Development of Activity Assays for High-Volume Evaluation of Human Immunodeficiency Virus (HIV) Protease Inhibitors in Rat Serum: Results with Ditekiren

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We showed previously that a commercially available synthetic tetradecapeptide, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser, produces authentic angiotensin I (Ang I) upon incubation with the HIV-1 protease (S. K. Sharma *et al., Anal. Biochem.* 198:363, 1991). Therefore, we developed an Ang-I based activity assay for HIV protease inhibitors based on the technology developed earlier (M. J. Ruwart *et al., Pharm. Res.* 7:407, 1990; S. K. Sharma *et al., Anal. Biochem.* 186:24, 1990) for tracking renin inhibitors in rat sera. Ditekiren was either extracted from sera with ethyl acetate or assayed after the interfering substances in sera were precipitated with acetonitrile. Purified recombinant HIV-1 protease was added to extracted rat serum and the enzymatic reaction was initiated in the presence of the tetradecapeptide substrate. The inhibition of Ang I production was measured by a commercially available RIA kit. The cleanup methodology also enabled a commercially available Proteinase Scintillation Proximity Assay (SPA, Amersham) to quantify ditekiren in rat serum through the addition of recombinant HIV-1 protease and cleavage of substrate from SPA beads. Results were confirmed by HPLC or by the renin assay for ditekiren, which inhibits both aspartyl proteases. These technologies should prove useful for assessing serum levels of HIV protease inhibitors in rat.

KEY WORDS: rat; serum; human immunodeficiency virus protease inhibitor; activity assay; scintillation proximity assay.

INTRODUCTION

Acquired immunodeficiency disease syndrome (AIDS) and its consequences are thought (1) to be caused by the human immunodeficiency virus (HIV). The hydrolysis of HIV-fusion proteins, the products of the *gag* and *pol* genes of HIV, is catalyzed by a virally encoded protease (2) that is essential to the replication of HIV type 1 virus (1,3,4). Therefore, the search for compounds that block the activity of the viral protease has been the subject of considerable interest for the last few years. Various methods to assay HIV-1 protease activity involve HPLC (5–9) or hydrolysis of chromogenic and fluorogenic substrates (10,11). The above assay techniques are useful for *in vitro* applications but are undesirable for rapid and high-volume pharmacokinetic evaluation of HIV-1 protease inhibitors in biological matrices, which frequently interfere in such assay systems.

Recently we reported (12,13) a high-volume bioassay for pharmacokinetic evaluation of renin inhibitory peptides (RIPs). The bioassay was developed around the inhibition of the enzyme renin, which catalyzes conversion of human angiotensinogen to Ang I. This novel approach obviates the need to design and develop specific HPLC, radioimmunoassay, or radiolabeling methods for each compound in order to obtain its pharmacokinetic profile. The above renin-based technology would be adaptable to allow quantitation of HIV-1 protease inhibitors in sera if one could find a substrate that (a) does not cross-react with the anti-Ang I in the RIA and (b) produces authentic Ang I upon incubation with the HIV-1 protease. In fact, the discovery of such a substrate, a tetradecapeptide (TDP) which is converted to authentic Ang I upon incubation with HIV-1 protease, has been reported recently (14,15).

In this report we describe a high-volume bioassay to evaluate HIV-1 proteinase inhibitors in rat sera. Additionally, another methodology (16) for measuring HIV-1 protease inhibitors in serum was pursued using an HIV-proteinase [^{125}I]SPA (scintillation proximity assay) kit (Amersham, IMK8939). Ditekiren, which exhibits dual renin and protease inhibitory activity, was administered to rats and peptide serum concentrations determined by both assays. These values were validated by comparing these results with those obtained by HPLC (17) or with the renin activity assay reported previously (12,13).

MATERIALS AND METHODS

Compounds

Ditekiren [bocPro-Phe-N-MeHis-Leuψ-(CHOCH$_2$)Val-IleAMP] was synthesized at the Upjohn Company as described previously (18) and had a chemical purity of ≥99%.

Formulation

Ditekiren (900 μg/mL) was formulated in a solution of 4% ETOH, 0.08% Tween 80, 8 m*M* HCl, 5% dextrose.

