Determination of L-6-Keto-piperidine-2-carbonyl-L-leucyl-L-proline Amide (RGH-2202), a New Analog of Thyrotropin-Releasing Hormone, in Plasma by Radioimmunoassay

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A sensitive and specific radioimmunoassay has been developed for the determination of L-6-keto-piperidine-2-carbonyl-L-leucyl-L-proline amide (RGH-2202), a new analog of thyrotropin-releasing hormone (TRH), in plasma. An antiserum was produced in guinea pigs immunized with RGH-2202 which was conjugated to bovine serum albumin by an N-succinimidyl ester method. Iodine-125 labeled L-6-keto-piperidine-2-carbonyl-L-leucyl-L-prolyltyramine ([125I]RGH-2202) was used as a tracer. Polyethylene glycol was used to separate bound and free [125I]RGH-2202 in the reaction mixture. The assay of RGH-2202 in plasma was possible over a concentration range from 0.1 to 6.4 ng/ml, using 0.1 ml of plasma without the need for an extraction procedure. The antiserum used for the assay was highly specific for RGH-2202, and did not cross-react with TRH and the hydrolyzed derivatives of RGH-2202 such as L-6-keto-piperidine-2-carbonyl-L-leucyl-L-proline, L-6-keto-piperidine-2-carbonyl-L-leucine and L-leucyl-L-proline amide, which were assumed to be present in plasma as metabolites. The coefficients of variation were 4.6—6.7% for within-assay and 6.0—8.8% for between-assay. Plasma levels of RGH-2202 in rats were determined after an intravenous administration of RGH-2202 at a pharmacologically effective dose (0.625 mg/kg).

**Keywords** — radioimmunoassay; L-6-keto-piperidine-2-carbonyl-L-leucyl-L-proline amide (RGH-2202); thyrotropin-releasing hormone analog; N-succinimidyl ester method; plasma level

**Introduction**

RGH-2202, L-6-keto-piperidine-2-carbonyl-L-leucyl-L-proline amide, is a new analog of thyrotropin-releasing hormone (TRH), having a structure shown in Fig. 1. Although TRH was first discovered as a hormone which stimulates the secretion of thyroid-stimulating hormone, it is now known to have various actions on the central nervous system (CNS) and is clinically used for the treatment of impaired CNS functions (e.g. unconsciousness).\(^1\) RGH-2202 is as potent as TRH in CNS actions but its endocrine activity is less potent (1/30 of that of TRH). From its characteristics in *in vivo* activities, RGH-2202 is expected to be a superior drug for CNS activation. Consequently, pharmacokinetic studies of RGH-2202 are necessary in order to elucidate the characteristics of the drug.

Recently, many reports concerning the determination of oligo-peptides in plasma by high-performance liquid chromatography (HPLC)\(^3\) or gas chromatography-mass spectrometry (GC-MS)\(^3\) have been published. How-

![Fig. 1. Structures of RGH-2202, Immunogen and Labeled Compound](image-url)
ever, it was difficult to prepare derivatives quantitatively for a highly sensitive assay for RGH-2202 in plasma by HPLC or GC-MS. Therefore, we attempted to obtain a specific antiserum and to develop a sensitive radioimmunoassay (RIA) for RGH-2202. In this paper, a sensitive and specific RIA method which can be useful for pharmacokinetic studies of RGH-2202 is described.

**Experimental**

**Chemicals** — RGH-2202, L-6-keto-piperidine-2-carboxylic acid, L-leucyl-L-proline amide, L-6-keto-piperidine-2-carboxyl-L-leucine, L-proline amide, L-6-keto-piperidine-2-carboxyl-L-leucyl-L-proline (Kpc-Leu-Pro) were kindly supplied by Gedeon Richter Ltd. (Budapest, Hungary). TRH was purchased from the Peptide Institute Inc. (Osaka, Japan), bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), Freund’s complete adjuvant from Difco Laboratories (Detroit, Mich., U.S.A.), bovine serum gamma-globulin (BGG) from Povite Producten N.V. (Amsterdam, Holland), polyethylene glycol #6000 (PEG) from Nakarai Chemicals, Ltd. (Kyoto, Japan), and Na\(^{125}\)I (carrier-free) from Amersham International plc. (Buckinghamshire, England). All other chemicals were of analytical reagent grade.

