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Note

Determination of ditekiren, a renin inhibitor peptide, in monkey serum using high-performance liquid chromatography with solid-phase extraction

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The renin–angiotensin system has a role in maintaining blood pressure, blood volume and normal electrolyte concentrations in the cardiovascular system [1–3]. Angiotensin-converting enzyme inhibitors are an effective approach to controlling hypertension and congestive heart failure [4]. Inhibitors of renin, another group of the proteolytic enzymes in the cascade, are an active research area [5–7], and most identified compounds are modified peptides. However, these compounds have a number of potential problems such as low systemic bioavailability, rapid systemic clearance and rapid proteolytic degradation which may limit their utility as therapeutic agents [8,9].

A potent inhibitor of human renin, ditekiren (Fig. 1), is being evaluated clinically for the treatment of hypertension. This modified peptide is a transition-state analogue of the normal human renin substrate [10] and has been shown to be resistant in vitro to proteolytic enzyme hydrolysis. Also, the candidate has produced in vivo hypotensive responses in rat and monkey pharmacological animal models after intravenous and oral dosing [11].

The evaluation of the adsorption and disposition pharmacokinetics of ditekiren requires the development of a specific, quantitative analytical method. Bioassay methods have been reported for the determination of this renin inhibitor peptide in rodent [12] and primate [13] serum. These assays measure pharmacologically active components present in the matrix, including the par-

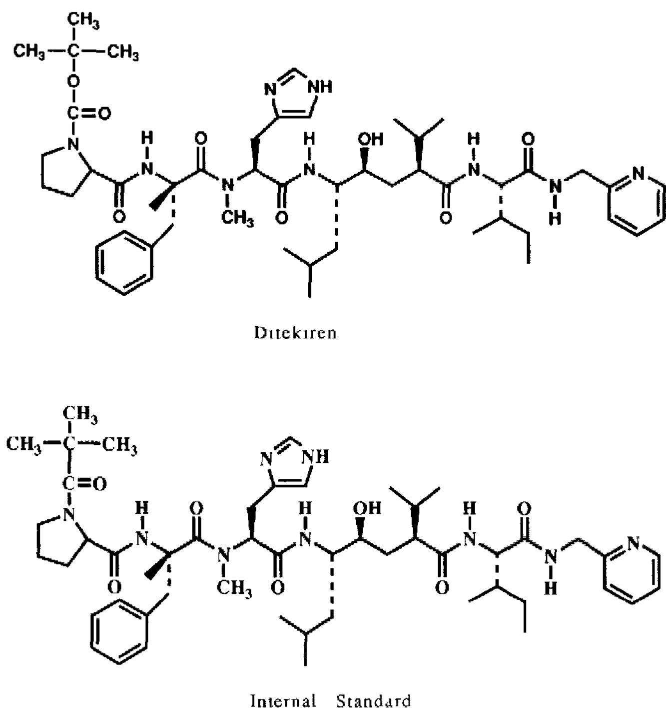

Fig 1. Structures of ditekiren and the internal standard.

ent compound and active metabolites, and thus may provide incorrect pharmacokinetic estimates on the absorption and disposition of ditekiren.

This report describes a high-performance liquid chromatographic (HPLC) analytical technique with solid-phase extraction sample preparation for the determination of ditekiren in monkey serum. The method has been employed to determine ditekiren serum concentrations in monkeys administered in 3-min intravenous infusion doses. Preliminary pharmacokinetic parameters were calculated.

EXPERIMENTAL

Chemicals and reagents

Ditekiren and the internal standard (I.S.), a structural homologue of ditekiren (Fig. 1), were provided by Upjohn (Kalamazoo, MI, U.S.A.). Acetonitrile, UV grade distilled-in-glass, was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Purified water was produced by a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.). Triethylamine (TEA), 99% (Aldrich, Milwaukee, WI, U.S.A.), was analytical reagent grade. Phosphoric

acid was purchased from Mallinckrodt (Paris, KY, U.S.A.). All other reagents used in these studies were of the highest purity available.

