In Vitro Evaluation of the Plasma and Blood Compatibility of a Parenteral Formulation for Ditekiren, a Novel Renin Inhibitor Pseudopeptide

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Ditekiren (U-71038; Boc-Pro-Phe-*N*-MeHis-Leu-Ψ[CHOHCH$_2$]-Val-Ile-(aminomethyl)pyridine) is a potent renin inhibitor peptide and was formulated for clinical intravenous administration in acidified dextrose. This formulation of ditekiren was evaluated *in vitro* with human and monkey plasma as to its potential for forming a precipitate either of drug or of plasma proteins. Analysis by centrifugation showed that no drug precipitation occurred in plasma from either species at concentrations 25 times higher than anticipated in clinical studies. Results obtained by turbidimetry indicated that formulated ditekiren did not cause aggregation of human platelets or flocculation of proteins at concentrations approaching the solubility limit of the drug in plasma. Ditekiren or vehicle also caused no detectable lysis of red cells at concentrations representing 10 times the maximum clinical level. Therefore, ditekiren solutions as formulated are judged completely compatible with blood and plasma upon clinical intravenous administration.

KEY WORDS: ditekiren; hemolysis; plasma compatibility; platelet aggregation; precipitation; renin inhibitor peptide.

INTRODUCTION

Ditekiren (U-71038; Fig. 1) is a highly potent inhibitor of human renin which has been demonstrated to exert prolonged hypotensive effects in animal pharmacologic models after both intravenous and oral administration (1). This compound, which is a transition-state analogue of the natural peptidic human renin substrate (2), is resistant to enzymatic hydrolysis *in vivo* (3) and is presently being clinically tested for the treatment of hypertension (4,5). Prior to initiating clinical studies involving the intravenous administration of this drug, a series of *in vitro* tests was undertaken to evaluate the compatibility of a parenteral formulation of ditekiren with plasma and erythrocytes. The potential for parenteral ditekiren to form precipitates of drug or protein when mixed with plasma, to effect the aggregation of platelets, and to induce hemolysis of red cells was evaluated for this pseudopeptide over a range of concentrations.

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MATERIALS AND METHODS

Materials

Ditekiren (Boc-Pro-Phe-*N*-MeHis-Leu-Ψ[CHOHCH$_2$]Val-Ile-(aminomethyl)pyridine) was synthesized at The Upjohn Company and had a chemical purity of ≥99%. [*Prolyl*-^{3}H]Ditekiren (Fig. 1) was prepared at NEN Research Products (Boston, MA) by incorporating Boc-[3,4-^{3}H]proline during the synthetic route developed by Thaisrivongs and co-workers (6). The labeled drug was purified at Upjohn to 98.3% radiochemical purity (HPLC) and had a specific activity of 35.2 mCi/mg.

A formulation of unlabeled ditekiren was prepared by dissolving 1000 mg of the drug in 89 ml of 5% dextrose solution (Injection, U.S.P.) to which had been added 1.92 ml of 1.0 *M* HCl. The resulting solution was diluted to 100 ml with water, giving a drug concentration of 10 mg/ml in 4.45% dextrose containing 0.0192 *M* HCl. This nearly isotonic solution was diluted with 5% dextrose to prepare solutions containing 5, 4, 2, 1, and 0.2 mg/ml. A placebo formulation with a buffering capacity similar to that of the 10-mg/ml ditekiren solution, but representing 0.0 mg/ml (placebo), was prepared by dissolving sodium acetate trihydrate (2.86 mg/ml) in the same vehicle. This placebo stock solution was diluted with isotonic saline or dextrose to prepare vehicle solutions having buffering capacities analogous to diluted ditekiren solutions. The pH of the 10-mg/ml ditekiren and placebo solutions was 3.3. [^{3}H]Ditekiren solution was separately prepared by dissolving in the same dextrose/HCl vehicle at a concentration of 61.4 μg and 2.16 mCi per ml.

Parenteral phenytoin solution (phenytoin sodium injection, USP; Dilantin; Parke-Davis, Morris Plains, NJ) as a 50-mg/ml aqueous solution containing 40% propylene glycol and 10% ethanol, v/v (pH adjusted to 12 with NaOH), was obtained commercially.