Animal Study

Three fasting male Upjohn Sprague–Dawley rats (600 g) were anesthetized with dial-urethane (0.6 m/kg, i.p.). Ditekiren (2 mg/kg) was administered intravenously through the tail vein. Blood samples were collected through a capillary tube from the orbital sinus prior to dosing and at 10 and 20 min after dosing. Serum was prepared by centrifuging whole blood at 12,000*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 12,000*g* for 5 min. Serum was aliquoted for the activity assays and HPLC and frozen at

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−80°C prior to analysis. All procedures in this study are in compliance with the Animal Welfare Act Regulations, 9 CFR Parts 1, 2, and 3, and with the Guide for the Care and Use of Laboratory Animals, DHEW Publication (NIH) 85-23, 1986.

Sample Preparation

Serum samples were either extracted with ethyl acetate (EtOAc) or treated with acetonitrile (ACN) to remove interfering substances.

Ethyl Acetrate Extraction. Serum samples containing ditekiren were mixed with EtOAc (1:2, v/v). The samples were then centrifuged for 3 min at 12,000*g* to separate the layers. The EtOAc layer was transferred to another tube and allowed to dry overnight. The next morning the samples were resuspended in 20% DMSO and assay buffer. Resuspended samples were stable upon freezing.

Acetonitrile Precipitation. Serum samples containing ditekiren were mixed with acetonitrile (1:2, v/v) to precipitate interfering proteins. The samples were then centrifuged for 3 min at 12,000*g* and the supernatant was assayed immediately. ACN-treated samples gelled upon storage.

Protease Activity Assay

The presence of HIV protease inhibitory activity was determined by the inhibition of the conversion of TDP substrate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) to Ang-I (14) and subsequent measurement of Ang-I by radioimmunoassay following the procedure described earlier (12,18) (Fig. 1). Briefly, 12 μL of extracted rat serum was added to 40 μL of assay buffer (0.1 *M* Na acetate, 10% glycerol, 5% ethylene glycol, 3 m*M* EDTA, 1 mg/ml BSA, pH 5.5), followed by the addition of 20 μL of HIV protease at a final concentration of 2.6 μg/mL. The reaction was initiated by the addition of 150 μL of the TDP substrate (in assay buffer) at a final concentration of 1.1 μ*M* and allowed to incubate for 2 hr at 37°C. After stopping the reaction by boiling for 5 min, the supernatant was collected by centrifugation at 12,000*g* for 5 min and diluted 1:10 with assay buffer prior to assay for Ang I by radioimmunoassay (12). Drug levels were quantitated by comparing enzymatic activity to a standard curve generated from nonlinear fitting of data obtained from solutions of known concentration. The activity assay had intra- and interassay coefficients of variation of 10.9 and 13.1%, respectively.

Scintillation Proximity Assay

Protease inhibitory activity was also determined by the inhibition of the cleavage of an ^{125}I substrate (Tyr-Arg-Ala-Arg-Val-Phe-Phe-Val-Arg-Ala-Ala-Lys) attached to a SPA (scintillation proximity assay) bead (16, SPA HIV Proteinase Enzyme Assay System, Amersham, IMK 8939) (Fig. 2). Briefly, rat serum extract (25 μL) was added to 96-well Pharmacia T-trays followed by 10 μL of protease (0.5 μg/mL) in enzyme buffer (2.3% acetic acid, 5% ethylene glycol, 10% glycerol, 0.005% NP_{40}, pH 5.5). The reaction was initiated by the addition of 100 μL of SPA beads to each well and allowed to incubate for 1 hr at room temperature. The reaction was terminated with 200 μL of STOP Reagent and the samples were counted using a Pharmacia 1205 Betaplate Scintillation Counter. The rate of reduction in signal was proportional to the activity of the enzyme. Drug levels were quantitated by comparing enzymatic activity to a standard curve generated from nonlinear fitting data obtained from solutions of known concentration. The activity assay had intra- and interassay coefficients of variation of 10.0 and 6.8%, respectively.

Additional Assays

Values generated by the two assays described above were confirmed using an UV-HPLC method developed for assaying ditekiren in serum (17) and the renin bioassay (12,19).

RESULTS

To determine the utility of the TDP substrate in quantifying HIV protease inhibitors in rat sera, standard curves generated from sera spiked with ditekiren were compared to standard curves generated from ditekiren in buffer. In the protease assay, the serum standard curve was shifted approximately one log (Fig. 3A). In the SPA assay, no radioactivity could be detected (Fig. 3B).

To remove the source of this interference, serum samples spiked with ditekiren were extracted with EtOAc or treated with ACN. To determine whether these treatments altered the amount of ditekiren in the sera, the standard curves generated from them were compared to the renin activity assay, which is not affected by the presence of sera.

