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Note

Determination of 4,5-dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone hydrochloride, a new cardiotonic, in plasma and urine by reversed-phase high-performance liquid chromatography

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4,5-Dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone hydrochloride (I, CI-914 hydrochloride), is a new cardiotonic [1] currently under phase 1 clinical investigation. This work describes reversed-phase high-performance liquid chromatographic (HPLC) methods for the determination of I in plasma and urine. Optimal conditions for drug extraction and chromatography were selected in order to achieve a satisfactory recovery and adequate sensitivity. The methods have been applied to pharmacokinetic and pharmacodynamic studies in laboratory animals.

EXPERIMENTAL

Chemicals

I and the internal standard, PD 111,908 (Fig. 1), were synthesized at Warner-Lambert/Parke-Davis Research Labs. ^{14}C-Labeled I was custom synthesized by Amersham (Arlington Heights, IL, U.S.A.). Dichloromethane, distilled in glass, was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); acetonitrile, distilled in glass, from Matheson, Coleman, Bell

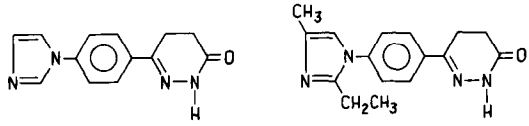

Fig. 1. Chemical structures of I (CI-914) and internal standard (PD 111,908).

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(Cincinnati, OH, U.S.A.); Ready-Solv MP® from Beckman Instruments (Fullerton, CA, U.S.A.); drug-free human plasma from Plasma Alliance (Knoxville, TN, U.S.A.). All other chemicals were of analytical grade. Water used in reagent preparation was purified using the Water-I system from Gelman Sciences (Ann Arbor, MI, U.S.A.).

Reagents

Stock solutions of I and the internal standard (1 mg/ml as the free base) were prepared in 0.1 mol/l hydrochloric acid. Appropriate aliquots of I stock solution were added to drug-free human plasma and dog urine to yield 200 and 1000 ng/ml working standards. A working solution of the internal standard was prepared weekly by diluting (1:400) the stock solution with 0.1 mol/l hydrochloric acid.

Preparation of plasma samples

Aliquots of plasma (0.1—0.5 ml) were pipetted into disposable glass tubes and drug-free human plasma was added to provide a final volume of 0.5 ml. A 0.2-ml aliquot of the internal standard solution (500 ng) was added to each tube. The samples were deproteinized with 2 ml of 10% trichloroacetic acid (TCA), vortexed for 30 sec, and allowed to stand 15 min at room temperature. After centrifugation, the supernatant was decanted into 16-ml glass-stoppered extraction tubes containing 0.7 ml of 2 mol/l sodium hydroxide and 6 ml of dichloromethane. The tubes were shaken for 5 min and centrifuged. The organic layer was transferred into disposable tubes and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.2 ml of 30% acetonitrile in water and transferred to autosampler vials containing limited volume inserts; 25 μl of this solution were injected for chromatography.

Preparation of urine samples

Aliquots of urine (0.1—1.0 ml) were pipetted into 16-ml glass-stoppered extraction tubes and drug-free dog urine was added to provide a final volume of 1.0 ml. A 0.4-ml aliquot of the internal standard (1 μg), 0.2 ml of 2 mol/l sodium hydroxide, and 6 ml dichloromethane were added to each tube. The tubes were shaken for 5 min and centrifuged. A 5-ml aliquot of the organic phase was back-extracted with 3.5 ml of 0.1 mol/l hydrochloric acid in a second set of tubes for 5 min. Following centrifugation, 3 ml of the aqueous phase was made alkaline with 0.2 ml of 2 mol/l sodium hydroxide and the mixture was extracted with 5 ml dichloromethane as described above. The organic layer was transferred into disposable tubes, evaporated to dryness under a stream of nitrogen, and analyzed as described for plasma samples.

Instrumentation

An automated HPLC system comprised a Model M-45 pump, a Model 441 ultraviolet (UV) detector operated at 280 nm, a Model 710B WISP sample processor (all from Waters Assoc., Milford, MA, U.S.A.), and a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The 5-μm LiChrosorb RP-2 column (stainless steel, 125 mm × 4 mm I.D.) was purchased from E. Merck (Gibbstown, NJ, U.S.A.).

