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MEASUREMENT OF URINARY GROWTH HORMONE (GH) LEVELS BY HIGHLY SENSITIVE ENZYME IMMUNOASSAY AS A POTENTIAL SCREENING TEST FOR GH SECRETION

(sensitive enzyme immunoassay / urinary GH / screening test)

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We set up a highly sensitive enzyme immunoassay of human growth hormone (hGH) using anti-hGH rabbit Fab'-peroxidase conjugate. IgG was obtained from anti-hGH rabbit serum with salting-out and diethylaminoethyl cellulose. IgG was digested by porcine gastric mucosa pepsin to $F(ab')_2$ which was reduced to Fab'. Fab' was conjugated with peroxidase by maleimide method. The minimum detectable amount of hGH was 0.3 pg/ml using 100 μl of dialyzed urine sample without any concentrating procedure.

Urinary GH was detectable in all normal subjects. In order to evaluate the measurement of urinary GH as a potential screening test of pituitary disorders, we examined 706 subjects in a population (19 to 80 years old). Urine and plasma samples were collected in the early morning after overnight fasting. Urinary creatinine and β_2-microglobulin levels were measured by autoanalyzer and specific radioimmunoassay, respectively. Urinary GH levels ranged from not detectable to 314 ng/g creatinine in these subjects. The logarithmus of these data showed a normal distribution. The normal range obtained from 58 male and 98 female subjects were 0.5 to 17.0 ng/g creatinine and 1.2 to 41.9 ng/g creatinine, respectively. Urinary GH levels were elevated in 48 out of the 706 subjects examined (6.8%). However, 13 out of these 48 subjects (1.8%) were acoompanied by increased urinary β_2-microglobulin (>200 μg/g creatinine), suggesting that urinary GH levels are influenced by renal tubular dysfunction. In contrast,

urinary GH was not detectable in 6 out of the 706 cases (0.8%), suggesting possible hypopituitarism.

These results indicate that measurement of urinary GH by highly sensitive enzyme immunoassay was useful for a potential screening test for pituitary dysfunction although the renal function should be considered simultaneously.

Plasma growth hormone (GH) levels fluctuate widely under the physiological conditions to reflect the pulsatile secretion from the pituitay gland. In order to assess the physiological secretion of GH, frequent blood sampling was required. Since blood GH is excreted into urine like other polypeptides, measurement of urinary GH levels might be useful for evaluation of spontaneous GH secretion. However, urinary excretion of GH was so small that urinary GH levels could not be detectable by conventional radioimmunoassay (RIA), in which the minimum detectable quantity of GH was 0.6 ng/ml. Previous investigators measured urinary GH levels by RIA after some concentrating (1-7). Recently, Hashida et al. (8,9) developed an enzyme immunoassay method that was sensitive enough to measure urinary GH levels without any concentration procedure. In this study, we set up a highly sensitive enzyme immunoassay by a slight modification of the method of Hashida et al. (9,10), and measured urine GH levels in a population in order to evaluate a potential use for screening of pituitary GH secretion.

SUBJECTS AND METHODS

Subjects

We examined 706 subjects (283 males, 423 females) in a population. The age distributed from 19 to 85 years with the mean of 54 years. Among them, 156 subjects (58 males, 98 females) were defined as normal judging from common blood cell count, blood chemistry, urinalysis, ECG, and detailed physical examination. None of the normal subjects took any medication known to affect GH secreion.

Samples

Ten ml urine samples were obtained in the early morning, mixed with 100 µl of 0.01M phosphate buffer, pH 7.0, containing 10% BSA and 10% NaN_3, and dialyzed against 0.01M sodium phosphate buffer, pH 7.0, at 4°C overnight using the multidialyzer (Spectrum Medical Industries, Inc., Los Angels, CA). The dialyzed urine samples were kept at 4°C until assayed. Blood samples were collected by venipuncture in the early morning after overnight fsting. Plasma was immediately separated and kept at -20°C until assayed.

