Radioimmunoassay for octapeptide analogs of somatostatin: Measurement of serum levels after administration of long-acting microcapsule formulations

(peptide hormone analog/hormone therapy/cancer therapy/drug delivery)

MEREDITH MASON-GARCIA*, MYRA VACCARELLA*, JUDIT HORIZTH*, TOMMY W. REDDING*, KATE GROOT*, PIERO ORSOLINI†, AND ANDREW V. SCHALLY*†‡§

*Endocrine, Polypeptide and Cancer Institute, Veterans Administration Medical Center, New Orleans, LA 70146; ‡Cytochem, S.A., Martigny, Switzerland; and †Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70112

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ABSTRACT The development of a long-acting delivery system for D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH₂ (RC-160), an octapeptide analog of somatostatin, required the establishment of a method for determining the concentration of this analog in serum during treatment. A sensitive and specific radioimmunoassay (RIA) for RC-160 was developed and used for following the rate of liberation of this peptide from microcapsules of poly(DL-lactide-coglycolide). Antibodies were generated in a rabbit against RC-160 conjugated to bovine serum albumin with glutaraldehyde. At an antiserum dilution of 1:100,000, the antibodies bound approximately 25% of added radiolabeled RC-160. Somatostatin octapeptide analogs that had a disulfide bridge showed crossreactivity with the antiserum, but analogs without the disulfide bridge and other peptides tested did not crossreact. The minimum detectable dose of RC-160 was 10 pg. Intra- and interassay coefficients of variation ranged from 9.1% to 12.8% and from 14% to 30%, respectively. The RIA was suitable for direct determination of RC-160 in serum. Eleven prototype batches of microcapsules were tested in rats, and the rate of release of the analog from the microcapsules was followed. An improved batch of microcapsules made from RC-160 pamoate maintained high serum levels of RC-160 for more than 30 days after intramuscular injection. The RIA should be of value for monitoring levels of this analog in serum during long-term therapy.

Thus, therapeutic application of these analogs in the treatment of various hormone-sensitive tumors is likely (6).

The use of RC-160 and related analogs for the therapy of hormone-dependent tumors and endocrine disorders would be greatly enhanced by delayed delivery systems capable of maintaining controlled levels of the peptide over an extended period of time. Consequently, we started the development of such a long-acting delivery system for RC-160 by using a formulation in microcapsules of poly(DL-lactide-coglycolide) similar to that successfully employed for [D-Trp⁴]LH-RH (7, 8). This polymer is biodegradable and biocompatible with living tissue. The injectable microcapsules were designed for a controlled release of the peptide over a 30-day period. This approach is convenient and efficacious and permits once-a-month administration of microcapsules. To monitor the release of RC-160 from these microcapsules, we have developed a rapid, sensitive, and specific RIA for this peptide. This paper describes the establishment of the RIA and the development of a delayed delivery system for RC-160.

MATERIALS AND METHODS

Analog and Microcapsules. RC-160 was synthesized by Nova Biochem (Laufelfingen, Switzerland) in the form of the acetate or pamoate salt. Microcapsules of RC-160 were prepared by a phase-separation process. The peptide was dispersed in a solution of poly(DL-lactide-coglycolide). A phase inducer for the copolymer was added to coacervate the polymer. The coacervate droplets, containing the drug, were hardened to obtain the microcapsules, which were sieved. Prototype batches were made by one of us (P.O.) at Cytochem (Martigny, Switzerland). The microcapsule product was a free-flowing powder of spherical particles consisting of RC-160 acetate (batches 1–10) or pamoate (batch 11) (2–6%, wt/wt), dispersed throughout a polymeric matrix of poly(DL-lactide-coglycolide) (94–98%). The microcapsules were sterilized with a 2.5-megarad (0.025-megagram) dose of γ radiation.

