Direct Double Monoclonal Immunoradiometric Assay of Urinary Human Growth Hormone

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Several reports indicate that urinary growth hormone (GH) excretion might reflect central release of the hormone, and that measurement of urinary GH shows promise in the investigation of physiological and pathological GH secretion. We have developed and evaluated a direct immunoradiometric assay (IRMA) in which two monoclonal antibodies are used to measure GH in the urine of children. The detection limit is ~0.018 pmol/L for a sample volume of 2 mL. Within- and between-run variations (CVs) were 5.6% and 14.2%, respectively. Analytical recovery and dilution experiments showed the specificity of the method for GH. In normal-stature prepubertal children ages 3–12 years, 24-h urinary GH excretion was 0.189 (SD 0.100) pmol and correlated well with the amount of GH in the first morning miction, which showed wide day-to-day variations. Like others, we found a strong correlation between GH concentrations in serum and urine during stimulation tests with GH-releasing hormone (somatostatin) and (or) during physiological nocturnal secretion, confirming that urinary GH measurement may be of help in investigating patients (particularly young children) with diseases in which GH secretion is impaired.

Additional Keyphrases: pediatric chemistry · somatostatin

Serum growth hormone (GH) concentrations fluctuate greatly in the absence of stimuli (1, 2), and a single blood collection, therefore, provides no useful information on GH secretion.2 Two or more pharmacological tests are required to measure GH secretory capacity and give evidence of a GH secretion deficiency. These tests do not always reflect endogenous secretion (3), which is determined as the integrated concentrations of GH in blood samples collected every 15–20 min for 12 or 24 h. However, this method is not suitable for use with large numbers of subjects (particularly young children), especially given the large day-to-day variability of physiological nocturnal secretion. Reliable measurement of GH secretion is necessary in several pathological settings in which secretion is stimulated and particularly in neurosecretory GH deficiencies (4, 5), in which the existence of a deficit is controversial. It would be useful to have a different approach based on serial measures of GH secretion, and Quattrin et al. (6) have suggested that a 12- or 24-h estimate of GH excretion in urine might reflect central GH secretion and provide semiquantitative measurements of GH in individuals of all ages. However, only an extremely small fraction of endogenously secreted or exogenously administered GH is excreted in urine (7, 8). Several groups recently developed highly sensitive assays for measuring urinary GH, including sandwich enzyme immunoassays of dialyzed urine (9, 10) and direct immunoradiometric assays (IRMAs) involving two polyclonal antibodies (11) or one monoclonal and one polyclonal antibody (12, 13).

The aim of this work was to develop and evaluate a sensitive quantitative assay for urinary GH by use of a direct IRMA method with two monoclonal antibodies.

Materials and Methods

Methods

Urinary GH. The IRMA technique we developed involves a protocol similar to that described by Erb et al. (14), with a two-step procedure. In the first step, non-sedimenting polyacrylamide beads are coated with the first monoclonal antibody, permitting the immunextraction of urinary GH; in the second step, an 125I-labeled monoclonal antibody that recognizes a different epitope of the GH molecule is used to detect and quantify GH.

Briefly, we washed 6 mL (0.2 g) of non-sedimenting polycrylamide beads (a kind gift from Baxter, Dunedin, Switzerland) with 0.03 mol/L sodium phosphate buffer, pH 6.3, resuspended the beads in 6 mL of the same buffer and mixed them with 100 μL (100 μl) of the first purified monoclonal anti-GH antibody and 20 μg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma Chemical Co., St. Louis, MO) at 4 °C overnight. After we washed the pellet with 0.05 mol/L sodium phosphate buffer, pH 7.5, we resuspended it in the same buffer containing also, per liter, 9 g of NaCl, 1 g of bovine serum albumin (BSA), 0.1 g of sodium ethimercurothioalcylate, and 1 mL of Triton X-100 (diluent buffer).