HPLC parameters

The isocratic HPLC system consisted of a ConstaMetric III pump [Laboratory Data Control (LDC), Riviera Beach, FL, U.S.A.], a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 50-μl loop mounted on an ISS-100 autoinjector (Perkin-Elmer, Norwalk, CT, U.S.A.), an LDC SpectroMonitor D variable-wavelength detector at 220 nm and a Linear Model 585 strip chart recorder (Linear Instruments, Reno, NV, U.S.A.). The analytical column was an Apex or IBM Cyano column, 5 μm, 250 mm$\times$4.6 mm I.D. (Jones Chromatography, Littleton, CO, U.S.A. or IBM Instruments, Danbury, CT, U.S.A.) protected with a Whatman Pelliguard LC-CN, 35 μm, 50 mm$\times$2.1 mm I.D. guard column (Whatman Inc., Clifton, NJ, U.S.A.). The chromatographic eluent was acetonitrile–water (32:68, v/v) containing 0.009 M (1.25 ml/l) TEA with a final pH of 7.0$\pm$0.1 (adjusted with concentrated orthophosphoric acid). The eluent was filtered (Nylon-66, 0.2 μm, Rainin Instruments, Woburn, MA, U.S.A.) and helium-degassed (10–15 ml/min for 30 min) prior to use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 1.0 ml/min. Quantification was accomplished using peak-height ratio analysis. Chromatographic peak heights were determined by a Harris (Fort Lauderdale, FL, U.S.A.) computer system. Reference standards and fortified serum standards over a concentration range were used to compute standard curves and relative weight response values.

Reference standard and fortified serum control preparation

Stock solutions of ditekiren and the I.S. were prepared by dissolving 10 mg of each (accurately weighed) in 0.5 ml of ethanol and diluting to 10 ml with acetonitrile. Working stock solutions were prepared by diluting the primary stock solutions with water. The stock solutions, stored at 4°C, were stable for a minimum of 30 days.

Reference standard concentration series were prepared by aliquoting ditekiren and I.S. working stock solutions into culture tubes and diluting to 1 ml with HPLC eluent. Fortified serum controls were prepared by aliquoting the working stock solutions into 1 ml control (drug-free) monkey serum. Each concentration series had a range of 0.1–20 μg/ml ditekiren and 5 μg/ml I.S. Fortified serum controls were carried through the sample preparation procedure prior to HPLC analysis.

Sample preparation

Fortified serum controls and unknown serum samples were prepared for chromatographic analysis using solid-phase extraction followed by concentration of the eluate. Bond Elut® C_{18} 3-ml columns (Analytichem International,

Harbor City, CA, U.S.A.) were activated with 2.5 ml acetonitrile followed by 2.5 ml water. Unknown serum samples (1 ml) and control serum (1 ml) were aliquoted onto a series of activated columns. The I.S. (5 μg) was added to each sample, and various amounts of ditekiren were added to the control serum specimens. The columns were allowed to drain using gravity flow and were then washed with 2.5 ml of water followed by 2.5 ml of 30% acetonitrile in water using slight vacuum (approximately 650 mm Hg). The ditekiren and I.S. were eluted with 2.0 ml of 70% acetonitrile in water using gravity flow, and the eluates were collected in Teflon®-lined screw-capped culture tubes (125 mm × 10 mm). The eluates were concentrated to approximately 1 ml on a 30°C hot plate under a gentle stream of nitrogen gas (10–15 ml/min). The concentrates were transferred to autoinjector vials, and 50 μl were injected onto the HPLC system.

Validation and application

The sample preparation procedure and HPLC analytical technique were validated using reference standards and fortified control monkey serum. The method was certified using monkey serum specimens obtained from four Rhesus monkeys (*Macaca mulatta*) administered 5 mg/kg ditekiren intravenously (3-min infusion). Blood samples were collected at 0 (predose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after dosing. After allowing the blood to clot at room temperature for 30 min, serum was harvested by centrifugation at approximately 2000 *g* for 20 min and transferred to a storage vial, frozen and maintained at −20°C until analysis. The ditekiren serum concentrations were evaluated using non-compartmental techniques [14] to obtain estimates of various pharmacokinetic parameters including the apparent terminal disposition rate constant (β), its half life $t_{\frac{1}{2}\beta}$, the area under the serum concentration–time curve (AUC_{∞}), the apparent volume of distribution (V_d) and the total body clearance (Cl_t).