Citrated fresh human plasma (American Red Cross, Lansing, MI) was pooled and pressure filtered (GF/F glass microfiber filter, 0.7 μm, Whatman Inc., Clifton, NJ), and the pH adjusted to 7.4 with 0.2 *M* HCl. Blood was collected from four cynomolgus monkeys, *Macaca fasicularis*, by venipuncture into syringes containing citrate phosphate dextrose anticoagulant solution (14 ml per 100 ml of blood) (7). Plasma was harvested by centrifugation for 30 min at 1200*g*. The pooled plasma was then filtered and the pH adjusted as described above.

Fresh human blood was collected from healthy volunteers by venipuncture and treated with sodium citrate (0.38%, w/v) to prevent coagulation. Platelet-rich human plasma was prepared by centrifuging fresh blood for 10 min at 200*g*. The resulting platelet count was found to be 2.6 × 10^5 platelets mm^3 of plasma.

Solubility Measurements in Plasma and Buffer

To 10 ml of plasma or isotonic phosphate-buffered saline (8) in a glass culture tube was added 1.0 ml of formulated ditekiren as well as an aliquot (50 μl) of the corresponding radioactive drug in vehicle. The mixture was thoroughly vortex agitated and then incubated for 30 min at 37°C. Immedi-

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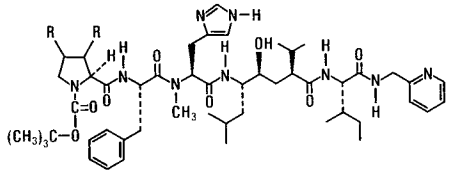

Fig. 1. Structure of ditekiren (R = H) and structure of [^{3}H] ditekiren, showing position of radiolabel (R = ^{3}H].

ately after reagitation, duplicate aliquots (200 μl) were placed in scintillation vials while a second set of duplicate aliquots (1.0 ml) was transferred to polypropylene microcentrifuge tubes and centrifuged for 5 min at 16,000g (Eppendorf Model 5415 microcentrifuge, Brinkmann Instruments, Westbury, NY). Aliquots (200 μl) of the supernatant were withdrawn, diluted with an equal volume of water, and assayed for radioactivity by liquid scintillation spectrometry (external standardization employed for quench correction).

Determination of Plasma Protein Precipitation and Platelet Aggregation

Samples (0.90 ml) of plasma, buffered saline, or platelet-rich plasma were incubated at 37°C and magnetically stirred (1000 rpm) in a glass cuvette of a Payton Aggregation Module (Payton Associates, Inc., Buffalo, NY). The attenuation of the instrument was adjusted to 50% transmittance to allow observation of either increasing or decreasing intensity of light passing through the cuvette. Aliquots (0.10 ml) of formulated ditekiren or its vehicle, formulated phenytoin (50 mg/ml), or aqueous trichloroacetic acid (10%, w/v) were then added and any changes in transmittance were monitored by a strip-chart recorder for 5 min.

Assessment of Potential Hemolytic Effects

To determine any alteration in the UV absorbance spectrum of hemoglobin induced by ditekiren, an aliquot of whole human blood (0.2 ml) was added to 5 ml of deionized water to induce hemolysis. After centrifugation of the hemolysate at 1300g for 5 min, an aliquot of the supernatant (0.5 ml) was combined with 2.5 ml of a formulated ditekiren solution (2.0 mg/ml). The ultraviolet absorption was scanned from 450 to 650 nm and the resulting spectrum was compared to that of hemoglobin exposed to placebo solution.

The hemolytic potential of the ditekiren and placebo formulations was assessed using methods previously described (9,10). Specifically, an aliquot of human whole blood (0.2 ml) was mixed with 5.2 ml of formulated drug or placebo having a concentration range of 0–2.00 mg/ml. The resulting suspensions were incubated at ambient temperature for 10 min and then centrifuged at 1300g for 5 min. Each supernatant was removed and its absorbance determined at 540 nm in a 1.0-cm cuvette. As negative control solutions (no hemolysis), whole blood (0.20 ml) was suspended in 5.2 ml of isotonic 0.9% saline and 5% dextrose solutions. As positive controls (complete hemolysis), aliquots of whole blood (5, 10, and 25 μl) were added to 5.4 ml of deionized water. The control solutions were then centrifuged and the absorbance read at 540 nm.