Immunogen Preparation. A modification of a coupling method described elsewhere (9) was used. One milligram of RC-160 in 1.0 ml of 0.01 M acetic acid was mixed with 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 5 mg of bovine serum albumin (BSA; Sigma, A 7888); 325 µl of 0.02 M glutaraldehyde (Sigma, G 5882) was then added with constant stirring. After 4 hr of stirring, 0.5 ml of 0.1 M sodium

Abbreviations: BSA, bovine serum albumin; Bₚ, percent of total added radiolabeled ligand bound to antibody in the absence of unlabeled ligand; IGF-I, insulin-like growth factor I (somatomedin C); LH-RH, luteinizing hormone-releasing hormone (luteinizing)
phosphate buffer (pH 7.0) and 3.0 ml of complete Freund's adjuvant (GIBCO, 660-5721) were added. The entire mixture was then emulsified.

**Immunization.** Two female New Zealand White rabbits were injected with freshly conjugated and emulsified BSA-peptide/Freund's adjuvant mixture. The inoculum (equivalent to 1 mg of RC-160 per rabbit) was injected intramuscularly, intraperitoneally, and subcutaneously. Booster preparations, composed of incomplete Freund's adjuvant (GIBCO, 660-5700) emulsified with 0.23–0.35 mg of RC-160 and 1.15–2.5 mg of BSA, were given after 3 weeks and thereafter monthly. The animals were bled prior to initiation of immunization and 2–3 weeks after each booster injection.

**Radioiodination.** RC-160 was iodinated by a modification of the chloramine-T method (10). To 5μg of RC-160 in 20μl of 0.1 M acetic acid were added sequentially 40μl of 0.5 M sodium phosphate buffer (pH 7.6), 1 μCi (37 MBq) of Na125I, and 10μg of chloramine T in 10μl of 0.05 M sodium phosphate buffer (pH 7.6). After 15 sec, the reaction was terminated by adding 100μl of a 25% (wt/vol) solution of human serum albumin.

**Purification of Labeled RC-160.** Labeled hormone was separated from free iodine by gel filtration on a 1×20-cm column of Sephacryl S-500 (Pharmacia) with 0.1 M acetic acid containing 0.25% BSA as eluant. One-milliliter fractions were collected.

**RIA Procedure.** Borosilicate glass tubes (Scientific Products, 12×75 mm) were used. The assay buffer consisted of 0.025 M EDTA, 0.01 M sodium phosphate (pH 7.6), 0.14 M NaCl, 0.35% human serum albumin, 0.88 unit of aprotinin per ml, and 0.4% 2-mercaptoethanol.

**Standards.** Aliquots of a solution of RC-160 (1μg/5μl of 0.1 M acetic acid) were pipetted into tubes, stored at −70°C, and diluted with buffer immediately before each assay. The range of the standard curve was 6.25–400 pg per tube.

**Assay Procedure.** The incubation mixture consisted of 100μl of standard, 350μl of assay buffer, and 50μl of pooled rat serum, or 450μl of assay buffer and 50μl of unknown serum; 10,000 cpm of 125I-labeled RC-160 in 100μl of assay buffer; and 100μl of antiserum to RC-160 (JH-426) at a working dilution of 1:12,250. The tubes were incubated at 4°C for 24 hr. Bound and free fractions were separated by a polystyrene glycol-facilitated double-antibody method. Five hundred microliters of 10% (wt/vol) polyethylene glycol (Mr, 3500) and 100μl of a 5% (vol/vol) solution of goat antiserum to rabbit γ-globulin (Antibodies, Inc.) were added per tube. After centrifugation at 2900 x g for 20 min at 4°C, the supernatants were decanted and the radioactivity of the precipitates was measured in a 10-detector γ scintillation counter (Micromedic Systems, Horsham, PA). All samples were assayed in duplicate.

**RIA Validation.** Assay sensitivity, specificity, accuracy, and precision, as well as the effects of the presence of serum in the assay system, were evaluated. The stability of the peptide in serum to repeated freeze–thaw cycles and to exposure to elevated temperature was also studied.

