**Radioimmunoassay of an Analogue of
Adrenocorticotrophic Hormone,
H-Met(O₂)-Glu-His-Phe-D-Lys-Phe-NH(CH₂)₈-NH₂ (Ebiratide)**

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Summary

A specific radioimmunoassay method was developed for H-Met(O₂)-Glu-His-Phe-D-Lys-Phe-NH(CH₂)₈-NH₂(ebiratide)., an analogue of adrenocorticotrophic hormone. The sensitivity was 30pg/ml. Recovery of ebiratide from plasma was almost 100%, and the intra- and inter-assay coefficients of variation were less than 15%. Plasma levels and urinary excretion of ebiratide were determined in six healthy male volunteers after intravenous administration of ebiratide (150μg). Ebiratide was eliminated from the plasma in a biphasic pattern, with half-lives of 3.2min (first phase) and 3h (second phase), and 0.76% of the dose was excreted in urine. These results indicate that this radioimmunoassay method is suitable for analyzing the pharmacokinetics of ebiratide.

Introduction

H-Met(O₂)-Glu-His-Phe-D-Lys-Phe-NH(CH₂)₈-NH₂ (ebiratide) is an adrenocorticotrophic hormone (ACTH) developed by Hoechst AG (FRG). This peptide has been reported to decrease the acetylcholine contents in different brain areas and to have effects on memory processes in mice and rats¹⁻³⁾, and it is expected to be effective for treatment of dementia. The N-terminal domain of dbiratide may have pharmacological activity⁴⁾, and so the determination method which has specificity to the N-terminal is required for pharmacokinetic studies of this peptide. In this study, we developed a sensitive and stable radioimmunoassay (RIA) method for ebiratide, and we used the method to determine the time course of plasma levels and urinary excretion of ebiratide in man.

Materials and Methods

1. Peptides

Ebiratide standard and fragments synthesized at Hoechst AG were used.

2. Preparation of anti-ebiratide serum

Ten milligrams of ebiratide and 50mg of bovine serum albumin (BSA, Wako Pure Chemical Industries, Ltd., Japan) were dissolved in 1.0ml of 0.05M phosphate buffer (pH 7.4), and the solution was allowed to react with 30mg of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-p-toluenesulfonate (Sigma Chemical Co., USA) at 4 °C for 24 h and then was freeze-dried.

One milligram of the conjugate thus obtained was dissolved in 0.5ml of distilled water and emulsified in an equal amount of complete Freund's adjuvant (Difco Laboratories, USA). The emulsion was injected in a volume of 1.0ml intradermally into multiple sites of the footpads of rabbits 4 times at 3-week intervals. Blood was collected after the last immunization, and the antibody titer was measured. The specificity of the antiserum was examined with ebiratide fragments.

3. Radioiodination of ebiratide

Ebiratide was radioiodinated by 30-sec reaction with chloramine-T⁵⁾. ¹²⁵I-Ebiratide was purified by high-performance liquid chromatography (HPLC; JASCO, Tokyo) and by gel filtration on Sephadex G-15 (Pharmacia Fine Chemicals, Sweden). The HPLC conditions were as follows: column, Protein C₄ 214TP104, 4.5 i.d. × 300mm (The Separations Group Co., Ltd, USA); eluant, a mixture of 1% phosphoric acid solution and acetonitrile (8:1) adjusted to pH 3 with triethylamine; and flow rate, 1.0ml/min. Gel filtration was performed with a 1 i.d. × 20cm column and 0.05M phosphate buffer (pH 7.4) as the eluant.

4. Assay buffer

The assay buffer consisting of 0.05M phosphate buffer (pH 7.4), 0.1% BSA and 0.1% mercaptoethanol was used.

5. Assay procedure

A mixture of 0.3ml of assay buffer, 0.1ml of diluted anti-ebiratide serum, and 0.1ml of ebiratide standard solution, plasma extract or urine was mixed with 0.1ml of ^{125}I -ebiratide in a 4-ml plastic test tube (Eiken Co., Ltd., Japan), and the tube was incubated at 4°C. Then, 0.1ml each of dilute normal rabbit serum (1:100) and goat anti-rabbit γ -globulin serum (1:10) (Daiichi Radioisotope, Labs., Ltd., Japan) was added. After further incubation at 4°C for 24h, the mixture was centrifuged, and radioactivity in resulting precipitate was determined with a γ -counter (Aloka, Tokyo).

To find the optimal conditions for RIA method, the following factors were changed variously, and their effects on the results were examined: i) dilution of antiserum; ii) amount of ^{125}I -ebiratide added; and iii) incubation time before and after addition of ^{125}I -ebiratide.