RESULTS AND DISCUSSION

HPLC characteristics

Evaluations on the HPLC conditions employed ODS and Cyano columns. Ditekiren had an absorption maximum below 200 nm, acceptable absorption at 220 nm and minimal absorption above 230 nm. Without an amine modifier, i.e. TEA, present in the eluent, no elution of ditekiren occurred from the column, and at levels above 0.005 *M* TEA, sharp peaks with acceptable retention volumes were obtained. The eluent acetonitrile content was critical with 27.5, 30 and 32% acetonitrile giving ditekiren retention volumes of 31, 17 and 10 ml, respectively. Initial evaluation of prepared serum samples showed late-eluting serum components on the ODS column whereas these components were eluted from a Cyano column within 20 min. The final chromatography system, de-

scribed in the Experimental section, gave sharp, symmetric and well resolved peaks for ditekiren and the I.S. with retention volumes ranging from 16 to 17 and 19 to 20 ml, respectively.

Sample preparation

Solid-phase extraction was the method of choice for isolation of ditekiren from serum components since relatively large numbers of unknowns can be processed with good precision and accuracy. Initial evaluations indicated that ditekiren was retained on C_{18} or Cyano extraction columns and could be eluted with acetonitrile. However, the elution did not appear to be quantitative (less than 80% recovery) and the HPLC chromatography of the eluate was unacceptable. The Cyano column eluates contained compounds which had HPLC elution volumes of over 40 ml. Thus, C_{18} columns were selected. The extraction columns could be washed with 2 ml of 20% acetonitrile in water, and ditekiren and I.S. were quantitatively eluted with 2 ml of 70% acetonitrile in water. However, the high organic content in the eluate, compared to the HPLC eluent, resulted in poor chromatography. Liquid–liquid extraction and eluate concentration, used to reduce the organic content, provided samples with acceptable chromatography. Eluate concentration was selected for simplicity. Fig. 2 presents typical HPLC profiles of a reference standard containing 5 μg ditekiren and 5 μg I.S. (A), an extract of control (drug-free) monkey serum (B) and monkey serum fortified with ditekiren at 5 μg/ml and I.S. at 5 μg/ml (C). The

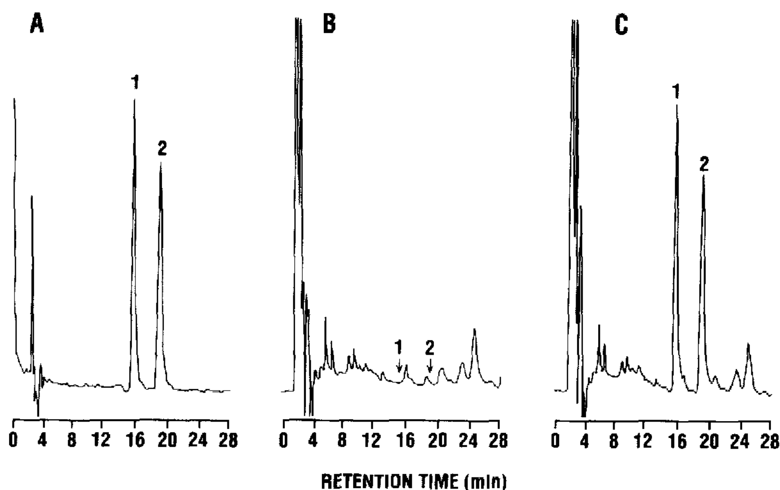

Fig. 2. Representative HPLC-UV (220 nm) profiles. (A) Reference standard containing 5 μg/ml ditekiren and 5 μg/ml I.S.; (B) blank monkey serum; (C) fortified (5 μg/ml ditekiren) monkey serum. Peaks: 1 = ditekiren; 2 = I.S.

control monkey serum chromatogram is free from extracted serum endogenous materials at the retention volumes of ditekiren and I.S.

Extraction efficiency

The absolute recoveries were determined at concentrations of 0.4, 1.0 and 5.0 μg/ml for ditekiren ($n=4$) and 5 μg/ml for I.S. ($n=12$). The final volumes of the extracted serums were adjusted to 1.0 ml for comparison with the peak heights obtained from corresponding reference standards. The average absolute recoveries were 100 ± 9, 101 ± 10 and 101 ± 13% for the 0.4, 1.0 and 5.0 μg/ml ditekiren concentrations, respectively, and 98 ± 12% for the I.S.