In the GH assay, we mixed 50 μL of immunosorbent with 2.5 mL of urine or hGH standard in diluent buffer (0–5000 pg/tube, calibrated against the first International Reference Preparation (IRP) MRC 66/217 and 1 mL of 0.1 mol/L Tris buffer, pH 7.5, containing (per liter) 9 g of NaCl, 0.1 g of sodium ethimercurothioalcylate, and 1 g of BSA. After 12–18 h of incubation at 4 °C with constant shaking, the pellet was washed with the diluent buffer and incubated once more for 12–18 h at 4 °C with 1 mL of 0.05 mol/L sodium phosphate buffer, pH

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2 Nonstandard abbreviations: GH, growth hormone; IRMA, immunoradiometric assay; BSA, bovine serum albumin; GHRH, growth-hormone-releasing hormone (somatostatin); IRP, International Reference Preparation; and IGF 1, insulin-like growth factor 1.

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7.4, containing 9 mg of NaCl and ~150,000 counts/min of the iodinated second purified monoclonal anti-GH antibody. We carried out the iodination with the conventional Chloramine T method, using 1 mCi of Na<sup>125</sup>I and 50 μg of antibody, and we separated the iodinated mixture on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). After a final washing with diluent buffer, we counted the radioactivity of the pellet in a gamma counter (Gamma Master; Pharmacia LKB Inc., Uppsala, Sweden).

The two monoclonal antihuman GH antibodies were kindly provided by CIS Biointernational (Gif sur Yvette, France).

Creatinine was determined by the conventional picric acid technique, adapted to an automated analyzer with a commercial kit (bioMérieux, Marcy l’Etoile, France). Urinary GH excretion was expressed as pmol/24 h for 24-h urine samples and as nmol/mol creatinine for 24-h nocturnal, and growth-hormone-releasing hormone (GHRH) test specimens.

**Plasma GH.** Plasma GH was measured with a competitive radioimmunoassay (RIA) by using a rabbit polyclonal antibody and a second polyclonal lamb anti-rabbit antibody coated onto the test tubes. Standards were calibrated with First IRP MRC 66/217. The mean within- and between-run CVs were 5.6% and 8.9%, respectively.

**Serum IGF 1.** For measurement of insulin-like growth factor 1 (IGF 1), we first passed serum samples through a column of Ultrogel ACA 54 (Pharmacia) under acid conditions, then lyophilized the samples and resolubilized them in albumin–phosphate buffer. IGF 1 was measured in duplicate by an equilibrium RIA technique (15).

**Subjects**

Seventeen strictly prepubertal normal-stature children (11 boys, 6 girls; height within 1 SD of age-related group means for stature, and having normal growth velocity), ages 3–10 years, served as the reference group. The first morning miction (i.e., the urine accumulated in the bladder during the night) was collected on five separate days, and two successive 24-h specimens were also collected. Ten prepubertal children (6 boys, 4 girls), ages 3.5–11.5 years, formed the complete GH-deficiency group, selected on the basis of the following criteria: growth failure by more than −2 SDs for chronological age by the French growth chart (16), abnormal response to two pharmacological tests (peak GH concentrations <8 μg/L), and abnormal nocturnal GH secretion (integrated GH secretion <2 μg·L<sup>−1</sup>·min<sup>−1</sup>). We collected 24-h urine specimens for this group. Twenty strictly prepubertal children (13 boys, 7 girls), ages 4–11 years, served as the normal-short control group on the basis of the following criteria: growth failure more than −2 SDs for chronological age by the French growth chart (16), normal growth velocity equal to or exceeding −2 SDs from the mean of bone age, and normal GH response to two pharmacological tests; these 20 patients were studied for their GH response to GHRH stimulation and their nocturnal GH secretion.

We injected GHRH-(1–44)-NH<sub>2</sub> (Sanofi, Toulouse, France) intravenously at 2 μg/kg of body weight. Blood was drawn into heparinized tubes starting 15 min before and at several intervals for 2 h after (−15, 0, 15, 30, 45, 60, 120 min) GHRH injection for analysis by the RIA of plasma GH. Blood was also collected at time 0 for IGF 1 measurement. Urine was collected for 5 h during the test (the patients were asked to urinate just before the test).

In the sleep test, a catheter was inserted at 2000 h with a slow saline drip. Samples for GH assay were taken every 20 min from 2100 to 0630; the children were fully informed as to the nature of the test and slept spontaneously for at least 6 h. We collected morning urine specimens from each child.