Preparation of IgG

Anti-human GH (hGH) rabbit serum was obtained by repeated subcutaneous injection of hGH emulsified in complete Freund's adjuvant. Four ml of 34% $NaSO_4$ were added to 4 ml of anti-hGH rabbit serum with continuous stirring at 25°C for 30 min. The mixture was then centrifuged at 11,500 rpm for 10 min at room temperature. The precipitate was dissolved in 4 ml of 0.015M sodium phosphate buffer, until no precipitation was formed by adding 1% $BaCl_2$. The solution was applied to a diethylaminoethyl (DEAE) cellulose (Sigma Chemical Co., St. Louis, MO) column (0.9 x 22 cm), which was equilibrated with 0.015M sodium phosphate buffer, pH 6.5, and eluted with linear gradient of 0.015 to 0.2M sodium phosphate buffer, pH 6.5.

Preparation of $F(ab')_2$

The purified IgG was dialyzed against 0.1M sodium acetate buffer, pH 4.5, at 4°C and added with 1/20 volume of 2M NaCl. The purified IgG was incubated with pepsin from porcine gastric mucosa (Sigma) (0.2 mg/10 mg IgG) at 37°C for 20 hours. The reaction was stopped with 0.1M NaOH to adjust the pH to 8.0. After concentration, $F(ab')_2$ fraction was separated from the digested IgG solution with an Ultrogel AcA 44(IBF, France) column (0.9 x 74 cm) using 0.1M sodium borate buffer, pH 8.0. $F(ab')_2$ was also obtained from normal rabbit IgG (Sigma) by the same procedure.

Preparation of Fab'

Four mg of $F(ab')_2$ was concentrated to 0.45 ml in 0.1M sodium phosphate buffer, pH 6.0, with a collodion bag (Sartorius, Gottingen, Germany). 0.05 ml of 0.1M 2-mercaptoethylamine (Sigma) dissolved in 0.1M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA, was added to the concentrated $F(ab')_2$ solution. The mixture was incubated at 37°C for 90 min. The reaction mixture was applied to an Ultrogel AcA 44 column (0.9 x 45 cm) to remove the reagents using 0.1M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA.

Introduction of maleimide groups into peroxidase

Four mg of horseradish peroxidase (Type XII, 335 U/mg solid, Sigma) was dissolved in 0.6 ml of 0.1M sodium phosphate buffer, pH 7.0. 3.2 mg of 4-(N-maleimidemethyl) cyclohexane-1-carboxylic acid N-succinimidlyl ester (Aldrich, Milwaaky, USA) was dissolved in 0.06 ml of N,N-dimethylformamide (Sigma). The peroxidase solution and the maleimide solution were individually incubated at 30°C for 1 min, then they were mixed and further incubated at 30°C for 30 min with continuous stirring. The peroxidase-maleimide conjugate solution was centrifuged at 11,500 rpm for 10 min and the supernatant was applied to a Sephadex G-25 (Pharmacia Fine Chemicals Co., Uppsala, Sweden) column (0.9 x 45 cm) using 0.1M sodium phosphate buffer, pH 6.0. The fractions showing absorbance at 403 nm were concentrated with a collodion bag.

Labeling

The concentrated Fab' was incubated with the concentrated peroxidase-maleimide conjugate at 30°C for 30 min. The mixture was applied to an Ultrogel AcA 44 column (0.9 x 95 cm) using 0.1M sodium phosphate buffer, pH 6.5, and Fab'-peroxidase conjugate was separated from unconjugates.

Preparation of affinity-purified Fab'-peroxidase conjugate

The Fab'-peroxidase conjugate was affinity-purified using CNBr-activated Sepharose 4B(Pharmacia). CNBr-activated Sepharose 4B (1g, dry weight) was coupled with 300 μg of hGH in accordance with the manual of Pharmacia (11), and packed in a column (0.5 x 2.5 cm). Fab'-peroxidase conjugate was concentrated and prepared in 1.2 ml of 0.1M sodium phosphate buffer, pH 6.5, containing 1g/l BSA. The Fab'-peroxidase conjugate solution was applied to a hGH-Sepharose 4B column using 0.1M sodium phosphate buffer, pH 6.5, at a flow rate of 1.0 ml/hr. After washing with the same buffer, the specific anti-hGH Fab'-peroxidase conjugate absorbed on the column was eluted using 50 mM glycine-HCl buffer, pH 2.9, and immediately neutralized with 0.5M Tris-HCl buffer, pH 8.0. BSA and thimerosal were added to the anti-hGH Fab'-peroxidase conjugate solution to give the final concentrations of 1 g/l and 50 mg/l, respectively. The affinity-purified Fab'-peroxidase conjugate solution was preserved at 4°C. At the time of the assay, the conjugate was diluted with 0.01M sodium phosphate buffer, pH 7.0, containing 1 g/l BSA.

