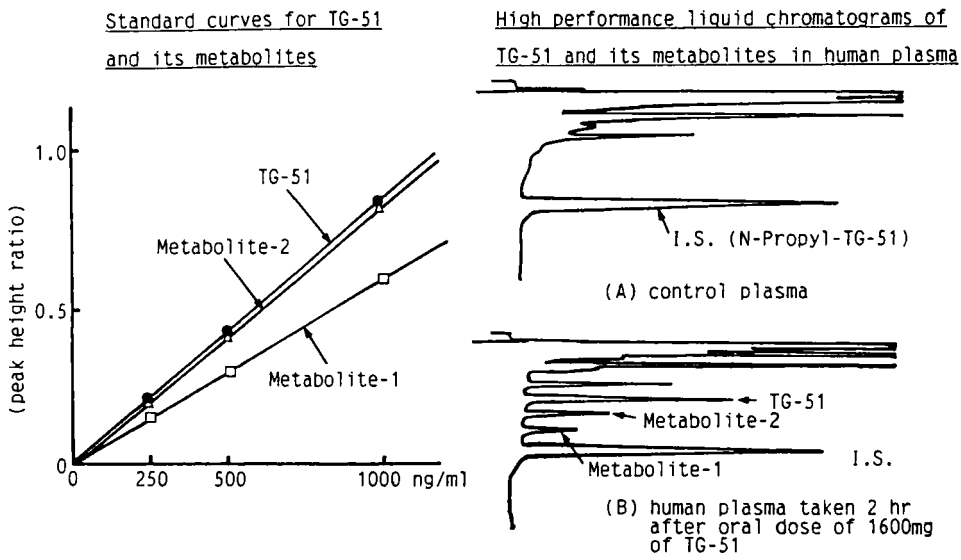

FIGURE 5. Standard curves and high performance liquid chromatograms of TG-51 and the metabolites.

effect of plasma samples on this assay system. Slight inhibition of the Ag-Ab reaction was observed due to non-specific interfering substances in plamsa. For this reason, the concentrations of TG-51 were measured using a calibration curve prepared by adding known amounts of TG-51 to TG-51 free plasma.

Recovery and Precision

Recovery studies are shown in Table 1. The intra-assay coefficient of variation ranged between 1.8% and 6.9%, while that of inter-assay between 2.5% and 12.5%.

HPLC Analysis and Correlation with Radioimmunoassay

The chromatogram recorded at 254 nm (Fig. 5) showed satisfactory separation of TG-51, M-1, M-2 and *N*-Propyl-TG-51

TABLE 1

Recovery Studies

Added (ng/ml)	Measured (ng/ml)	Obtained (ng/ml)	Recovery (%)
0	12.5 ± 0.2	-	-
3.1	15.2 ± 0.2	2.7 ± 0.2	86.3
6.3	18.8 ± 0.3	6.3 ± 0.3	100.8
12.5	24.5 ± 0.4	12.0 ± 0.4	96.0
25.0	37.0 ± 1.0	24.5 ± 1.0	98.0
50.0	64.0 ± 0.9	51.5 ± 0.9	103.0
100.0	120.0 ± 4.6	107.5 ± 4.6	107.5
200.0	220.0 ±10.6	207.5 ±10.6	103.8
		Mean	99.3 ± 6.9

Authentic TG-51 was added to control plasma. TG-51 was measured by the proposed radioimmunoassay and calculated for recovery %. Each value is represented as the mean ± S.D. of five determinations.