We collected urine in polypropylene containers and stored it in 10-mL samples at −20 °C after noting the volume. We thawed the specimens, centrifuged them at 1000 × g to remove particulate material, and used the supernate for the assay.

**Data Analysis**

The night GH profiles were analyzed with the PULSAR program developed by Merriam and Wachter (17) and adapted by S. Rosberg and Albertsson-Wickland (PC-PULSAR; 1987, personal communication and gift). The PULSAR program identifies secretory peaks by height and duration from a smoothed baseline with the assay SD as a scale factor.

Peak selection criteria appropriate for our own assay conditions and data set were established before the nocturnal GH profiles and were analyzed by the PC-PULSAR program. For our RIA, the assay noise was derived from the equation

\[
SD = (0.00063x^2 + 0.0477x + 0)/100
\]

where \(x\) is any given GH concentration. The assay SD was calculated from duplicate nocturnal GH profiles. The smoothing time was set to one-half the total nocturnal profile time. The splitting parameter was set to 2, and the weight assigned to each peak was 0.02.

The following values were extracted from the PULSAR analysis: number of peaks, overall mean, maximal value, mean of the baseline, mean peak height, mean peak amplitude, mean peak area, and mean peak length. The area under the curve was estimated above zero and above the calculated baseline.

**Results**

**Analytical Evaluation**

**Standard curve, linearity, and detection limit.** Figure 1 shows the correlation between GH content per tube and counts per minute (i.e., those corresponding to bound <sup>125</sup>I-labeled antibody). The curve was linear up to 1000 ng/tube (equivalent to 400 ng/L in urine, i.e., 18.182 pmol/L) (Figure 1, top), and the detection limit was <2 pg/tube (Figure 1, bottom); a precision profile
Recovery and serial dilution. The accuracy of the method was evaluated with analytical recovery and dilution tests. We assayed 81 urine samples before and after the addition of a known amount of GH (31.2 pg/tube), and four urine samples before and after the addition of increasing amounts (7.8 to 125 pg/tube) of GH (IRP 66/217). In the first assay, the amount recovered was 32.9 (SD 2.45) ng/tube, giving a percentage recovery of 105.0% (SD 7.8%). In the second, the percentage recovery ranged from 98% to 113% (Table 2). In the dilution test, we diluted four urine samples with high concentrations of urinary GH (collected from one to four-day-old full-term infants) stepwise twofold in 0.05 mol/L phosphate buffer, pH 7.5, containing (per liter) 1 g of BSA. The mean CV for each dilution was −15% to +20% (Table 3).

Clinical Evaluation

Normal control group. Urinary GH excretion, measured in the first morning miction, varied severalfold from night to night for each child. Figure 2 shows the intra-individual variation of GH excretion for the five collections from each of the 17 children. Urinary GH excretion in the normal control group was 0.055 (SD 0.020) nmol/mol creatinine (range 0.017–0.098) for the morning collection and 0.057 (SD 0.020) nmol/mol creatinine (range 0.029–0.091; i.e., 0.189 ± 0.100 pmol), for the 24-h collection. Nocturnal (x) and 24-h (y) GH excretions were well correlated in all 17 children: y = 0.67x + 0.02 (r = 0.62, P < 0.01, S² = 2.47 · 10⁻⁶).

Complete GH-deficiency group. Urinary GH was undetectable in five children, concentrations being <0.018 pmol/L. In the other five children, urinary GH excretion was 0.009 (SD 0.005) nmol/mol creatinine (i.e., 0.038 ± 0.021 pmol/24 h). These values were significantly less than those obtained in the normal control group (P < 0.001), with no overlap between these groups.

Normal-short control group. Nocturnal urinary GH excretion (y) correlated positively with the plasma GH values during the overnight collection test, expressed as the area under the curve (x): y = 0.00063x − 0.062 (r = 0.91, P < 0.001, S² = 1.671). Urinary GH values (y) were also positively correlated with plasma GH values (x) during the GHRH test: y = 0.0086x − 0.11 (r = 0.85, P < 0.001, S² = 1.526).