**Preparation of Immunogen** — 1) Synthesis of L-6-Keto-piperidine-2-carboxyl-L-leucyl-L-proline Succinimide Ester (I): To a solution of Kpc-Leu-Pro (1 g) and N-hydroxysuccinimide (327 mg) in 25 ml of absolute tetrahydrofuran (THF) was added N,N’-dicyclohexylcarbodiimide (584 mg) dissolved in 5 ml of absolute THF at room temperature with stirring. The mixture was stirred for 3 h at room temperature and then filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was dissolved in 20 ml of dioxane and filtered. Ether was added to the filtrate, and the resultant precipitate was collected by filtration and dissolved in 25 ml of CH\(_3\)CN. Activated charcoal was added to the solution, and the mixture was then filtered. The filtrate was concentrated in vacuo, the resultant precipitate was recrystal-

lized from acetone-ether to give 950 mg of I (72.5%), mp 120—123 °C. Anal. Calcd for C\(_{21}\)H\(_{28}\)N\(_4\)O\(_7\)·3/4H\(_2\)O: C, 54.36; H, 6.84; N, 12.07. Found: C, 54.21; H, 6.74; N, 12.06.

2) Synthesis of RGH-2202-BSA Conjugate (Immunogen): To a solution of BSA (500 mg) in 195 ml of 0.05M phosphate buffer (pH 7.4), I (154 mg) dissolved in 5 ml of absolute dioxane was added at room temperature with stirring. After stirring for 3 h, the reaction mixture was filtered. The filtrate was dialyzed against running water for 5 d at 4 °C and lyophilized to give 560 mg of fluffy immunogen powder.

The conjugation of the hapten to BSA was confirmed by an amino acid analysis. The apparent degree of derivatization calculated from the amount of aminoadipic acid in the conjugate, was 12—13 hapten residues per BSA molecule.

**Immunization** — Male Hartley guinea pigs weighing 300—350 g were used for immunization. One milligram of the immunogen was dissolved in 0.2 ml of physiological saline and emulsified with an equal volume of Freund’s complete adjuvant. The emulsion, about 0.05 ml per site, was injected intradermally at several different sites on the back of each guinea pig. The guinea pigs were injected once every 2 weeks for 10 weeks. Blood which was obtained by cardiac puncture 6—13 d after the booster injections, allowed to clot overnight at 4 °C, and centrifuged at 500 × g for 20 min to obtain the serum. The antiserum used for the assay was collected 10 d after the sixth injection.

**Preparation of \(^{125}\)I-Labeled Hapten** — Iodine-125 labeled L-6-keto-piperidine-2-carboxyl-L-leucyl-L-prolyltyramine ([\(^{125}\)I]RGH-2202) was used as a tracer. By reacting I with tyramine, L-6-keto-piperidine-2-carboxyl-L-leucyl-L-prolyltyramine (RGH-2202-tyramide) was synthesized, mp 163—165 °C. Anal. Calcd for C\(_{37}\)H\(_{35}\)N\(_4\)O\(_5\)·1/2H\(_2\)O: C, 62.35; H, 7.74; N, 11.63. Found: C, 62.62; H, 7.93; N, 11.71.

RGH-2202-tyramide was iodinated by a modification of the method of Hunter and Greenwood as follows: To a small glass test tube containing 0.224 μg of RGH-2202-tyramide (11.2 μg/ml in 0.05M phosphate buffer (pH 7.4)—ethanol (19:1)), 1.64 mCi of Na\(^{125}\)I (9.65 mCi/ml in 0.05M phosphate buffer (pH
7.4)) and 15.8 μg of chloramine T (1.58 mg/ml in H₂O) were added. After stirring gently for 90 s at room temperature, 21.2 μg of sodium metabisulfite (1.06 mg/ml in H₂O) were added to terminate the reaction. [¹²⁵I]RGH-2202 was purified by anion exchange column chromatography and reversed-phase high-performance liquid chromatography.