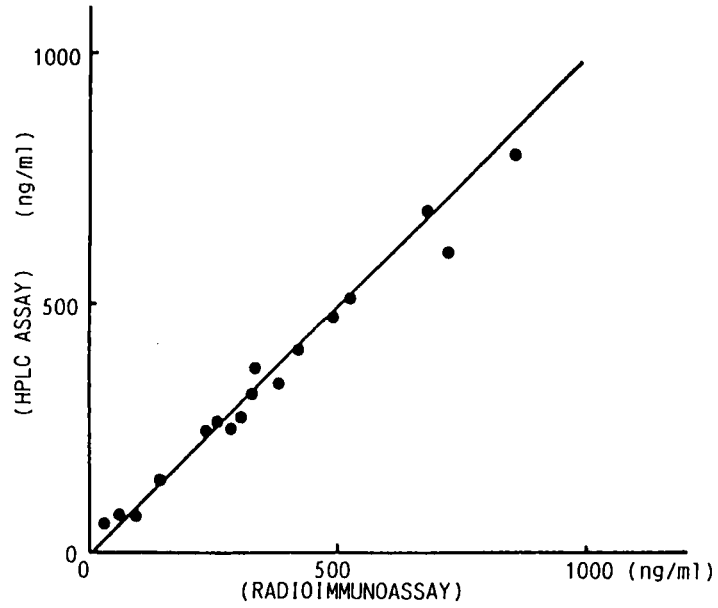

FIGURE 6. Correlation between TG-51 concentrations measured by the proposed radioimmunoassay and high performance liquid chromatography (HPLC) method.

(internal standard). Fig. 5 also shows chromatograms of a plasma blank and the HPLC pattern after addition of three standards. The detection limits of TG-51, M-1 and M-2 were respectively 80, 100 and 80 ng/ml using 0.5 ml of samples.

Fig. 6 shows good agreement between parallel determinations of TG-51 by radioimmunoassay and HPLC.

Plasma Levels of TG-51 in Rats after a Single Oral Administration

The radioimmunoassay was applied to the detemination of plasma levels of TG-51 in rats which were treated with a single oral dose of 200 mg/kg. Fig. 7 shows the time course of plasma concentration of TG-51. The maximum concentration (approximately 560 ng/ml of plasma) was observed 30 min after

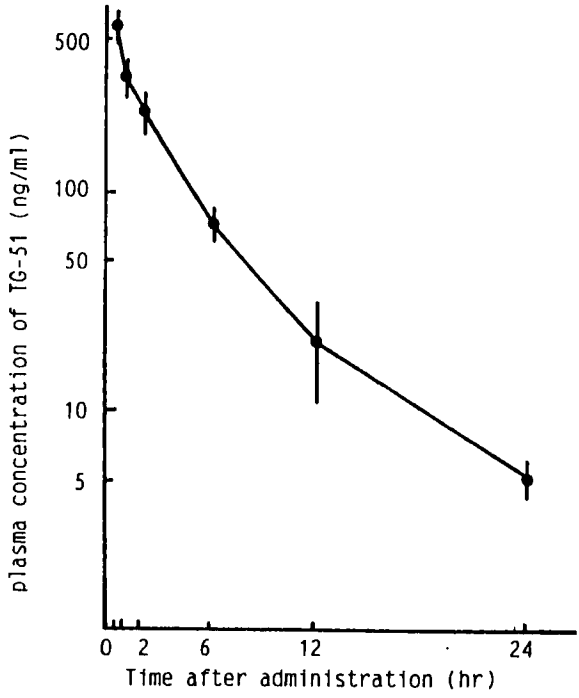

FIGURE 7. Plasma levels of TG-51 in rats after a single oral administration (200 mg/kg). Each point represents the mean ± S.E. of three male animals.

administration. Thereafter, the levels rapidly declined. 24 hr after administration, the plasma levels were lower than 1/100 of the maximum concentration. The half life of TG-51 in plasma was estimated to be approximately 4.9 hr ($T_{1/2}\beta_{6\text{-}24\ hr}$).

Plasma Levels of TG-51 in Healthy Volunteers after a Single Oral Administration

By use of this radioimmunoassay, the plasma levels of TG-51 were also examined after a single oral administration of 200, 400,