Separately, to examine the effect of urine, the urine from a nontreated subject was added to ebiratide standard solution when the standard curve was prepared.

6. Pharmacokinetic study of ebiratide in man

Ebiratide (150 μg) was intravenously injected to six healthy male volunteers. Blood was taken at scheduled time intervals (0.083, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0h), and plasma was separated. Urine was collected for 24h after the compound injection.

As untreated plasma was not suitable for RIA because of its nonspecific binding, the plasma extract was prepared as follows with Sep Pak-C18 (Waters Associates, USA). Sep Pak-C18 was washed with 3ml of methanol and 4ml of distilled water in that order. Then, 1.0ml of plasma was added to the column, washed with 3ml of assay buffer and eluted with 4ml of methanol. The eluate was dried under reduced pressure, and the residue dissolved in the assay buffer was used as the sample (plasma extract) for RIA. Urine was subjected to RIA without any treatment.

Results

1. Specificity of anti-ebiratide serum

Table I shows the cross-reactivities of ebiratide fragments with prepared rabbit anti-ebiratide serum. The antiserum was found to recognize a wide domain of the N-terminal

Table I Cross-reactivities of ebiratide and ebiratide fragments with rabbit anti-ebiratide serum

Peptide	Cross-reactivity (%)
Ebiratide	100
H-Met(O ₂)-Glu-His-Phe-D-Lys-Phe-OH (Ebiratide (1-6))	100
H-Met(O ₂)-Glu-His-Phe-OH (Ebiratide (1-4))	14.8
H-Met(O ₂)-Glu-His-OH (Ebiratide (1-3))	<0.01
NH ₂ -(CH ₂) ₈ -NH ₂	<0.01
Etoc-ACTH-(6-23)	<0.01
Phe-D-Lys-Phe-NH (CH ₂) ₈ -NH ₂	<0.01

of ebitatide.

2. Purification of ^{125}I -ebiratide

Fig. 1 shows the HPLC chromatogram of ^{125}I -ebiratide. The hatched peak, immunoreacting with the antiserum, was isolated by gel filtration on Sephadex G-15. The specific radioactivity of ^{125}I -ebiratide was 1.1mCi/ μg .

Fig. 1 Purification of ^{125}I -ebiratide by HPLC. The volume of each fraction was 0.5 ml, and a hatched peak showed immunoreactivity.

3. Evaluation of assay procedure

1) Dilution of antiserum and amount of ^{125}I -ebiratide

Table II shows the ratio of radioactivity of ^{125}I -ebiratide bound to antibody (Bo) to total radioactivity (T) determined with a 1 : 800 or 1 : 1,600 dilution of the antiserum and with 5,000 or 10,000cpm/100 μl of ^{125}I -ebiratide. The postincubation time (i.e., period after addition of the tracer) was 24h (preincubation was not performed). The use of 5,000cpm/100 μl of ^{125}I -ebiratide and a 1 : 1,600 antiserum dilution gave the Bo/T value of 33.4%.

Table II Effect of amount of ^{125}I -ebiratide and dilution of antiserum on radioimmunoassay of ebitatide

Amount of ^{125}I -ebiratide (cpm/100 μl)	Dilution of antiserum	Bo/T(%)
10,000	1 : 800	48.2
10,000	1 : 1,600	30.7
5,000	1 : 800	51.4
5,000	1 : 1,600	33.4

The postincubation time was 24 h.

2) Incubation time

Table III shows the effect of the preincubation time (i.e., delayed addition of ^{125}I -ebiratide^{6,7)} and postincubation time on assay results. The dilution of antiserum and amount of ^{125}I -ebiratide were fixed (1:800 and 10,000cpm/100 μl , respectively). The delayed addition of ^{125}I -ebiratide increased the Bo/T value from 48.2% to 63.6%. The Bo/T value was larger as the postincubation time was prolonged.

Based on the above results the following RIA conditions were selected so that the whole procedure could be completed within a week (5 days): antiserum dilution, 1:1,600 (final, 1:9,600); amount of ^{125}I -ebiratide added, 5,000cpm/100 μl ; preincubation time, 24h; and postincubation time, 48h. Fig. 2 shows the standard curve obtained under these conditions. The lower limit of detectability of ebiratide with 95% confidence was 3pg per tube, which corresponds to 30pg/ml.

Table III Effect of incubation time on radioimmunoassay of ebiratide

Preincubation time (h)	Postincubation time (h)	Bo/T (%)
24	24	63.6
0	24	48.2
0	48	50.3
0	72	53.3

The dilution of antiserum was 1:800, and the amount of ^{125}I -ebiratide was 10,000 cpm/100 μl .