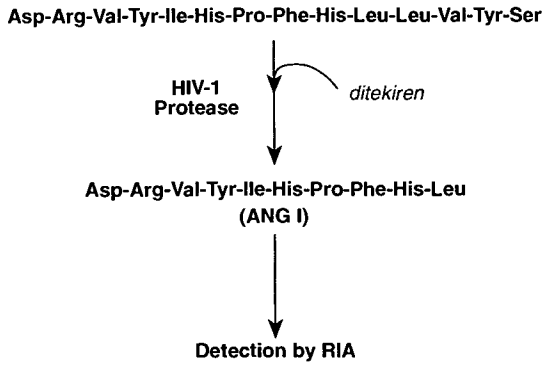

Fig. 1. Strategy for following inhibition of HIV-1 protease using a TDP substrate.

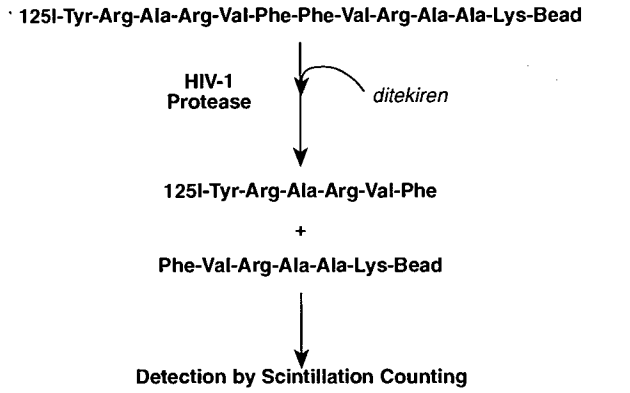

Fig. 2. Strategy for following inhibition of HIV-1 protease using SPA assay principle.

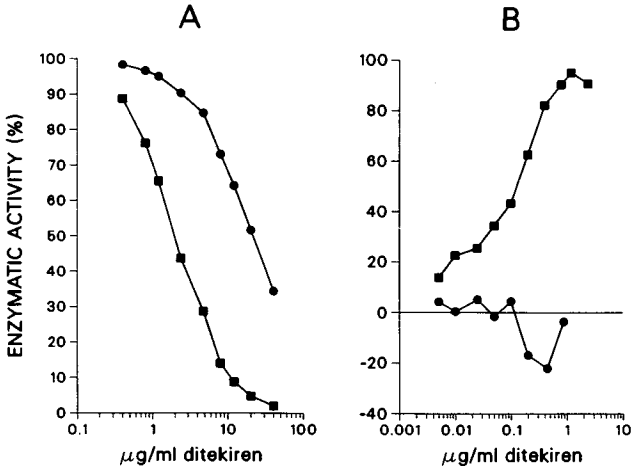

Fig. 3. Standard curve of ditekiren enzymatic activity for serum (●) or buffer (■) in the (A) protease and (B) SPA assays.

As expected, ditekiren standard curves in buffer and sera were identical in this assay (Fig. 4). EtOAc extracts produced a standard curve the same as the buffer and serum curves, suggesting that ditekiren was completely extracted with this solvent. The standard curve generated from supernatants after ACN precipitation were similar to standard curves generated from buffer spikes, although the low end of the curve was not completely superimposable. Thus, neither treatment greatly altered the ditekiren measurements in the renin activity assay.

Ditekiren in EtOAc extracts of sera gave identical standard curves in the protease activity assay to those generated from buffer (Fig. 5), suggesting that the interference from sera was effectively dealt with by this technique. Supernatants from ACN precipitations gave similar curves as well.

The data were analyzed using an equation (20) for competitive inhibition in which inhibitor concentrations were of the same order of magnitude as the protease concentration. Good agreement was observed between the theoretical curve and the experimental values (data not shown). Calculations showed that ditekiren is a purely competitive inhibitor with a K_i of 3.7 ± 0.12 μg/mL. This implies that [E] ≪ [I], and therefore, one does not have to consider that ditekiren would simply titrate the protease. Based on the variability in the activity of the HIV protease preparation (20–90% activity), the assay conditions described herein provide an approximate lower limit of detection of 0.04–0.2 μ*M*. For inhibitors of greatly different potencies, the protease concentration might need to be adjusted.