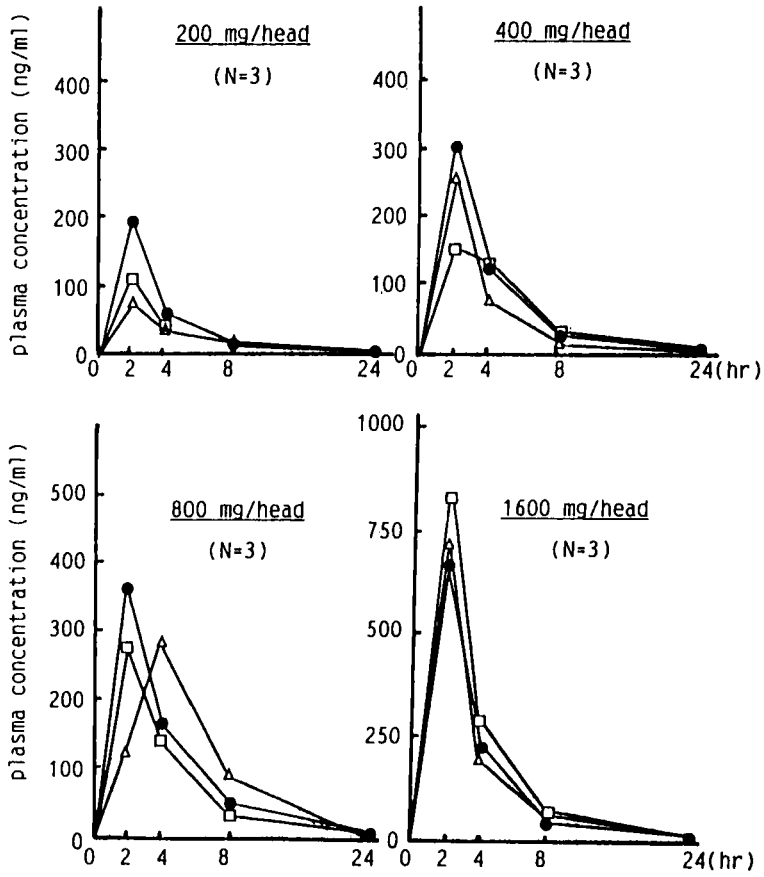

FIGURE 8. Plasma levels of TG-51 in healthy volunteers after a single oral administration.

800 and 1600 mg of the drug to each of three healthy volunteers. The maximum levels were achieved 2 hr after administration. (C_{max}; 200 mg: 122.3 ± 35.3, 400 mg: 238.0 ± 45.8, 800 mg: 310.0 ± 25.2, 1600 mg: 748.3 ± 55.3 ng/ml, mean ± S.E.). Thereafter, the levels of plasma TG-51 rapidly declined. These results are shown in Fig. 8.

DISCUSSION

TG-51 is metabolized to M-1 and M-2 through oxidative deamination of the amino group or β-oxidation of the side chain in rats and dogs (6). The low plasma levels of TG-51 necessitate a sensitive assay in order to evaluate pharmacokinetics in humans at a clinical dose.

In this study, we have developed a highly sensitive radioimmunoassay and quantitative analysis by high-performance liquid chromatography. The radioimmunoassay system made it possible to measure 3 ng/ml of TG-51 in plasma using 100 μl of samples, and the values obtained correlated with the HPLC method.

We have determined plasma TG-51 levels in rats and humans after a single oral administration of the drug. In rats (200 mg/kg), the maximum plasma level of approximately 560 ng/ml was achieved at 30 min after administration, and the half life of TG-51 in plasma was estimated to be 4.9 hr. The pharmacokinetic patterns of TG-51 in plasma correlated well with those obtained by animal studies using ^{14}C-TG-51. In human volunteers, the maximum plasma TG-51 level was approximately 120 ng/ml at 2 hr after oral dosage (200 mg).

The specificity and sensitivity of this radioimmunoassay system is due largely to the following reasons.

1) It is possible to prepare a uniform conjugate by blocking the amino group of TG-51.
2) TG-51 in buffer, plasma or urine is immediately and quantitatively converted to *N*-Acetyl-TG-51 by the addition of acetylating reagent.

3) Among TG-51 and its metabolites in biological fluids, only TG-51 is converted to *N*-Acetyl form that has strong binding affinity to the antibody.

4) The tritiated labelled compound of *N*-Acetyl-TG-51 is easily obtained by acetylating TG-51 with ^{3}H-Acetic anhydride, but the synthesis of ^{3}H-TG-51 is rather difficult.

The present radioimmunoassay will be applied to further investigation of TG-51, for instance, the evaluation of bioavailability or bioequivalence of TG-51 in humans.

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