Assay Procedure — The following solutions were prepared prior to assay.

1) 0.075M Phosphate buffer (pH 7.4) containing 0.2% BSA (buffer A).
2) 0.075M Phosphate buffer (pH 7.4) containing 1% BGG (Buffer B).
3) Standard solutions containing 10, 20, 40, 80, 160, 320 and 640 pg of unlabeled RGH-2202 in 0.1 ml of buffer A.
4) A solution of [¹²⁵I]RGH-2202 in buffer A containing 30000 dpm/0.1 ml.
5) PEG solution containing 30 g of PEG in 100 ml of 0.075M phosphate buffer (pH 7.4).

To each of duplicate plastic tubes, 0.1 ml of plasma samples or control plasma, 0.1 ml of buffer A or standard solutions, 0.1 ml of buffer B, 0.1 ml of [¹²⁵I]RGH-2202 solution, and 0.1 ml of diluted antiserum (diluted 1:15000 with buffer A) were added. The contents of the tubes were mixed on a vortex mixer and allowed to stand for 16 h at 4 °C. Then, 0.5 ml of 30% PEG solution was added to the mixture. The tubes were vortexed vigorously, allowed to stand for 5 min at 4 °C, and centrifuged at 1000 × g for 30 min at 4 °C. The supernatant fluid was removed by aspiration and the radioactivity of the precipitate was counted in a well-type scintillation counter (ARC-360, Aloka Co., Ltd., Tokyo, Japan). A standard curve was constructed by a logit-log plot of the relative percentage of bound labeled drug (B/B₀) against the amount of unlabeled drug added.

Stability of RGH-2202 in Plasma — Fresh plasma samples from humans, rabbits, rats and mice were spiked with 0.5 ng/ml RGH-2202 and incubated under various conditions. After incubation, RGH-2202 was extracted with acetone according to the procedure for stabilization of TRH in blood. The extract was evaporated in a centrifugal evaporator (Model RD-21, Yamoto Scientific Co., Ltd., Tokyo, Japan) at 40—50 °C, and the residue was dissolved in buffer A. Then, RGH-2202 was determined according to the assay procedure described above.

Animal Study — Male Wistar rats weighing 250—255 g were used in the experiment. A solution of RGH-2202 in physiological saline was administered (0.625 mg/2 ml/kg) intravenously to the rats. Blood was drawn from the carotid artery (a cannula was inserted on the day preceding drug administration) at 2, 4, 6, 8, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min after administration. Immediately after blood collection, plasma was separated at low temperature and stored frozen at −20 °C until assayed.

Results

Antiserum Titer

The titer of antiserum obtained was determined according to the assay procedure. A typical titer curve for [¹²⁵I]RGH-2202 (8 pg) with anti-RGH-2202 antiserum at different dilutions is shown in Fig. 2. An antiserum dilution of 1:75000 (final dilution; the final dilution was 75000-fold when 0.1 ml of the 15000-fold diluted antiserum was added to the assay system)

Fig. 2. Dilution Curve of Antiserum

The percentage of [¹²⁵I]RGH-2202 bound to the antibody is plotted against the dilution of the antiserum represented on a semilogarithmic scale.
The relative percentage of $[^{125}]$RGH-2202 bound to the antibody in the presence of various amounts of RGH-2202 is plotted on a logit-log scale.

Fig. 3. Standard Curve for RGH-2202

A typical standard curve for the RGH-2202 RIA using the logit transformation plot is shown in Fig. 3. The linear portion of the curve extends from 0.01 to 0.64 ng of RGH-2202 per assay tube, which indicates that RGH-2202 in plasma can be reliably assayed in a concentration range from 0.1 to 6.4 ng/ml if 0.1 ml of plasma samples are used.

