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Report

Development of a Sensitive Activity Assay for High-Volume Evaluation of Human Renin Inhibitory Peptides in Rat Serum: Results with U-71,038

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A sensitive activity assay for high volume evaluation of human renin inhibitory peptides (RIPs) in rat sera (range 2–80 ng/ml) was developed based on the low affinity of RIPs to rat renin and their high affinity to human renin. The utility of this activity assay was tested by measuring concentrations of a human RIP, U-71,038 (BOC-Pro-Phe-N-MeHis-Leu Ψ [$CHOHCH_2$]Val-Ile-Amp), in rat sera, determined by the activity assay, by a sensitive radioimmunoassay (RIA), and by tracking tritiated drug. Rats were given radiolabeled drug as an intravenous bolus, and blood samples were collected at various times after dosing. The serum level of U-71,038 equivalents was determined by the three techniques. Whole blood was also counted for total radioactivity to evaluate the potential for U-71,038 incorporation into red blood cells. Results from the three serum assays indicate good agreement between the calculated U-71,038 equivalents for the 30 min and 1 hr collection times. The 2 and 4 hr collection times show excellent agreement for the activity assay and RIA; [^{3}H]-U-71,038 determinations gave substantially higher values. Serum levels for U-71,038 determined 30 min after dosing averaged less than 300 ng equivalents/ml suggesting that less than 1% of the administered dose was in the systemic circulation at that time. Thus, U-71,038 was rapidly cleared. At the 4 hr collection time, the level of U-71,038 equivalents, as determined by activity assay and RIA, was ten times the in vitro IC_{50} for the renin inhibitory activity of U-71,038. Analysis of whole blood levels of ^{3}[H]-U-71,038 indicated little or no incorporation of drug related material into red blood cells. In addition to predicting pharmacological response, the activity assay can be used to quantify human RIPs in rat serum when biotransformation is absent.

KEY WORDS: human renin inhibitory peptide; rat; activity assay; angiotensin I; serum.

INTRODUCTION

Human renin (EC 3.4.99.19) is a physiologically important aspartyl protease that catalyzes the formation of angiotensin I from angiotensinogen (1) which is the rate-determining step in the biogenesis of angiotensin II (ANG II). The octapeptide ANG II, produced by this cascade, has been shown to be involved in blood-pressure regulation and electrolyte balance (2–4). Inhibitors of angiotensin-converting enzyme, which catalyzes the second enzymatic step in the production of ANG II, are being synthesized and tested *in vitro* (5–10).

RIPs which are effective against human renin tend to be manyfold less active against the renin from rat and other common laboratory species (11). Therefore, *in vivo* evaluation of promising agents has been restricted to primate models (12) or, more recently, to the hog-renin infused rat model (12), which can measure their hypotensive effects. Quantification of serum levels of RIPs effective against human renin in common laboratory animals has, until now, required time-consuming development of specific HPLC, radioimmunoassay, or radiolabeling methods for each new compound. Consequently, very few RIPs can be evaluated. These cumbersome, time-consuming blood-pressure models and quantification techniques have greatly retarded the development of a RIP for clinical treatment of hypertension.

This paper describes a simple activity assay for the detection of human RIPs in rat sera. This assay is based on the low affinity of these peptides for rat renin and their high affinity for human renin and utilizes previously reported improvements (13) in the *in vitro* methodology for measuring renin activity. It allows high-volume evaluation of RIP analogues in small laboratory animals and should, therefore,

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accelerate development of these compounds into useful pharmaceutical agents.

MATERIALS AND METHODS

Compounds

U-71,038 (ditekiren: Boc-Pro-Phe-N-MeHis-LeuΨ-[$CHOHCH_2$]Val-Ile-AMP) was synthesized at The Upjohn Company as previously described (14) and had a chemical purity of ≧99%.

[Prolyl-^{3}H]U-71,038 was prepared by New England Nuclear Research Products [Boston, MA; sp act, 37.3 mCi/μmol; radiochemical purity (by HPLC), 98.6%] by incorporating Boc-[3,4-^{3}H]proline during the synthesis.