Serum values for IGF 1 were 59.8 (SD 37.0) μg/L and showed no correlation with (a) nocturnal GH secretion.

Table 1. Within- and Between-Run Precision for Urinary GH (pmol/L) in Urine Samples and Control Solutions

<table>
<thead>
<tr>
<th>Urine</th>
<th>Mean (SD)</th>
<th>CV, %</th>
<th>Control solutions</th>
<th>Mean (SD)</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td>0.078 (0.009)</td>
<td>12.5</td>
<td>L1</td>
<td>0.169 (0.023)</td>
<td>14.2</td>
</tr>
<tr>
<td>U2</td>
<td>0.246 (0.023)</td>
<td>9.3</td>
<td>L2</td>
<td>0.338 (0.032)</td>
<td>9.5</td>
</tr>
<tr>
<td>U3</td>
<td>0.459 (0.036)</td>
<td>7.9</td>
<td>L3</td>
<td>0.468 (0.059)</td>
<td>12.6</td>
</tr>
<tr>
<td>U4</td>
<td>0.622 (0.045)</td>
<td>5.6</td>
<td>L4</td>
<td>0.750 (0.050)</td>
<td>6.6</td>
</tr>
<tr>
<td>U5</td>
<td>1.586 (0.100)</td>
<td>6.2</td>
<td>L5</td>
<td>3.377 (0.400)</td>
<td>10.8</td>
</tr>
<tr>
<td>U6</td>
<td>3.096 (0.190)</td>
<td>5.7</td>
<td>L6</td>
<td>3.377 (0.400)</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Table 2. Analytical Recovery of GH In Four Urine Samples Supplemented with Increasing Concentrations of GH (IRP 66/217)

<table>
<thead>
<tr>
<th>GH, pg/tube</th>
<th>Added to urine</th>
<th>Mean measured</th>
<th>Mean recovery, %</th>
</tr>
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<tbody>
<tr>
<td>7.8</td>
<td>7.8</td>
<td>8.2</td>
<td>105</td>
</tr>
<tr>
<td>15.6</td>
<td>15.6</td>
<td>17.1</td>
<td>110</td>
</tr>
<tr>
<td>31.2</td>
<td>31.2</td>
<td>35.4</td>
<td>113</td>
</tr>
<tr>
<td>62.4</td>
<td>62.4</td>
<td>67.8</td>
<td>108</td>
</tr>
<tr>
<td>124.8</td>
<td>124.8</td>
<td>127</td>
<td>98</td>
</tr>
</tbody>
</table>

Fig. 1. Linear correlation between GH content per tube (pg) and counts per minute measured
Top to bottom: successive expansions of the standard curve
gave an estimate of ~1 pg/tube (i.e., 0.4 ng/L urine or 0.018 pmol/L).
Precision test. Repeatability studies on six urine samples (0.073–3.036 pmol/L) measured 10 times in the same assay gave a CV <10%, except for the lowest concentration (0.073 pmol/L), for which the CV reached 12.5%. We estimated between-run reproducibility by measuring five control solutions (0.159–3.164 pmol/L) in triplicate in 28 different assays (February '90 to January '91). The CV ranged from 6.6% to 14.2% (Table 1).
Table 3. Analytical Linearity of GH in Four Urine Samples Successively Diluted Twofold in Buffer

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Expected (pg/tube)</th>
<th>Mean measured (pg/tube)</th>
<th>Mean variation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>160</td>
<td>181</td>
<td>+13</td>
</tr>
<tr>
<td>1/2</td>
<td>80</td>
<td>88</td>
<td>+10</td>
</tr>
<tr>
<td>1/4</td>
<td>40</td>
<td>43</td>
<td>+8</td>
</tr>
<tr>
<td>1/8</td>
<td>20</td>
<td>17</td>
<td>-15</td>
</tr>
<tr>
<td>1/16</td>
<td>10</td>
<td>8.5</td>
<td>-15</td>
</tr>
<tr>
<td>1/32</td>
<td>5</td>
<td>6.0</td>
<td>+20</td>
</tr>
</tbody>
</table>

Fig. 2. Intra-individual variation of urinary GH excretion (nmol/mol creatinine) in five first-morning urine samples from each of 17 healthy children. Bars indicate SD

expressed as the area under the curve (r = 0.11), (b) nocturnal urinary GH excretion (r = 0.14), (c) peak GH during the GHRH test (r = 0.09), or (d) urinary GH excretion during the GHRH test (r = 0.10).