Preparation of anti-hGH IgG coated polystyrene balls

Polystyrene balls (3.2 mm in diameter, Ichiko Co., Aichi, Japan) as solid phase were incubated with anti-hGH IgG (0.1 mg/ml) in 0.1M sodium phosphate buffer, pH 7.5, at 37°C for 30 min and then at 4°C for 24 hrs. The polystyrene balls were washed with 0.1M sodium phosphate buffer, pH 7.5, and then, washed three times with 0.01M sodium phosphate buffer, pH 7.0, containing 0.1% NaN_3 BSA and 0.1M NaCl. The balls was preserved at 4°C and used after 2 weeks.

Assay procedure

Assay procedure was shown in Fig. 1. Anti-hGH IgG coated polystyrene balls were incubated with 100 μl of standard GH or 100 1 urine samples, and 50 μl of 0.1M sodium phosphate buffer, pH 7.0, in duplicate at 37°C for 6 hrs with continuous shaking. After removal of the supernatant, the polystyrene balls were washed twice with 2 ml of

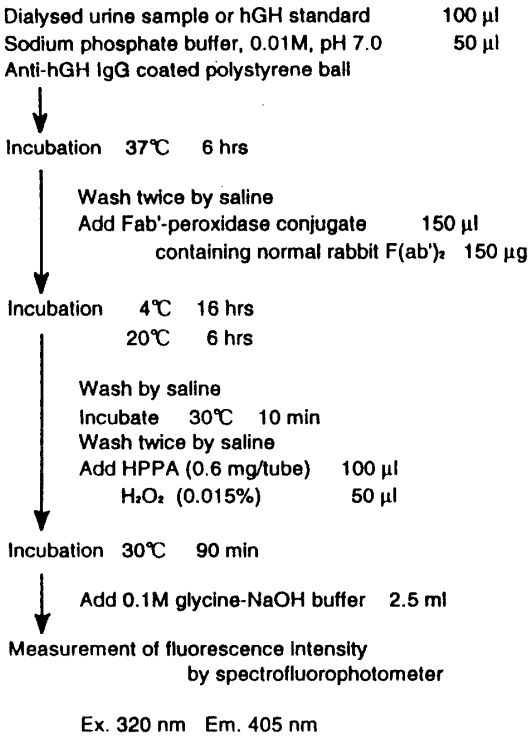

Fig. 1. Assay procedure of a highly sensitive enzyme immunoassay.

150 mM saline and then incubated with affinity-purified Fab'-peroxidase conjugate solution and 150 µg normal rabbit $F(ab')_2$ at 4°C for 20 hrs and then at 20°C for 6 hrs. After removal of the supernatant, the polystyrene balls were washed with 2 ml saline and incubated with 1 ml saline at 30°C for 10 min and washed with 2 ml saline, and then incubated with 100 µl of 3-(p-hydroxyphenyl) propionic acid (0.6 mg/tube) recrystallized as substrates and 50 µl of 0.015% H_2O_2 at 30°C for 90 min. The reaction was stopped by adding 0.1M glycine-NaOH buffer, pH 10.3. Fluorescence intensity was measured by spectrofluorophotometer (Shimazu RF-5000, Shimazu Co., Kyoto, Japan). The exitation and emission wave length were 320 nm and 405 nm, respectively. The exitation and emission slit width were 3 nm and 30 nm, respectively. Urinary GH levels were corrected by urinary creatinine concentrations.

Urinary β_2-microglobulin and creatinine measurments

Urinary β_2-microglobulin levels were measured by a solid phase RIA

kit (β_2-MICRO RIABEAD, Dainabot Co., Tokyo, Japan). The minimal detectable quantity was 12 μg/l. Urinaly creatinine levels were measured by an autoanalyser (TBA50s, Toshiba, Tokyo, Japan). Urinary β_2-microglobulin levels were corrected by urinary creatinine concentrations.