RESULTS AND DISCUSSION

Solubility of Ditekiren in Plasma and Buffer

Ditekiren (Fig. 1) is a renin inhibitory peptide which is currently under clinical evaluation for the treatment of hypertension. Ditekiren is weakly basic and was formulated in acidified dextrose solution at approximately pH 3.3 to prepare concentrations of up to 10 mg/ml. As the pH approaches physiologic or neutral values, the solubility diminishes rapidly to about 200 μg/ml in buffer at pH 7.4 at room temperature. This compound also exhibits an inverse solubility/temperature relationship, being somewhat more soluble in aqueous solutions at lower temperatures. Because of the limited solubility of ditekiren at physiologic pH and temperature, the potential exists that drug precipitation could occur as the formulation mixes with blood at the site of infusion. In one safety study in monkeys (11), intravascular drug emboli were observed at the higher dose levels and rates of infusion. In order to assess the possibility of formulated ditekiren to intravascularly precipitate during clinical tolerance trials, the solubility in human plasma, monkey plasma, and buffer was investigated *in vitro*.

We first estimated the maximum local ditekiren concentration expected in human blood at the site of infusion. The average arterial blood flow (12) to the human forearm is 3.1 ml/min per 100 ml of forearm volume. Since the average forearm volume is ca. 1200 ml, this represents a total venous forearm blood flow of 37 ml/min. In turn, the flows of individual antecubital veins can be estimated as about 12 ml/min by dividing the number of major veins across the elbow into the forearm blood flow. The maximum concentration of formulated drug to be used clinically is 0.2 mg/ml, which is to be infused at a maximum rate of 1.2 ml/min (5). Thus, at the site of infusion for each 1-min interval, 0.24 mg of drug will be mixed with a total fluid volume of about 13 ml (12 ml blood + 1.2 ml vehicle), giving an estimated maximum concentration of about 18.5 μg/ml. For defining a drug concentration range to be used in compatibility studies, this approximate concentration value was rounded upward to 20 μg/ml. Based on the above reasoning, the volume ratio of vehicle added to blood per minute would be forecasted as about 1.2:12 (1:10) at the maximum infusion rate.

To define and compare the limits of drug solubility in plasma and in isotonic buffer, solutions of radiolabeled ditekiren in the dextrose/HCl vehicle were mixed at increasing concentrations with pooled fresh human and monkey (cynomolgus; *Macaca fasicularis*) plasma and with isotonic phosphate-buffered saline, keeping the volume ratio of formulated drug to plasma or buffer constant at 1:10. After incubation (37°C for 30 min), the mixture was agitated to resuspend any precipitates, and then an aliquot was removed for radioactivity determination. Further aliquots were then centrifuged to remove any suspended material, and the radioactivity in the supernatant was compared to that prior to centrifugation. The results (average of experiments run in duplicate) of these solubility measurements are presented in Fig. 2. The change in ditekiren concentration after centrifugation is presented as a percentage of the original, uncentrifuged concentration. Data are presented for increasing drug concentrations in human plasma, monkey plasma, and buffered saline.

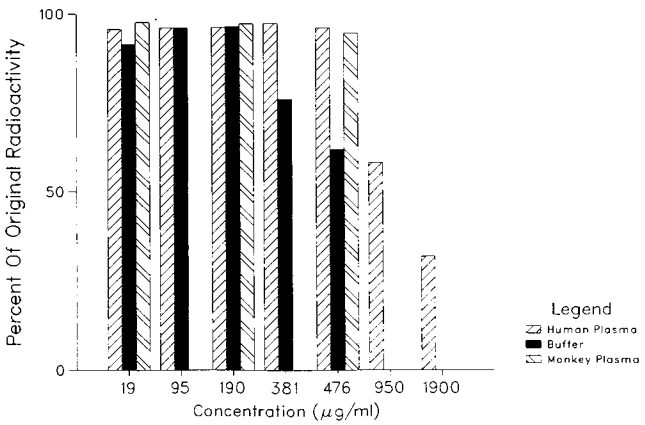

Fig. 2. Change in ditekiren concentration after centrifugation. An aliquot of formulated ditekiren was mixed (1:10, v/v) with human plasma, isotonic buffer, or monkey plasma and incubated at 37°C for 30 min. Each vertical bar represents an average of duplicates.