Likewise, both ACN and EtOAc treatment removed most of the interference from the SPA assay (Fig. 6). Standard curves from treated sera did not match the buffer curves quite as well as with the protease activity assay. The SPA assay was somewhat more sensitive than the protease activity assay and had a higher background of 20–30% bound

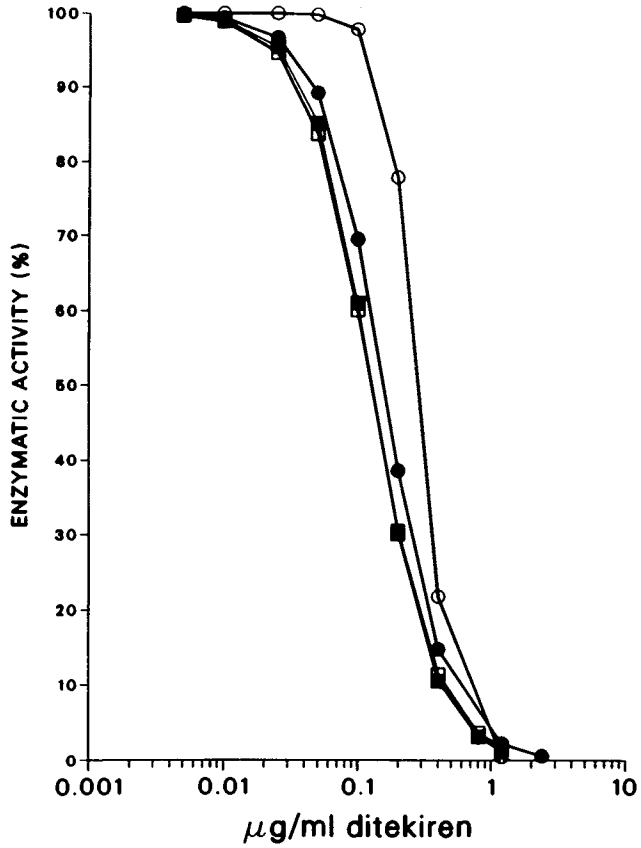

Fig. 4. Standard curve of ditekiren enzymatic activity in the renin assay for buffer (■), serum (●), EtOAc extract (□), and ACN precipitation supernatant (○).

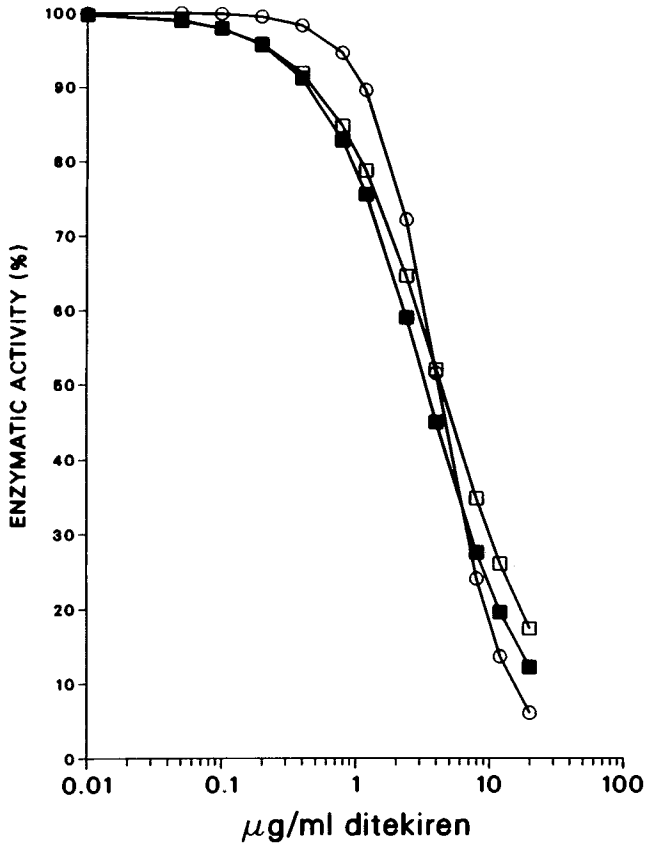

Fig. 5. Standard curve of ditekiren enzymatic activity in the protease assay for buffer (■), EtOAc extract (□), and ACN precipitation supernatant (○).