RESULTS

Fig. 2 shows the elution profile of IgG from a DEAE cellulose column. The first major peak indicates the purified IgG. We obtained 12.38 mg of IgG from 4 ml of anti-hGH rabbit serum. Fig. 3 shows the elution profile of $F(ab')_2$ from a Ultrogel AcA 44 column. The first major peak, the second small peak and the third small peak indicate $F(ab')_2$, Fc portion and small peptides, respectively. We obtained 6.91 mg of $F(ab')_2$ from 12.38 mg of IgG. Fig. 4 shows the elution profile of Fab' obtained by reduction of $F(ab')_2$ with 2-mercaptoethylamine. The first small peak indicates $F(ab')_2$ and the second major peak indicates Fab'. We obtained 6.28 mg of Fab' from 6.91 mg of $F(ab')_2$. 6.28 mg of Fab' was concentrated to 350 μl and 5.65 mg of peroxidase-maleimide conjugate was concentrated to 200 μl. They were mixed and incubated at 30°C for 30 min. Fig. 5 shows the elution profile of Fab'-peroxidase conjugate from a Ultrogel AcA 44 column. The arrows indicate the eluted points of BSA and peroxidase, respectively. The first major peak and the second major peak of absorbance at 280 nm indicate Fab'-peroxidase conjugate, free Fab' and peroxidase, respectively. It was shown that the conjugation of Fab' and peroxidase was monomeric (Fab': peroxidase=1.00 : 1.07). The labeling efficiency in the different concentrations of Fab', 1.13, 1.79 and 2.83 mg/100 μl were 52.6, 60.6 and 70.7% respectively. The elution profile of the Fab'-peroxidase conjugate from a hGH-Sepharose 4B column was shown in Fig. 6. The second small peak indicates the affinity-purified Fab'-peroxidase conjugate.

The representative standard curve using the assay system is shown in Fig. 7. The minimal detectable quantity was 0.3 pg/ml. The intraassay and interassay coefficients of variation were 6.0% and 9.8%, respectively. The mean recovery of GH was 95%.

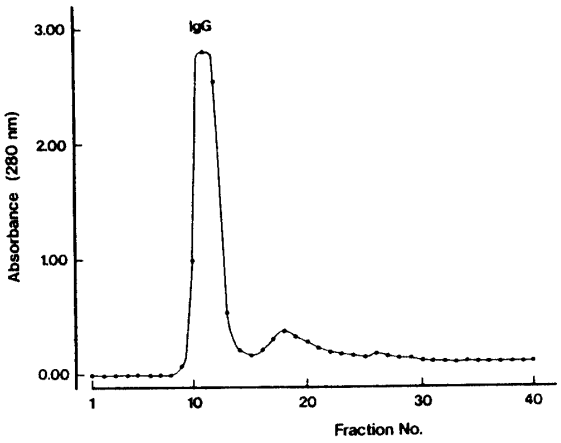

Fig. 2. Elution profile of IgG from a DEAE cellulose column (0.9x20 cm) eluted with linear concentration gradient of sodium phosphate buffer, pH 6.5, 0.015M-0.2M. The volume of each fraction was 1.3 ml.

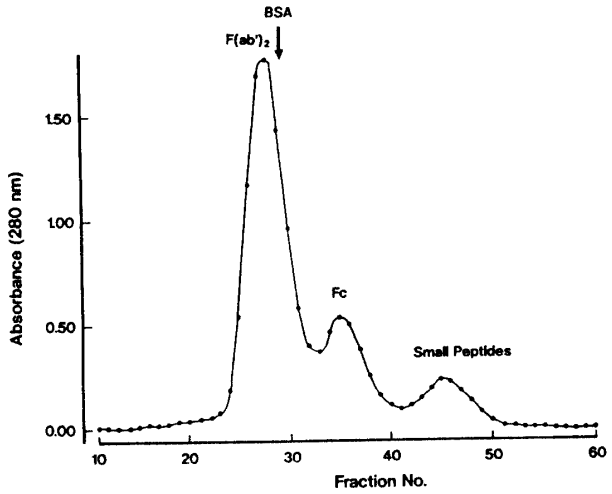

Fig. 3. Elution profile of digested IgG from an Ultrogel AcA 44 column (0.9x75 cm) using 0.1M sodium borate buffer, pH 8.0. The volume of each fraction was 1.3 ml.