Formulation

[^{3}H]U-71,038 was combined with unlabeled U-71,038 to prepare the formulation for dosing the rats. The final solution contained 0.001 *M* citric acid, 0.8% sodium chloride, and 4.7% ethanol with a U-71,038 concentration of 511.2 μg/ml and a specific activity of 48.3 μCi/μmol. The concentration was determined using an HPLC stability indicating assay (15) which quantifies the concentration of U-71,038. The specific activity was measured with scintillation counting. Aliquots of the formulation were distributed to the various assay laboratories for preparation of standard curves for calculation of the concentration of U-71,038 equivalents in dosed animals.

Assays

Determination of Drug-Related Radioactivity in Rat Blood and Serum

Blood and serum specimens collected from rats at various times after intravenous administration of [^{3}H]U-71,038 were counted on a Packard Tri-Carb 2000CA after combustion using a Packard D-306 oxidizer. The efficiency of recovery after combustion of serum samples (100 μl) was calculated using standard ^{3}H-pellets oxidized with the samples. The radioactivity in the samples was converted to nanogram equivalents per milliliter using the appropriate counting efficiency, the specific activity (nCi/ng), and dilution factors. Approximately 20 ng U-71,038 equivalents/ml could be quantitated.

Radioimmunoassay of U-71,038 in Rat Serum

Preparation of Antisera. U-71,038, a small peptide, required conjugation to a larger protein prior to immunization. Since U-71,038 has no functional end groups for attachment to a carrier protein, the carboxyterminal aminomethylpyridine (AMP) of U-71,038 was removed, leaving a free carboxyterminus on isoleucine. This compound was conjugated to bovine serum albumin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Mature New Zealand white rabbits were immunized every 6 weeks. Serum was harvested 11 days after the fifth immunization and used for optimal determination of assay characteristics. The antiserum did not cross-react with U-71,038 analogues that had *N*-terminal modifications; C-terminal analogues were unavailable.

Iodination of U-71,038. The histidine of U-71,038 was iodinated with Na[^{125}I] using Pierce Iodo-Beads incubated for 15 sec on 0.2 *M* sodium phosphate buffer (pH 7.0). The reaction was terminated by the addition of 0.1% trifluoracetic acid (TFA) in water and the immediate separation of the reaction mixture from the Iodo-Bead. The free iodine and incorporated iodine were separated by passing the reaction mixture through a Waters C-18 Sep-Pak column and washing with 10 ml of 0.1% TFA in water. The labeled and unlabeled peptide was eluted off the Sep-Pak with 3 ml of 0.1% TFA in 70% acetonitrile/30% water. Labeled U-71,038 was then separated from unlabeled compound by reversed-phase HPLC.

Analysis of Sera. Serum samples were diluted with control rat sera whenever U-71,038 concentrations were higher than the upper limit of the assay. Equal volumes (100 μl) of acetonitrile were used to precipitate interfering proteins. The supernatant was dried and reconstituted with 100 μl of phosphate-buffered saline, then incubated overnight at 4–8°C in the presence of [^{125}I]U-71,038 and rabbit antisera. Goat anti-rabbit IgG antiserum was used to precipitate the rabbit antibody by overnight incubation at 4–8°C. The reaction mixture was diluted with saline and centrifuged at 4–8°C. The supernatant was decanted, and the sedimented material counted. The RIA had a linear range of 0.2–20 ng/ml U-71,038 in sera and intra- and interassay coefficients of variation of 19.4 and 2.3%, respectively.