**Sensitivity.** Twenty replicates of the zero standard were assayed in one assay run.

**Specificity.** Various analogs and fragments of somatostatin were assayed at concentrations varying by 4 orders of magnitude. Several other peptide hormones were also tested.

**Accuracy.** Fractional-recovery studies were performed with rat and human serum by adding RC-160 to a pool of serum at various dose levels and assaying the mixture. Dilutional parallelism was evaluated by making serial dilutions of a serum pool containing approximately 250 pg of RC-160 per 100 μl.

**Precision.** Ten replicates of pools of rat serum containing RC-160 at three levels (low, medium, and high pools) were assayed in one run to determine intraassay precision. Three replicates of each pool were also assayed in five assay runs to evaluate interassay precision.

**Effect of serum.** Various amounts (10, 25, or 50 μl) of rat serum were added to the standard-curve mixtures to test the effect of serum volume in the assay system.

**Stability of peptide.** A serum pool was subjected to five freeze–thaw cycles and assayed for RC-160 activity. A similar pool was assayed after incubation at 37°C for 15 min, 30 min, or 1, 2, or 3 hr.

**Experiments in Rats.** Adult male Sprague–Dawley rats received single intramuscular injections of various batches of RC-160 microcapsules. The microcapsules, in 25- to 50-mg portions calculated to release approximately 50 μg per day for 30 days, were suspended in disposable syringes in 0.7 ml of injection vehicle [2% (wt/vol) CM-cellulose and 1% (vol/vol) Tween 80 in distilled water] (7, 8). The suspension was mixed thoroughly on a Vortex mixer and injected through an 18-gauge needle into the thigh muscle. Control rats were injected with the vehicle alone. Six to eight rats were used per group.

Blood samples of about 0.5 ml were taken from the jugular vein daily for the first 7 days and periodically thereafter for 20–40 days after the injection. The serum was collected and stored frozen at −20°C until assayed for RC-160.

Some serum samples were also assayed for IGF-I by a modification of the method of Daughaday et al. (11). Five microliters of rat serum was extracted with 200 μl of 0.3 M HCl in ethanol. The supernatant was diluted with 0.2 M Tris buffer (pH 7.4), and an aliquot equivalent to 0.2 μl of serum was taken for the assay. IGF-I (a gift from C. H. Li) was iodinated by the chloramine-T method and purified by HPLC; the same antigen was used for the standard curve at concentrations from 16 to 4000 pg per tube. Rabbit anti-IGF-I antiserum (provided by the National Hormone and Pituitary Program) was used at an initial dilution of 1:4000. The reactants were incubated overnight at room temperature, and bound and free antigens were separated by a polystyrene glycol-facilitated double-antibody method.

Statistical evaluations of all data were made with Duncan’s new multiple range test (12).

**RESULTS**

**Antiserum.** Of the two rabbits immunized, only one produced a useful antiserum. The sixth bleeding of this animal yielded an antiserum (JH-426) with a titer of 1:99,750 (final dilution), suitable for use in the RIA. At this dilution, the antibodies could bind 25% of added labeled antigen (B0).

![Fig. 1. Representative standard curve of the RC-160 RIA.](image-url)
Radioiodination. By using the chloramine-T method to iodinate RC-160, we were able to achieve an average specific activity of 351 μCi/μg (range, 281–460 μCi).

Purification of Labeled RC-160. The fractions of the portion of the peak containing the [125I]-labeled RC-160 eluted from the Sephadex G-50 column [elution volume/void volume (V/Vo) = 4.85] were pooled and stored at 4°C. Under these conditions, the labeled antigen was found to be stable for at least 3 weeks with no apparent decrease in immunoreactivity. Repurification of the [125I]-labeled RC-160 during this interval was found to be unnecessary; however, further purification by HPLC using a C18 column (Waters Associates) and a 0.1% trifluoroacetic acid/acetonitrile mixture phase could be used to improve the specific activity and immunoreactivity of the labeled peptide.