Method validation

The sample preparation procedure and HPLC analytical technique were validated by preparing and analyzing concentration series of fortified serum extracts and reference standards containing 0.1–20 μg/ml ditekiren on four different days. The results are summarized in Table I. The 0.1 μg/ml fortified serum samples and reference standards had unacceptable results with the calculated concentrations being 10% above theory and the relative standard deviation (R.S.D.) being greater than 12% ($n=4$). Thus, the quantification limit was considered to be 0.2 μg/ml. Both curves were linear from 0.2 to 20 μg/ml; the least-squares regression equations and correlation coefficients for the fortified serum samples and reference standards were: $y = (1.011 \pm 0.004)x +$

TABLE I

LINEARITY AND PRECISION OF DITEKIREN HPLC–UV (220 nm) SERUM METHODS

Ditekiren added (μg per sample)	Ditekiren found						
	Reference standards			Fortified serums			
	n	Average (μg per sample)	R.S.D. (%)	n	Average (μg per sample)	R.S.D. (%)	Recovery[a] (%)
0				4	N.D[b]	–	–
0.11	3	0.12	11.9	4	0.12	40.8	112
0.22	4	0.21	5.8	4	0.22	2.9	98
0.43	4	0.43	3.4	4	0.44	7.1	103
0.64	4	0.64	2.8	4	0.63	2.2	98
1.07	4	1.06	3.3	4	1.03	1.9	96
2.15	4	2.18	1.8	4	2.21	0.4	103
5.37	4	5.46	0.6	4	5.46	1.2	102
10.74	4	10.83	0.9	4	10.91	1 6	102
21.49	4	21.26	1.2	4	21.71	2.3	101

[a]Recovery (%) = (average found/added) × 100

[b]N.D = not detectable, <0.025 μg per sample.

(0.004 ± 0.038), $r = 0.999$, $n = 4$; $y = (0.992 \pm 0.003)x + (0.039 \pm 0.026)$, $r = 0.999$, $n = 4$. The intercepts were not significantly different from zero ($P > 0.05$) for each curve and the slopes were equal. Thus, relative weight response and either fortified serum samples or reference standards can be used to quantify ditekiren in serum. A chromatographic peak (signal-to-noise ratio of 3) for ditekiren was detectable in a serum sample fortified at 0.025 μg/ml which was defined as the minimal detectable level.

Stability

The stability of ditekiren in serum at 23, 4 and −20°C, in prepared serum samples at 23°C and of ditekiren stock solutions was evaluated. No apparent degradation of ditekiren in serum was noted when stored at 23°C for two days, at 4°C for three weeks and at −20°C for three months. Prepared serum samples were stable for a minimum of seven days. No significant decrease in ditekiren stock solution potency was observed after storage at 23°C for thirty days.

Application

The developed method was employed to determine the ditekiren serum concentrations in samples obtained from Rhesus monkeys receiving 5 mg/kg 3-min intravenous infusion doses. The monkeys were fasted for 16 h prior to dosing. The ditekiren serum concentration–time curves for each monkey are shown in Fig. 3. These profiles show that ditekiren is rapidly distributed and eliminated from systemic circulation. At the 4-h sampling time, only trace levels (between 0.025 and 0.2 μg/ml) of ditekiren were detectable in the serum. Selected pharmacokinetic parameters were calculated and are listed in Table II along with the average ($n=4$), standard deviation (S.D.) and R.S.D. for each parameter. The average V_d of 1.7 l suggests that ditekiren is distributed

TABLE II

DISPOSITION KINETICS OF DITEKIREN IN RHESUS MONKEYS

Dose: 5 mg/kg 3-min intravenous infusion.

Monkey No.	Dose (mg)	$V_{d,area}$ (l)	β (min^{-1})	$t_{1/2\beta}$[a] (min)	AUC_{∞} (μg/ml per min)	Cl_t (ml/min)
M9C	26.0	1.6	0.03	20	495	53
M10C	21.0	1 2	0.02	28	704	30
M11C	26.5	2.2	0.04	20	340	78
M13C	28.5	1.8	0 02	28	633	45
Average[b]		1.7	0.03	23	543	52
S.D.		0.4	0.01		161	20
R.S.D. (%)		14	19		30	39