As shown in Fig. 2, in human plasma, no decrease in ditekiren concentration was observed at levels of 19 (approximate maximum concentration expected in blood), 95 (5 times the maximum), 190 (10× higher), 381 (20× higher), or 476 μg/ml (25× higher). At 950 (50×) and 1900 (100×) μg/ml, decreases in the original drug concentrations were observed, indicating that the solubility limit in human plasma lies approximately 25- to 50-fold higher than the maximum blood concentrations anticipated in clinical studies. Even at the 950 μg/ml level, ca. 60% of the drug remained soluble in human plasma. Therefore, from these data, intravascular precipitation of ditekiren would not be anticipated during clinical administration.

The solubility of ditekiren was found to be comparable in monkey and human plasmas. Therefore, the rates of infusion and drug concentrations in vehicle that were established as safe from preclinical studies (11) should also pertain to human administration. The observation (Fig. 2) that ditekiren is more soluble in plasma than in isotonic buffer at the same pH suggests that plasma proteins bind or help solubilize the drug in aqueous media.

The concentrations of ditekiren after centrifugation in polypropylene tubes were slightly less than 100% (Fig. 2). Previous experience with this drug (13) indicated a tendency to adsorb nonspecifically to some plastic surfaces and the 2–3% losses of the original concentrations were attributed to surface adsorption during the centrifugation procedure. To investigate the influence of adsorption during the method described above, control experiments were conducted by incubating spiked plasma or buffer in polypropylene tubes and letting stand without centrifugation; approximately 2–4% decreases in radioactivity were observed in these controls. This minor adsorptive loss appeared to be constant over the drug concentration range shown in Fig. 2 and accounts for the percentage values being slightly less than 100% at the lower concentrations.

Plasma Protein Precipitation

The degree to which formulations of ditekiren could possibly cause the precipitation or flocculation of proteins from plasma was studied by turbidimetry. In these experiments, formulated drug of increasing concentrations was mixed with either human or monkey plasma (keeping a constant volume ratio of 1:10 for formulated drug to the final mixture) and any changes in visible light transmitted through the sample were recorded continuously for 5 min. A decrease in light transmission would represent an increase in turbidity in the sample resulting from increased scattering of the incident light. The instrument used in these experiments was a turbidimeter designed to measure the aggregation of platelets in plasma samples. The instrument photoelectrically measures transmitted light intensity through a cuvette maintained at 37°C and stirred magnetically to keep particulates in suspension. Control experiments (data not shown) were conducted by adding formulated phenytoin (an example of a relatively insoluble drug) or aqueous trichloroacetic acid (a protein denaturant) to plasma. In both cases, a precipitate of either insoluble drug or protein was readily observed by the turbidimetric procedure.

Figure 3 presents the results obtained upon the addition of ditekiren formulations to plasma. In these studies, the instrumental setting for the optical density of the plasma was adjusted to 50%, and then the test substance was added. A setting of 50% (rather than 100%) allowed observation of both increased and reduced light transmission through the plasma. At final drug concentrations of 20, 200, or 500 μg/ml, either no change or a slight increase in incident light transmission was measured for both human and monkey plasmas, indicating no significant precipitate (either protein or drug) formed during the 5-min observation period after ditekiren addition. At 1000 μg/ml, however, an increased turbidity was observed, indicating that particulates had formed. Since the limit of solubility of ditekiren in plasma is between 476 and 950 μg/ml (see above), the particulates in the 1000-μg/ml solution most likely may represent drug precipitate. Therefore, it can be concluded from these data that neither significant protein precipitation nor flocculation occurred at ditekiren concentrations of up to 25-fold higher than the maximum levels anticipated in clinical studies (ca. 20 μg/ml).

Induction of Platelet Aggregation

Changes in turbidity upon the addition of formulated

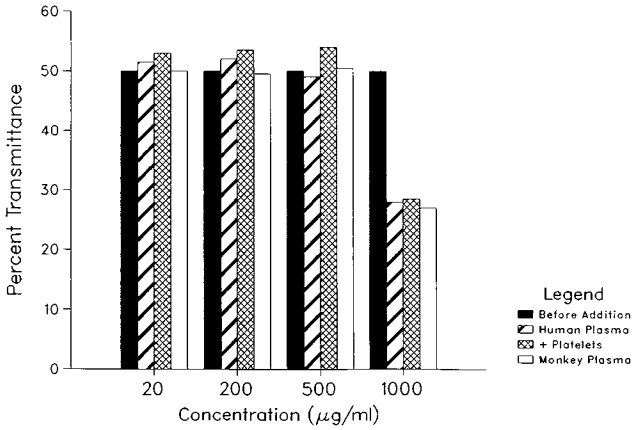

Fig. 3. Change in visible light transmission through plasma after addition of formulated ditekiren or placebo. Average of duplicates.