Fig. 2 Typical standard curve for radioimmunoassay of ebiratide. The detailed method is described in Materials and Methods.

4. Extraction of ebiratide from plasma

As shown in Table IV, almost 100% of ebiratide added was recovered from the plasma.

5. Effect of urine on standard curve

As shown in Fig. 3, the standard curve prepared with the urine-added ebiratide standard solution was good, though it was slightly affected. Accordingly, urine was used as the sample for RIA without any treatment. Instead, the curve prepared with the urine-added ebiratide standard solution was used as the standard curve for RIA.

Table IV Extraction of ebitatide from human plasma by Sep Pak-C18

Concentration (ng/ml)			
0.05	0.1	1.0	10.0
Recovered concentration (ng/ml)			
0.058 $\pm$ 0.005	0.103 $\pm$ 0.013	0.90 $\pm$ 0.06	10.17 $\pm$ 0.64

Each value shows the mean $\pm$ S.D. (n=5).

Fig. 3 Effect of fresh ebitatide-free urine on the standard curve for radioimmunoassay of ebitatide.

Typical standard curve is shown. Amount of urine added (100 μ l) : $\triangle \cdots \triangle$, none : $\bullet \cdots \bullet$

6. Validity of assay

Ebitatide (0.05–10.0 ng/ml) was added to plasma and urine from a nontreated subject, and intra- and inter-assay coefficients of variation were determined. All assays showed good results, and the intra- and inter-assay coefficients of variation were less than 15% (data not shown).

7. Pharmacokinetic behavior of ebitatide in man

Fig. 4 shows the time course of plasma ebitatide levels determined by the established RIA method after intravenous administration of ebitatide (150 μ g) to six healthy male subjects. The plasma level of ebitatide declined in a bihasic pattern, the half-lives in the first and second phases being 3.2 ± 0.2 min and 3.0 ± 0.8 h, respectively. In the urine, 0.76% of the dose was excreted.

Discussion

We developed a specific RIA method for ebitatide, an ACTH analogue, and determined plasma levels and urinary excretion of ebitatide in man using this method.

The antibody prepared hardly recognized the C-terminal of ebitatide, but widely recognized the N-terminal domain which is reported to have pharmacological activity⁴⁾. The antibody had 100% cross-reactivity with ebitatide (1–6) (H-Met (O₂)-Glu-His-Phe-D-Lys-Phe-OH), but showed little cross-reactivity with any other ebitatide fragments. This

Fig. 4 Plasma levels of ebitatide after intravenous administration to six male volunteers. Each point with a bar shows the mean $\pm$ S.D.

antibody, which recognizes the pharmacologically active site of ebitatide, in other words, is specific for the active site, is available for its pharmacokinetic study.

Since untreated plasma could not be used in this RIA method due to its nonspecific binding, the plasma extract prepared with Sep Pak-C18 was used as the sample. The recovery of ebitatide from the plasma was almost 100%. Urine, having little effect on the standard curve, could be served as the sample without any treatment.

The delayed addition of ^{125}I -ebitatide and prolongation of the postincubation time increased the detection sensitivity of the method. The lower detection limit was 30pg/ml and appeared to be sufficient for determination of ebitatide in human plasma and urine.

When ebitatide was intravenously administered to healthy male subjects, the declining pattern of its plasma levels showed two phases, the first phase lasting until about 30min after administration (half-life, 3.2min) and the second phase thereafter (half-life, 3.0 h). This half-life in the second phase is very long for the peptide, compared with the value of natural ACTH (10–15min)⁸⁾ and with the values of ACTH (4–10) showing two phases (0.38 and 3.4min)⁹⁾.

A possibility is considered that the long half-life in the second phase of ebitatide shown in this study may be attributed to metabolites of ebitatide. If ebitatide (1–6) showing 100% cross-reactivity with the antiserum used (Table I) was present in a high concentration, it cannot be said that this method detected the exact level of ebitatide. However, it has been reported that the His-Phe bond was most labile when a substitution of 8-Arg by 8-D-Lys in the sequence of natural ACTH (4–9) was incubated with serum

¹⁰⁾, and it is presumed from this finding that the main metabolite of ebitatide is Phe-D-Lys-Phe-NH (CH₂)₈-NH₂. This fragment, if it was present in the plasma, showed little cross-reactivity with the antiserum used (Table I) and, therefore, could hardly be detected as ebitatide. The actual amounts of metabolites present in human plasma have not yet been determined, thus, further study will be necessary.

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