Samples were eluted isocratically at a flow-rate of 0.8 ml/min with a pressure of 6.89 MPa. The mobile phase used consisted of a mixture of 0.005 *M* octanesulfonic acid in 0.1% acetic acid—acetonitrile (70:30).

A Hewlett-Packard Model 1040A high-speed scanning diode array UV spectrophotometric detector was used to determine the specificity of the plasma assay under identical chromatographic conditions described above.

Radioactivity determinations were performed using a Model 3255 liquid scintillation spectrometer (Packard, Downers Grove, IL, U.S.A.).

Calibration and precision

Since preliminary experiments showed that drug-free animal and human plasma yielded equivalent results, human plasma was used for all subsequent plasma validation studies and calibration standard preparations.

Six calibration standards containing 100, 200, 400, 600, 800, and 1000 ng I per ml drug-free human plasma or dog urine were processed daily with each set of unknowns. Calibration curves were constructed by plotting peak height ratios of I to the internal standard as a function of the concentration of I. The best-fit straight line was determined using reciprocal concentration weighted least-squares linear regression. To determine the precision of the assay procedures, triplicate plasma and urine standards were analyzed on three separate days to yield nine replicate values for each of the six concentrations.

Selectivity

Selectivity of the plasma method was determined by examining the homogeneity of the eluted I peak in the extract of a 4-h postdose dog plasma sample. Absorbance of the peak was measured as a function of wavelength and time simultaneously.

Recovery determination of plasma

^{14}C-Labeled drug was used to assess the recovery of I for both the protein precipitation step and the extraction of drug into dichloromethane. The minimum volume of 10% TCA required to provide an optimum recovery of I from plasma was determined by treating 0.5-ml aliquots of a 2 μg/ml plasma standard with 0.5—2.5 ml of 10% TCA. In a separate study, the extraction efficiency of [^{14}C]I from alkalinized TCA supernatant (pH 14) into dichloromethane was also determined. Radioactivity was measured by liquid scintillation counting using Ready-Solv MP® as the scintillation cocktail and external standardization for quench correction.

Recovery determination of urine

Recovery of I from urine was determined by extraction of triplicate 500 ng/ml urine standard solutions and comparison to aqueous standards injected directly.

Quality control and stability studies

Quality control samples in the concentrations of 300, 500, and 700 ng/ml were prepared in drug-free human plasma or dog urine, and aliquots were stored frozen in disposable glass vials. Individual samples at the three concen-

trations were then analyzed daily with each set of unknowns to determine the reliability of the day's analyses and the stability of I in frozen plasma and urine.

RESULTS AND DISCUSSION

Representative chromatograms obtained from plasma and urine analyses are depicted in Figs. 2 and 3, respectively. Retention times for I and the internal standard were 3.0 and 4.9 min, respectively, and no interfering peaks were detected at either retention time.

Homogeneity of the I peak in a dog plasma extract was determined by multiple UV scans at the upslope, apex, downslope, and baseline over the range 211—400 nm. The resulting concentration normalized and background-cor-