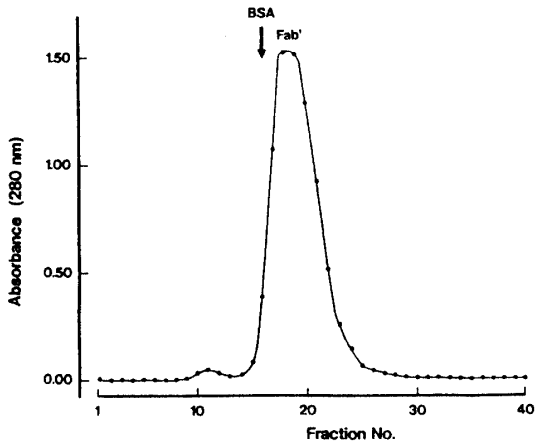

Fig. 4. Elution profile of Fab' from an Ultrogen AcA 44 column (0.9x45 cm) using 0.1M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. The volume of one fraction is 1.3 ml. The arrow indicates the eluted point of BSA.

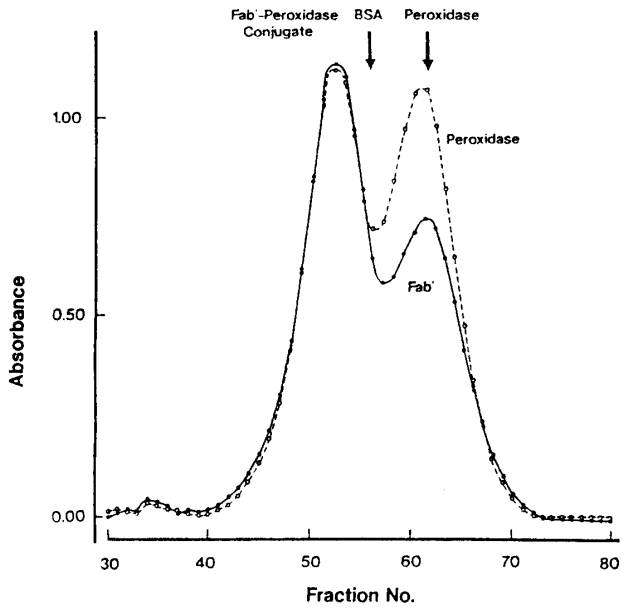

Fig. 5. Elution profile of Fab'-peroxidase conjugate from an Ultrogel AcA 44 column (0.9x95 cm) using 0.1M sodium phosphate buffer, pH 6.5. The volume of each fraction was 0.65 ml. The arrow indicates the eluted point of BSA and peroxidase, respectively. Open and closed circles indicate absorbance at 280 and 403 nm, respectively.

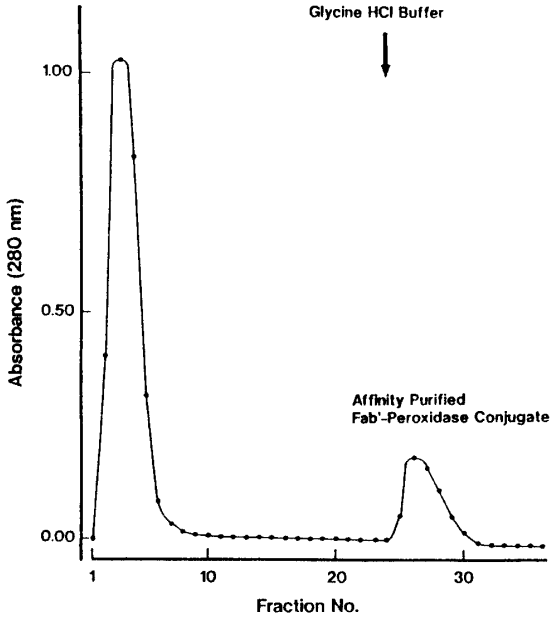

Fig. 6. Elution profile of affinity chromatography of Fab'-peroxidase conjugate from a hGH-Sepharose 4B column (0.5x2.5 cm). The volume of each fraction was 0.65 ml.