[a] $t_{1/2\beta}$ calculated from average β

[b] Average values from $n=4$

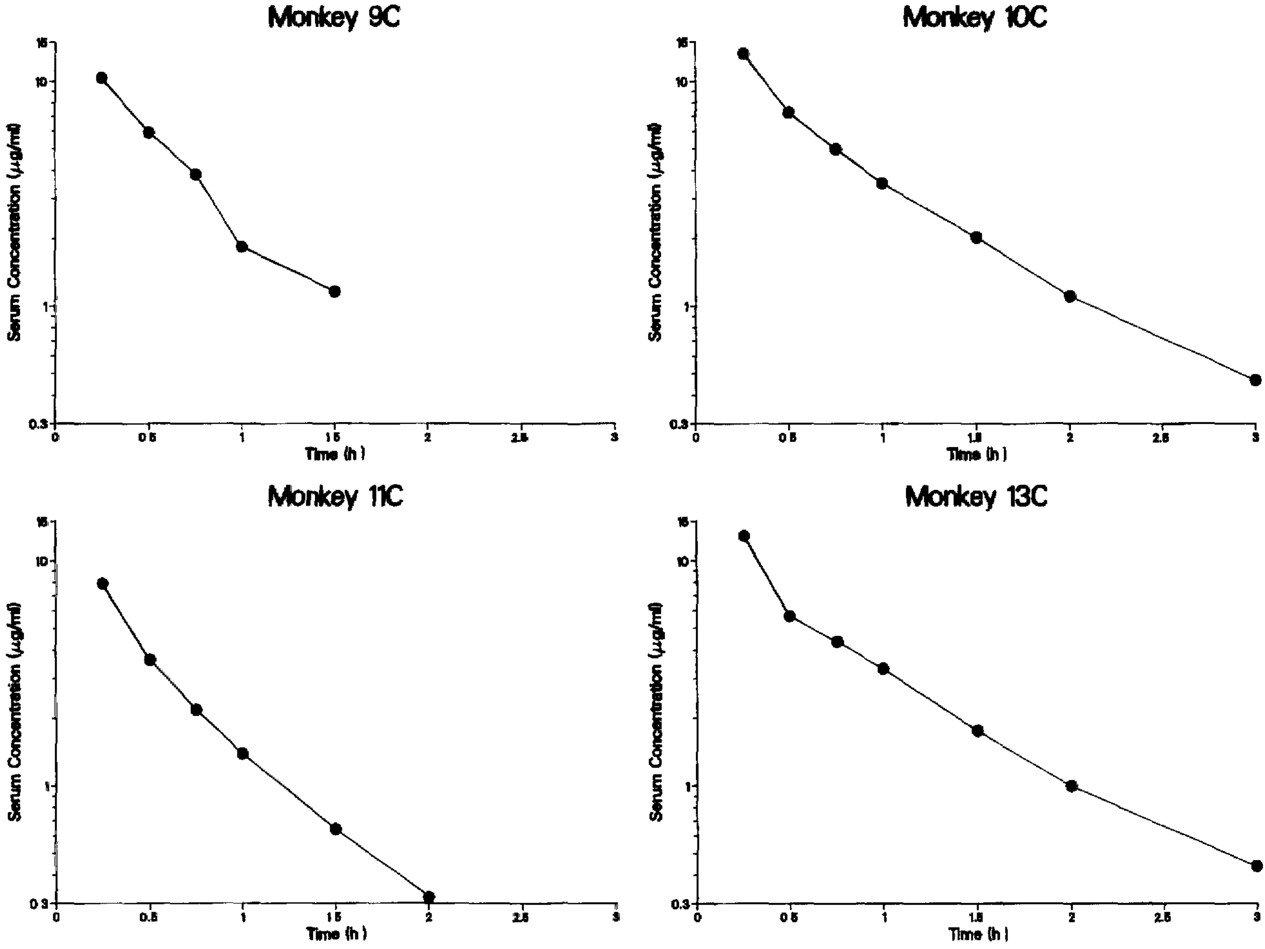

Fig. 3. Serum concentration-time profiles in monkeys each administered 5 mg/kg 3-min intravenous infusion doses of ditekiren.

more widely than the plasma volume, 220 ml in a 5-kg monkey [15] and thus may reach some extravascular spaces. The $t_{\frac{1}{2}\beta}$ of 23 min indicates that ditekiren is removed from systemic circulation rapidly after an intravenous dose. The Cl_t-average of 52 ml/min is about one half the 90 ml/min plasma flow-rate to the liver [15].

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