Discussion

In the past few years, several reports have indicated that urinary GH excretion might reflect GH secretion, such that its measurement shows promise in the investigation of physiological and pathological GH secretion (13). Hashida et al. (9) and Okuno et al. (18) found that urinary GH concentrations clearly reflected serum GH concentrations during stimulatory tests, and concluded that it should be easy to distinguish between patients with normal and those with low physiological GH secretion and to distinguish between responses to pharmacological tests. Others later suggested that such measurements could also be useful to screen for patients with neurosecretory dysfunction (19), to obtain additional insight into the role of GH in diabetes (20), and to assess the correct replacement dose in GH-treated children (21).

However, the measurement of GH in urine has been hampered by the very low concentrations of GH in normal subjects, and many attempts to measure GH in urine have therefore involved the concentration and (or) dialysis of the urine sample (6, 9). More recently, direct IRMAs, based on the use of either one or two polyclonal antibodies, have been developed (11–13). To increase the sensitivity and specificity of this assay and to avoid the use of polyclonal antibodies with their highly variable affinity and specificity, we developed a direct IMA with two monoclonal antibodies. The method is very sensitive and precise, and the specificity, as studied by recovery and dilution tests, is excellent.

In a normal control group of prepubertal children, the 24-h GH excretion values (0.189 ± 0.100 pmol/24 h) were of the same order of magnitude as those reported by other authors studying groups of prepubertal and pubertal subjects. The 24-h and nocturnal urinary GH excretion values were well correlated, although both varied widely on a day-to-day basis for any given patient (14, 20). Despite the fact that the morning collection is far more convenient, our results with this small population suggest that the 24-h value is more reliable. In agreement with Kohno et al. (22), we recommend that at least two 24-h collections be analyzed for an accurate evaluation of GH secretion.

Interestingly, we found a correlation between GH concentrations in serum and urine during both the GHRH test and physiological nocturnal secretion; our results thus confirm that urinary GH concentrations reflect GH serum concentrations, as suggested by Hashida et al. (9, 18, 19) and Suzuki et al. (20). Moreover, urinary GH concentrations were undetectable or very low in subjects with a complete GH deficiency, as previously reported (21, 22).

We expressed our GH results as pmol/24 h urine or as nmol/mol creatinine. At present, there is a lack of uniformity in the expression of results by different authors (ng per collection period, ng per kg body weight, ng per m² body surface area, ng or pmol per g or nmol of creatinine or, finally, ng of GH relative to another protein such as β₂-microglobulin). Given the wide variations in diuresis in children, together with large differences in renal excretion with growth, a correction factor for urinary GH values appears to be necessary. Creatinine clearance is one of the most useful approaches for determining such a correction factor, although other studies are necessary to find more specific markers of renal function.

One problem remaining with urinary GH assay is the precise nature of the immunoreactive GH in urine. The major form of GH has a molecular mass of 22 500 Da; heterogenic forms have so far not been detected in unextracted and unconcentrated urine (12). Baumann and Abramson (8) have described the presence of a 20 000-Da form and an unidentified acidic form in extensively concentrated urine. Moreover, it is possible that oligomeric forms of GH, by virtue of their molecu-
lar size, escape renal filtration and do not appear in urine in quantifiable amounts (12, 23, 24). The 22 500-Da form should therefore represent virtually all the GH in urine. This seems to be confirmed by Albini et al. (10), who showed that the immunoreactivity of HPLC fractions of urinary GH was similar in a double-monoclonal IRMA and in a standard GH polyclonal assay.

In conclusion, the double monoclonal antibody GH assay we propose here appears to be accurate enough to measure urinary GH. Further clinical studies will be necessary to show its validity in various pathological disorders in which GH secretion can be altered.

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References