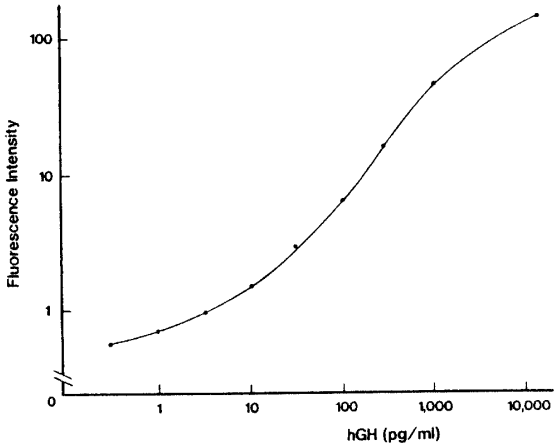

Fig. 7. A standard curve of the highly sensitive enzyme immunoassay of hGH.

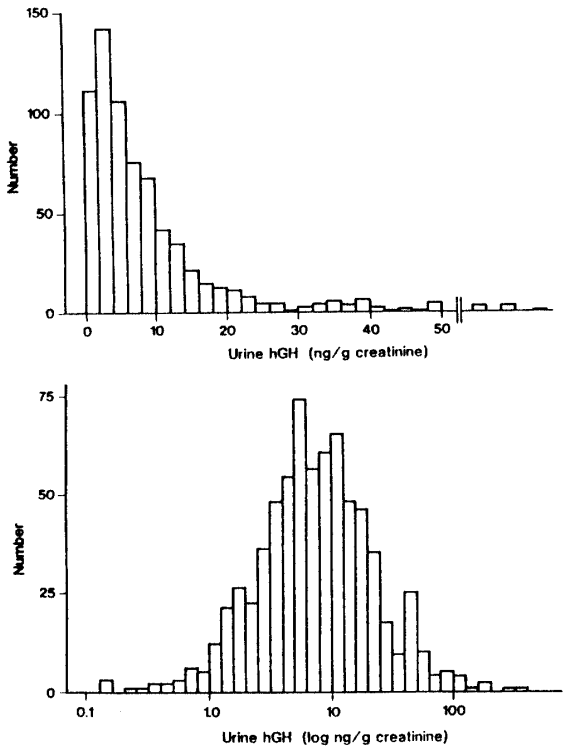

Fig. 8. The distribution of urinary hGH levels measured by the highly sensitive enzyme immunoassay in a population. Urinary hGH levels were shown on a logarhythmic scale in a lower panel.

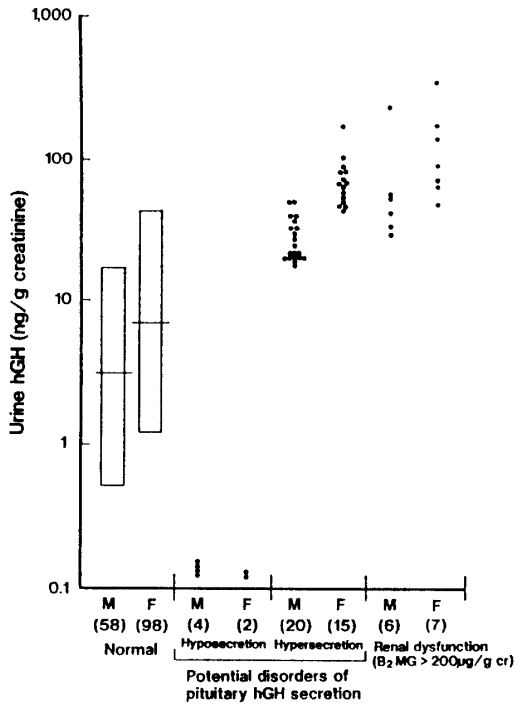

Fig. 9. Urinary hGH levels in normal subjects and potential disorders of GH secretion in a population of 706 subjects. In normal subjects, the mean values and the range (-2SD, +2SD) are shown. Numbers of the subjects are shown in parentheses.

Urinary GH ranged from not detectable to 314 ng/g creatinine in a population. The urinary GH levels transformed into logarithms showed a normal distribution with the mean value of 5.8 ng/g creatinine (Fig. 8). Urinary GH levels were detectable in the all normal subjects and ranged from 0.38 to 46.8 ng/g creatinine. The mean (-2SD - +2SD) values were 5.1 (0.7 - 35.2) ng/g creatinine. Urinary GH levels in normal male subjects were significantly lower than those in normal female subjects (3.1 (0.5 - 17.0) ng/g creatinine vs 7.01 (1.2 - 41.9) ng/g creatinine, $p < 0.01$).