**Specificity**

The cross-reactivities of various compounds

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<th>Name</th>
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<th>% cross reactivity</th>
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<td>L-Proline amide</td>
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<tr>
<td>Thyrotropin-releasing hormone (TRH)</td>
<td><img src="image" alt="Structure" /></td>
<td>&lt;0.01</td>
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The values are expressed in percentage cross-reactivity which is defined as $X/Y \times 100$, where $X$ is the amount of unlabeled RGH-2202 and $Y$ is the amount of the test compound required to produce a 50% inhibition of binding of $[^{125}]$RGH-2202 to the antiserum.
Radioimmunoassay of RGH-2202

Fig. 4. Stability of RGH-2202 in Plasma

RGH-2202 was dissolved in freshly obtained human plasma to give the concentration of 0.5 ng/ml. After incubation, extraction of RGH-2202 with acetone was performed to avoid degradation of RGH-2202.

with the antiserum are shown in Table I. The antiserum was highly specific for RGH-2202 and showed substantially no cross reaction with endogenous TRH or the RGH-2202 related compounds, some of which were believed to be metabolites of RGH-2202 in plasma.

Precision

Within- and between-assay variations were assessed by repeated assays of pooled human plasma. The coefficients of variation were 4.6—6.7% (0.4—6.1 ng/ml) for within-assay and 6.0—8.8% (0.4—6.1 ng/ml) for between-assay.

Stability of RGH-2202 in Plasma

The residual amounts of RGH-2202 in human plasma after incubation at 4, 25 and 37 °C are plotted against incubation period in Fig. 4. At the three temperatures studied, practically no degradation of RGH-2202 took place during the incubation period. Similar results were obtained in rabbit plasma. Although a slight degradation of RGH-2202 was found in rat and mouse plasma after 2 h incubation at room temperature, it was prevented by a 10-fold dilution of plasma samples with 0.075M phosphate buffer (pH 7.4).

Plasma Levels of RGH-2202 in Rats

Fig. 5 shows the mean plasma levels of RGH-2202 in rats after a single intravenous administration of RGH-2202 at a dose of 0.625 mg/kg. The plasma level showed a biphasic elimination pattern, with elimination half-lives in α and β phases (t_{1/2α}, t_{1/2β}) being 1.6 min and 18.0 min, respectively.

Discussion

A RIA was developed for RGH-2202 in plasma with a high sensitivity and specificity. Accurate determinations of RGH-2202 are possible with 0.1 ml of plasma samples over a concentration range from 0.1 to 6.4 ng/ml.
The antiserum was obtained by immunizing guinea pigs with the immunogen prepared by coupling an RGH-2202 derivative (Kpc-Leu-Pro) to BSA. The antiserum specificity was determined by its cross-reactivity with the RGH-2202 related compounds, some of which were believed to be metabolites of RGH-2202. The antiserum did not cross-react with any of the RGH-2202 related compounds tested. The fact that the antiserum did not cross-react substantially with Kpc-Leu-Pro, which was formed by a cleavage of the amide bond at the carboxyl side of proline in RGH-2202, suggested that the antiserum is highly specific for the unchanged RGH-2202.

When TRH is measured in plasma, extraction with organic solvent is needed prior to assay because degradation of TRH in plasma occurs quickly, even at low temperatures. Therefore, the stability of RGH-2202 in plasma was investigated. No degradation of RGH-2202 was found in human and rabbit plasma, while a slight degradation was found in rat and mouse plasma after 2 h incubation at room temperature. When plasma samples were diluted 10 times or above with the buffer solution, the degradation of RGH-2202 was not observed in rat and mouse plasma after 2 h incubation at room temperature. Consequently, the RIA for the determination of RGH-2202 in plasma can be performed without an extraction procedure.

Plasma levels of the unchanged drug in rats after intravenous administration of RGH-2202 were determined by RIA. Although the detection limit was 1 ng/mL in rat plasma because plasma samples were diluted to prevent the degradation of RGH-2202, plasma levels of RGH-2202 in rats after intravenous administration of RGH-2202 at a pharmacologically effective dose were sufficient to be determined.

Mean plasma levels of RGH-2202 showed a biphasic elimination pattern. The elimination half-lives in $\alpha$ and $\beta$ phases ($t_{1/2\alpha}$, $t_{1/2\beta}$) were 1.6 min and 18.0 min, respectively. Thus, the plasma levels of RGH-2202 in rats were successfully determined by the RIA. The present method would be useful for further pharmacokinetic studies of RGH-2202 in animals and humans.

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References