RIA Characteristics. A typical standard curve (Fig. 1) showed these characteristics: nonspecific binding, 10%: Bso, 28.7%; slope, −0.99475; ED20, 463 pg; ED50, 113 pg; ED80, 28 pg (ED20, ED50, and ED80 are the effective doses measured at 20%, 50%, and 80% of the Bso value). The interassay coefficient of variation for the standard curve was 9.2% (n = 10).

RIA Validation. Sensitivity. The minimal detectable dose of RC-160 was found to be 10 pg per tube, based on the dose estimated by the 95% confidence limits for the cpm of the zero standard (n = 20).

Specificity. The specificity of the antisera is given in Table 1. RC-121 showed a crossreactivity of 100%. Other octapeptide analogs that have the disulfide bridge, including Sandoz analog SMS-201995, reacted with the antisera to a lesser degree. The fragments of RC-160 without the disulfide bridge and other peptides including somatostatin and Veber analog 1 (13) only had 0.005–1% crossreactivity. Substitution of phenylalanine for tyrosine at position 3 and of threonine for valine at position 6, as in analog RC-102, reduced the crossreactivity (Table 1). The heptapeptide fragment of RC-98-I or RC-102, without the N-terminal amino acid, showed 24% crossreactivity.

Table 2. Recovery of RC-160 added to rat or human serum and assayed directly

<table>
<thead>
<tr>
<th>Serum</th>
<th>RC-160, pg</th>
<th>Recovery, %</th>
<th>Code no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Added</td>
<td>Recovered</td>
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</tr>
<tr>
<td>25</td>
<td>30.6</td>
<td>122</td>
<td>RC-160</td>
</tr>
<tr>
<td>50</td>
<td>59.2</td>
<td>118</td>
<td>RC-121</td>
</tr>
<tr>
<td>100</td>
<td>136.8</td>
<td>136</td>
<td>RC-98-I</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>35.0</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>61.0</td>
<td>122</td>
<td></td>
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</tbody>
</table>

Accuracy. In the fractional-recovery study (Table 2), the recovery of RC-160 from rat serum was 118–136%, and from human serum it was 122–140%. In the dilutional-parallellism study (Fig. 2), good parallelism was demonstrated between the regression line for the RC-160 standard and the regression line for the RC-160-containing serum.

Precision. The precision study (Table 3) showed intrassay coefficients of variation that ranged from 9% to 12%. Interassay variance ranged from 14% to 30%.

Effect of serum. The addition of 50 μl of serum to the incubation mixture produced a shift to the right in the standard curve and a decrease in Bso (Fig. 3).

Stability of peptide. There were no significant differences in the mean levels of RC-160 measured in pooled serum (52.0 ± 7.3 pg, mean ± SD, n = 10), after five freeze–thaw cycles (46.4 ± 13.3 pg, n = 10) or after incubation at 37°C for up to 3 hr (52.5 ± 9.2 pg, n = 10).

RC-160 Levels After Administration of Long-Acting Microcapsules to Rats. Eleven batches of microcapsules containing RC-160 have been evaluated for release of the peptide by monitoring serum levels over a period of several weeks in rats injected with these microcapsules. In several of the batches tested, there was a pronounced "burst" effect, with large amounts of peptide being released from the microcapsules during the first 3–4 days after injection. Serum levels of RC-160 then declined from these peak values, reaching nearly...
Table 3. Intra- and interassay variance of the RIA

<table>
<thead>
<tr>
<th>Serum pool</th>
<th>RC-160 measured, pg (mean ± SD)</th>
<th>Coefficient of variation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraassay</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>46.3 ± 4.5</td>
<td>9.7</td>
</tr>
<tr>
<td>Medium</td>
<td>66.8 ± 8.5</td>
<td>12.8</td>
</tr>
<tr>
<td>High</td>
<td>278 ± 25.2</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Interassay</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>29.4 ± 8.9</td>
<td>30</td>
</tr>
<tr>
<td>Medium</td>
<td>52.0 ± 7.3</td>
<td>14</td>
</tr>
<tr>
<td>High</td>
<td>300 ± 55</td>
<td>18.3</td>
</tr>
</tbody>
</table>