Activity Assay

The concentration of U-71,038 in rat sera was determined by adding 20-μl aliquots to 250 μl of incubation buffer (0.15 *M* sodium phosphate, 3 m*M* EDTA, 0.16 *M* NaCl, 0.1% bovine serum albumin, pH 6) and 10 μl of phenylmethylsulfonyl fluoride prepared from a Gammacoat [^{125}I] plasma renin activity radioimmunoassay kit (Dade-Baxter-Travenol Diagnostics, Inc). Recombinant human renin (0.04 μg; Ref. 16) was then added, followed by 50 μl of processed human plasma substrate (i.e., the lyophilized "renin activity control" reconstituted with 15 ml of water). The reaction mixture was incubated at 37°C for 1 hr. The amount of angiotensin I produced was measured by radioimmunoassay and compared to a standard curve generated from nonlinear fitting of data obtained with solutions of known concentrations of U-71,038. The activity assay had intra- and interassay coefficients of variation of 13.2 and 11.1%, respectively.

Animal Study

Twelve fasting female Upjohn Sprague-Dawley rats (250 g) were anesthetized with sodium pentobarbital (35 mg/kg, i.p.). [^{3}H]U-71,038 (2 mg/kg) was administered intravenously over 1 min to six rats through the tail vein. Blood samples were collected through a capillary tube from the orbital sinus at 0.5, 1, and 2 hr, and another sample was obtained at 4 hr when the animals were exsanguinated at sacrifice. Aliquots of whole blood (50 μl) from each collection period were pipetted into combustor cones for isotope determination by sample oxidation.

Serum was prepared by centrifuging whole blood at 1500*g* for 5 min. A glass ball was then added to the tubes to

aid in the separation of the serum; samples were recentrifuged at 1500*g* for 15 min. Serum was aliquoted for the activity assay and for the RIA and stored frozen at −80°C prior to analysis. Aliquots of serum (100 μl) were pipetted into combustor cones for isotope determination.

RESULTS

The standard curve for the activity assay (Fig. 1) had an acceptable linear range, which allowed quantitative detection of U-71,038 from spiked serum samples (Table I).

Throughout the time course of the study, the activity assay and RIA gave virtually identical results (Fig. 2). The results from the radiolabel paralleled the results of the other two assays for the first hour. Thereafter, blood levels of U-71,038 determined by radiolabel declined more slowly than the other two assay values had indicated. The level of radiolabel in whole blood was approximately one-half that of serum. Since approximately half of the whole blood volume is serum, there was probably little, if any, RIP associated with the red blood cells.

DISCUSSION

The activity assay is based on the observation that renin inhibitors are species specific (11). Since rat renin is not expected to bind RIPs that interact with human renin, rat serum may be considered an inert vehicle for these peptides. Thus, the rat sera can be added directly to the reaction mixture without an extraction, isolation, or other purification step (Table I).

The serum data (Fig. 2) shows excellent agreement for all three assays during the first hours of sampling, providing strong evidence that all three methods measure parent drug during that period. A metabolite would be likely to show different cross-reactivity to the RIA antibody, different renin suppression in the activity assay, or different clearance properties to alter the serum levels of isotope. These results are consistent with earlier observations that 95% of an intravenous dose of U-71,038 is excreted essentially intact (17). However, drug related material in the serum at 2- and 4-hr collections is higher when estimated by isotope recovery than the values obtained by activity assay or RIA. Since both these amounts represent less than 0.1% of the administered dose and the readiochemical purity was 98.6%, this difference may be due to either the presence of a radiochemical impurity which has different disposition characteristics

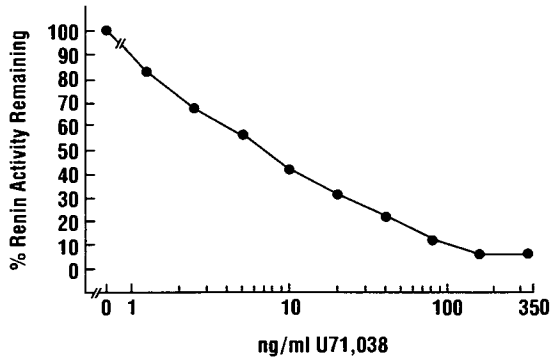

Fig. 1. Standard curve of U-71,038 in the activity assay.