ditekiren to platelet-rich human plasma were observed for 5 min after mixing by the method described above and these results are also shown in Fig. 3. No aggregation was observed over the concentration range of up to 500 μg/ml. The slight increase in transmission observed in platelet-rich plasma was attributed to dilution of the platelet count after mixing. At a concentration level of 1000 μg/ml, the plasma containing platelets formed particulates, resulting in diminished light transmission of the same approximate magnitude as observed for platelet-depleted human or monkey plasma.

Hemolytic Effects of Formulated Ditekiren

Assessment of the potential for ditekiren solutions to cause hemolysis was based on measuring the ultraviolet absorbance at 540 nm to detect the release of hemoglobin from erythrocytes (9,10). The data are summarized in Table I. A control experiment (data not shown) indicated that the absorbance spectrum of hemoglobin was unaffected by ditekiren and vehicle over the range of drug concentrations that were employed in these investigations. A slight absorbance (ca. 0.03 AU) was observed with isotonic control solutions containing dextrose or sodium chloride after mixing with red cells which was attributed to blood protein absorbance. No significant increase in A_{540} was evident after mixing erythrocytes with ditekiren or placebo formulations with concentrations up to 1.0 mg/ml. Addition of a blood aliquot to both ditekiren and placebo solutions at 2.0 mg/ml caused an increase in absorbance, indicating that some rupture of red cells had occurred at this highest concentration.

Table I. Supernatant Absorbances (A_{540}) Following Exposure of Whole Blood to Ditekiren and Placebo Formulations[a]

	A_{540}
Negative controls	
5.9% dextrose	0.027
0.9% saline	0.034
Ditekiren solutions (mg/ml)	
0.01	0.026
0.05	0.023
0.20	0.028
0.50	0.036
1.00	0.041
2.00	0.166
Placebo solutions (mg/ml)	
0.01	0.031
0.05	0.032
0.20	0.028
0.50	0.038
1.00	0.037
2.00	0.076
Positive controls[b]	
25 μl whole + DI water	0.526
10 μl whole blood + DI water	0.216
5 μl whole blood + DI water	0.108

[a] Whole blood (0.2 ml unless stated otherwise) was combined with 5.2 ml test solution for 10 min and centrifuged. The supernatant absorbance represents mean values: controls, $n = 4$; drug and placebo solutions, $n = 2$.

[b] Positive controls were prepared by dilution of the indicated volumes of whole blood in deionized water to a total volume of 5.4 ml.

When increasing volumes (5–25 μl) of whole blood were added to deionized water, a proportionate increase in A_{540} was found (Table I). The increase in absorbance induced by the ditekiren solution at 2.0 mg/ml corresponded to the hemolysis of a 6-μl aliquot of blood under the conditions of the experiment. Since 200 μl of blood was added to the 2.0 mg/ml ditekiren solution, the degree of hemolysis could be estimated at about 3% at this drug concentration. By similar reasoning, approximately 1% of the red cells were lysed when suspended in the 2.0 mg/ml placebo formulation.

Ditekiren solutions for clinical intravenous use cover a concentration range of 0.005–0.2 mg/ml (5) and have the same composition as the solutions examined in this study. It was found that ditekiren and placebo concentrations exceeding the maximum clinical concentration by 10-fold cause only slight hemolysis. The data indicate that neither drug nor placebo solutions prepared for clinical investigation pose significant risk for causing hemolysis.

The experimental conditions for observing hemolytic potential were nonphysiologic in that a small volume of whole blood was suspended in a large excess of drug solution for a 10-min incubation. Upon intravenous administration of parenteral formulations, a small volume of drug solution is diluted into a large volume of blood and extended exposure of red cells to undiluted drug solution does not occur. Therefore, this assay probably magnifies the hemolytic potential of a drug or placebo formulation. Nonetheless, the lack of hemolysis under these experimental conditions accentuates the margin of safety of the drug. For other compounds that have a greater hemolytic effect than ditekiren, this assay may require modification to test the hemolytic potential under more physiologic conditions.

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