undetectable levels by days 5–16. Thus, in the case of earlier batches, therapeutic serum levels of RC-160 were maintained for only 4–15 days. This burst effect was diminished but not eliminated in later batches by modifying the polymer. The use of RC-160 pamoate instead of acetate further reduced the initial high release of the analog. Serum levels of RC-160 in rats injected with batch no. 11 of microcapsules remained in the range 600–2000 pg/ml for 30 days after injection and then gradually declined (Fig. 4). Thus, for this improved batch (no. 11), made with RC-160 pamoate, the therapeutic levels of the analog were maintained for about 1 month. No immunoreactivity related to RC-160 was detected in the serum of the control rats. Serum IGF-I (somatomedin C) levels declined 1–2 days after the injection and remained suppressed for at least 45 days.

DISCUSSION

The development of long-acting delivery systems for peptide hormones must be accompanied by the development of sensitive and specific techniques for monitoring serum levels. The RIA for RC-160 described here fulfills the criteria for such a technique.

Since RC-160 is a small peptide, it can be bound to macromolecular components of serum as are LH-RH and its analogs (14, 15). Therefore, to measure RC-160 in serum requires strategies designed to overcome interference in the RIA produced by those peptide-binding proteins. There are several possible approaches to this problem. The first of these is extraction, in which ethanol or methanol is used to precipitate serum proteins, followed by assay of the peptide in the evaporated and reconstituted alcoholic extract. This did not prove feasible for use in this RIA, as RC-160 is not easily soluble in neutral-buffered solutions, making reconsti-

ution of the extract difficult and resulting in low recoveries (data not shown). The second strategy involves the use of a small sample volume to reduce the interference from serum binding proteins. This method was successfully used in our RIA for the d-Trp₆ analog of LH-RH (15), but the sensitivity of the RC-160 RIA was not sufficient to permit its use. The third approach to solving this problem consists of adding an aliquot of pooled serum, equivalent to the volume of the unknown samples, to the standard-curve tubes. This is the most common method used to overcome this problem in the case of commercially available RIA kits that permit “direct” assay of steroids, various drugs, or small peptides (16). This tactic is useful only if the pooled serum is free of crossreacting peptides. As RC-160 is an exogenous peptide, we were not concerned with interference from endogenous materials in serum. In addition, there was virtually no crossreactivity with somatostatin or other naturally occurring peptides. Therefore, we chose to use this method for our RIA system.

The rates of release of RC-160 from the earlier batches of
microcapsules were uneven, and a marked fall in the serum levels of the peptide occurred earlier than the targeted 30-day interval. The release pattern of the improved batch (no. 11) showed the liberation of acceptable serum levels of RC-160 in a continuous fashion over the time period studied (Fig. 4). The patterns of release were similar to those seen with related formulations of an analog of LH-RH (15). The serum levels of the peptide occurred earlier than the discontinuous administration (Fig. 4). Diminished IGF-I levels reflect suppression of pituitary release of growth hormone (17) and have also been seen in previous studies with RC-160 (5). The present formulation of the microcapsules, which can be conveniently injected at monthly intervals, should have a higher efficacy than discontinuous administration and would ensure patient compliance. This use of microcapsules might allow the establishment of continuous therapeutic levels of RC-160 and permit new approaches in the management of pancreatic cancer and other malignancies and conditions (6, 18, 19). Microcapsules of RC-160 could also be tried in combination with LH-RH agonists for the treatment of breast and prostate cancer (5, 6, 18).

We thank the National Hormone and Pituitary Program for the gift of the antiserum used in the IGF-I RIA, and we thank Dr. Valer Csernus for performing the IGF-I RIA. The late Prof. C. H. Li supplied IGF-I. The work described in this paper was supported by National Institutes of Health Grants DK07467 and CA40077 (to A.V.S.) and by the Medical Research Service of the Veterans Administration.