Table 1. Recovery of U-71,038 from Spiked Serum Samples

Spike (ng/ml)	Activity assay % recovery
1.25	92.6
2.50	95.1
5.00	94.2
10.00	98.6
20.00	99.3
40.00	97.6
80.00	101.6
160.00	104.7
320.00	101.2
640.00	97.8
$\bar{X}$ ± SE	98.3 ± 1.2

than [^{3}H]U-71,038 or a small amount of U-71,038 which has been biotransformed to less active species (e.g., amino acids, free radiolabel). The excellent agreement between the activity assay and RIA levels indicates that the activity assay can be used to quantitate RIPs that do not generate active metabolites.

In addition to the serum level determinations, whole-blood samples were analyzed at each collection time to provide preliminary information on the potential for drug related material to be incorporated into red blood cells. Figure 2 illustrates parallel radiolabel curves for blood and serum, with the blood level about one-half the serum level. These data suggest that U-71,038 is not extensively incorporated into circulating cells and that serum level determinations will provide a direct measure of the circulating RIP.

Results from this study can also be used to provide preliminary information on the disposition and clearance of drug-related material in the rat. A total dose of 500 μg U-71,038 was administered intravenously; the level of drug-related material at 30 min was between 200 and 300 ng/ml as determined by the three assay methods. The rats in this study weighed about 250 g and, therefore, had a serum volume of approximately 10 ml (18). Thus, between 2 and 3 μg of U-71,038, or less than 1% of the administered drug, was

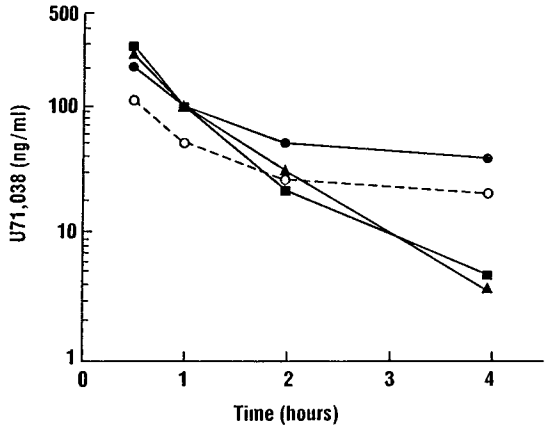

Fig. 2. Mean values of U-71,038 in collected rat sera and blood. Activity assay (serum), ▲; RIA (serum), ■; radiolabel (serum), ●; and radiolabel (blood), ○. SE (ng/ml) for these respective assays were 30 min (9, 38, 18, 8), 60 min (4, 16, 13, 8), 120 min (4, 4, 6, 3), and 240 min (1, 4, 2, 2).

present in the serum 30 min after dosing, suggesting that the drug is rapidly cleared from systemic circulation. The fate of U-71,038 once removed from the circulation is the focus of continuing studies.

The serum level of active drug at 4 hr was 2–4 ng/ml or 10 times the *in vitro* IC_{50} for the inhibition of human renin by U-71,038 (14). The ability to detect a 2 mg/kg dose after 4 hr suggests that the activity assay is a more sensitive method of detecting systemic RIPs than the hypotensive effects in the hog-renin infused rat. In that model, blood-pressure lowering effects of a 5 mg/kg dose of U-71,038 had dissipated within 1.5 hr of administration (12).

The activity assay, therefore, has several advantages over the traditional blood-pressure models and HPLC/RIA detection techniques for evaluation of RIP analogues. Like the blood-pressure models, the activity assay reflects the ability of the RIPs and their metabolites to lower renin activity; however, the activity assay has better sensitivity and does not require surgical intervention (i.e., nephrectomy, jugular cannulation). Since many RIPs, unlike naturally occurring peptides, are constructed to survive degradation in the gut, they are frequently metabolically stable also, allowing the activity assay to be used for quantification without necessitating novel RIAs or HPLC methodology for each RIP. Thus, the activity assay is a high-volume alternative for laboratories involved in frequent evaluation of RIP analogues.

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