When the upper limit of normal urinary GH levels was defined as the mean±2SD, 17.0 ng/g creatinine for male and 41.9 ng/g creatinine for female subjects, respectively, 48 cases (6.8%) were screened as hypersecretion of GH into urine (Fig. 9). Among them, urine β_2-microglobulin levels were increased (more than 200 μg/g creatinine) in 13 cases (1.8%). On the other hand, urinary GH levels were not detectable in 6 cases (0.8%).

DISCUSSION

In the present study, we set up a highly sensitive enzyme immunoassay with a slight modification of the method described by Hashida et al. (9,10), and measured urinary GH levels in a population to determine the normal range for adults. We modified the labeling conditions to find that better efficiency was obtained with high concentrations of Fab' and peroxidase reacted at higher temperature of 30°C for a shorter period of 30 min. Furthermore, in order to reduce the non-specific binding of Fab'-peroxidase conjugate to the solid phase, incubation procedure at 30°C for 10 min was added between washing procedures.

Hassen (3) reported that urinary hGH levels in 9 normal subjects ranged from 28 to 53 ng/24h using a double antibody radioimmunoassay. However, the urine sample had to be dialyzed and concentrated 50 fold with lyophilization and the detection limit was 4 pg/ml. Baumann and Abramson (5) extracted GH from approximately 35 liters urine by diafiltration and concentration, and showed that immunoreactive GH excreted in urine consisted primarily of 22K, small quantities of 20K and an unidentified acidic form and that 0.01% of circulating GH was excreted into urine. Since these assay methods required the complexed extraction methods of such as ultrafiltration, lyophilization and diafiltration, it was difficult to use for routine examinations.

Sensitive enzyme immunoassay was enable to measure urinary GH levels using dialyzed urine samples without any concentrating method and recognized 22K GH which was the main compornent in urine (8). Furthermore, it was shown that there was no cross-reaction with hPL, prolactin, LH, FSH and TSH in urine (8,11). Because the Fab'-peroxidase conjugate was stable at least for 9 monthes, it is not necessary to label frequently. The detection limit was 0.3 pg/ml, and urinary GH levels in normal subjects were detectable within this sensitivity.

Urinary GH levels are known to correlate with plasma GH changes, not only by pharmacological stimulations, but also by physiological conditions (9,12,13). It was confirmed that patients with acromegaly had excess excretion of urinary GH and patients with pituitary dwarfism had low concentrations of urinary GH (9,19). It is

suggested, therefore, that the measurement of urinary GH levels is useful for assessment of pituitary GH secretion.

In order to determine the distribution of urinary GH levels in a general population, we measured GH levels in 706 urine samples using the highly sensitive enzyme immunoassay. The normal range obtained from 156 subjects in the present study was 0.5 - 17.0 ng/g creatinine in males and 1.2 - 41.9 ng/g creatinine in females. These values were lower than those in the previous reports. The difference may be explained by the sensitivity. Urinary GH levels were increased over the normal range in 48 (6.8%) of a population, and not detectable in 6 (0.8%) of a population.

When we assess GH excretion into urine, renal function and day-to-day variation must be considered. GH in plasma is filtered at the glomerulus and reabsorbed at renal tubulus (14,15). As previously reported, urinary GH levels are positively correlated with urinary β_2-microglobulin in patients with renal insufficiency (16). There is no correlation among urinary GH, albumin and α_1-microglobulin during the night-time in normal subjects (17). Urinary β_2-microglobulin levels were increased in 13 of 48 cases with increased urine GH levels in the present study, indicating that the remaining 35 (5%) of a population could be screened as hypersecretion of GH. Increased plasma GH values (up to 15.2 ng/ml) were shown in 4 of the 35 cases. However, since it was reported that mean day-to-day variation of urinary GH levels was 49% in normal men (17) and 30.1% in normal but short children (18), two or more urine samples should be measured when individual GH secretion was assessed. Although urine GH levels were detectable in all normal subjects, 6 cases (0.8%) showed no detectable urine GH values, indicating potential hyposecretion of GH. In fact, further studies revealed two cases with hypopituitarism due to pituitary tumors.

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