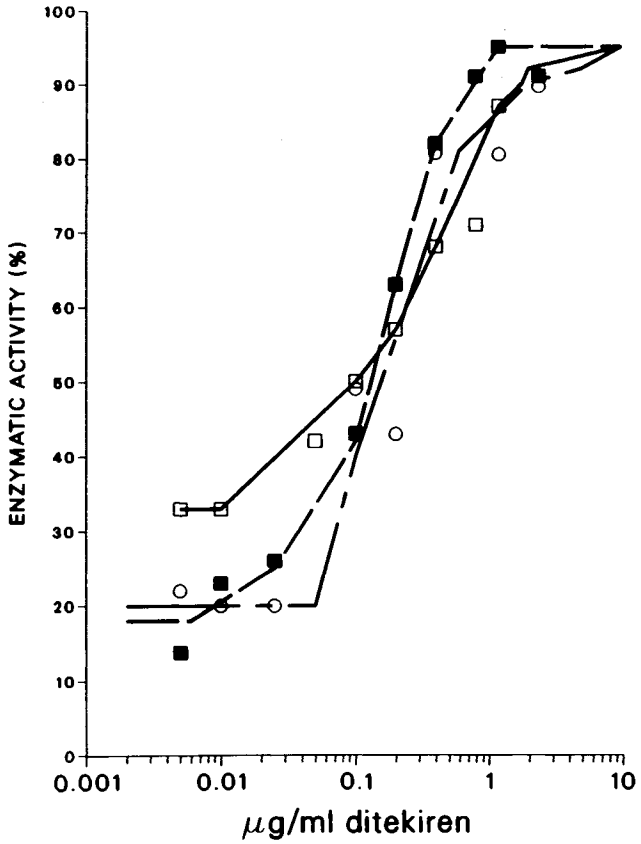

Fig. 6. Standard curve of ditekiren enzymatic activity in the SPA assay for buffer (■), EtOAc extract (□), and ACN precipitation supernatant (○).

counts. Both assays had ranges of approximately two logs in concentration.

To verify the utility of these assays, ditekiren was administered to rats as described above. Ditekiren was quantified by HPLC, renin activity assay, protease activity assay, and SPA assay for all time points. No ditekiren was detected in the predose samples. Standard curves for assays using EtOAc or ACN were generated in sera treated by the same process as the samples. Good agreement was seen between all assays (Table I).

DISCUSSION

In an earlier report, other investigators found that serum interfered with quantification of HIV protease inhibitors using the TDP substrate (15). We were able to confirm this observation, for both the protease activity assay, which utilizes the TDP substrate, and the SPA assay. The nature of this interference was not determined.

Both the EtOAc and the ACN treatments appeared to remove most of the observed interference. Utilizing standard curves that received the same treatment as the samples presumably corrected for any residual interference, such as the small differences observed between standard curves generated from buffer and those generated from ACN-treated sera (Figs. 4–6). Thus, regardless of which assay was utilized for ditekiren, good agreement was found when measuring the concentration of this peptide in sera from rats given iv ditekiren. The iv results are consistent with those determined by a different activity assay (12) and are reported elsewhere (21,22).

Not all HIV inhibitors are extractable with EtOAc. Thus, the optimum process for each peptide must be determined independently. To date, we have assayed over 100 inhibitory peptides and found only 3 that were neither extractable in EtOAc nor retained in the supernatant after ACN precipitation. Therefore, one of these two processes is usually adequate cleanup for quantitation of HIV protease inhibitors in these assays.

In conclusion, two methods of quantifying HIV protease inhibitors in rat serum can be utilized if interfering substances are first removed with EtOAc extraction or ACN precipitation. Either of these assays then provides a rapid methodology for measuring HIV protease inhibitors in sera. Hopefully, these techniques will simplify the drug disposition studies of these promising compounds for the treatment of AIDS.

Table I. Ditekiren Equivalent Serum Concentration (μg/mL)

	HPLC Solid phase extraction	Renin bioassay No extraction	Protease bioassay		Scintillation proximity assay	
			EtOAc extraction	ACN extraction	EtOAc extraction	ACN extraction
			10-min samples			
Rat 1	2.6	2.4	2.3	2.6	2.2	2.0
Rat 2	2.7	2.1	2.6	2.9	2.0	1.6
Rat 3	2.6	1.8	3.5	2.5	2.4	2.2
Mean	2.6	2.1	2.8	2.7	2.2	1.9
SE	0.03	0.2	0.4	0.1	0.1	0.2
			20-min samples			
Rat 1	1.4	1.3	0.9	1.5	1.5	0.8
Rat 2	1.1	1.1	0.7	No sample	1.1	0.7
Rat 3	1.1	1.0	0.8	1.1	1.2	1.5
Mean	1.2	1.2	0.8	1.3	1.3	1.0
SE	0.1	0.1	0.1	0.2	0.1	0.3

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