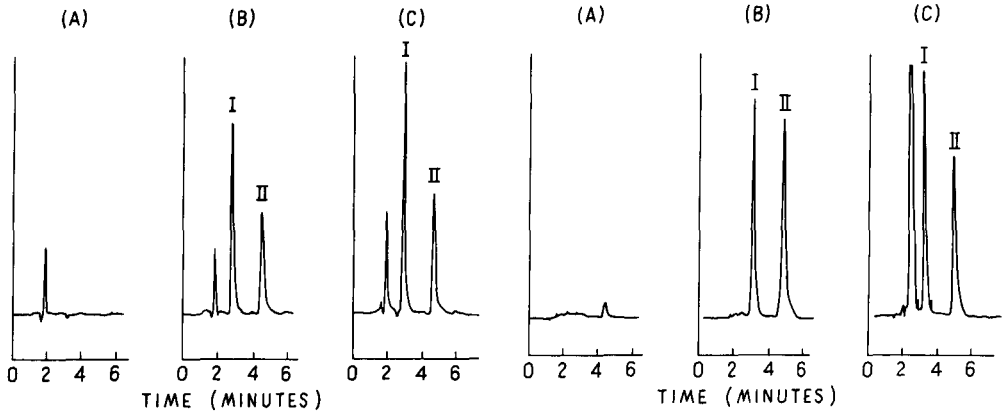

Fig. 2. Chromatograms of an extract of (A) drug-free human plasma; (B) 600 ng/ml plasma calibration standard; (C) 3-h postdose dog plasma sample (1 mg/kg oral dose) containing 690 ng I per ml. Peaks: I = CI-914; II = internal standard.

Fig. 3. Chromatograms of an extract of (A) drug-free dog urine; (B) 600 ng/ml urine calibration standard; (C) 8—12 h postdose dog urine sample (0.5 mg/kg oral dose) containing 770 ng/ml I. Peaks: I = CI-914; II = internal standard.

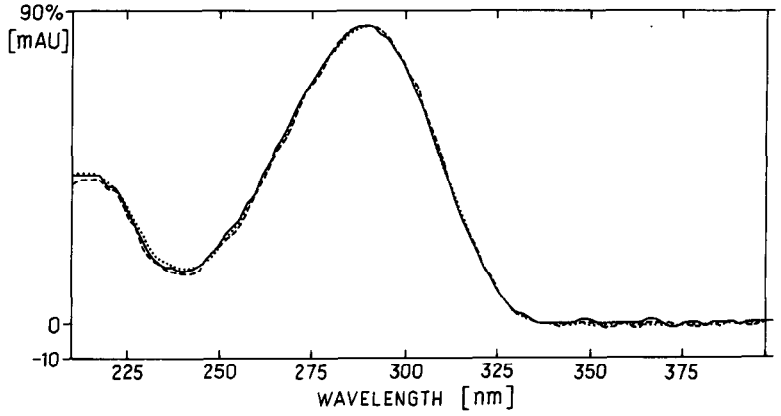

Fig. 4. Spectra of I peak in a postdose dog plasma extract. —, Upslope; • • • • •, apex; - - - -, downslope.

rected spectra (Fig. 4) were superimposable with that of a standard I solution, indicating that the I peak was homogeneous and that the plasma assay procedure is specific for unchanged drug.

Based on peak height ratios of six replicate sequential injections of 100 and 1000 ng/ml standards, the HPLC system reproducibility had a relative standard deviation (R.S.D.) of 0.7% and 0.5%, respectively.

The relationship between peak height ratio (I/internal standard) and I concentration was linear over the concentration range studied (100—1000 ng/ml) and yielded a correlation coefficient of 0.999 or greater for both the plasma and urine assays.

The assay precision and accuracy varied from 1.8% to 4.9% for plasma analyses and from 0.8% to 5.4% for urine analyses (Table I).

For the plasma assay, recovery of I for the protein precipitation step ranged from 54%, when equal volumes of plasma and TCA were used, to 86% when the ratio of plasma to TCA was 1:4. Further increases in TCA volume failed to improve the recovery. The extraction efficiency into dichloromethane was 90%, giving a total recovery of 77% for the entire plasma assay procedure. The urine assay showed 97% recovery of I.

Plasma and urine quality control samples in the concentrations of 300, 500, and 700 ng/ml I were frozen and assayed over a three-month period (Table II).

TABLE I

PRECISION AND ACCURACY OF THE METHODS APPLIED TO I CALIBRATION STANDARDS (n = 9)

I added (ng/ml)	I found			
	Plasma		Urine	
	ng/ml	R.S.D. (%)	ng/ml	R.S.D. (%)
100	101.4	3.0	99.5	5.4
200	194.7	1.9	200.6	3.0
400	403.2	4.9	399.4	1.7
600	607.5	3.5	602.4	0.8
800	796.5	2.1	797.4	2.8
1000	996.7	1.8	1000.0	2.7

TABLE II

STABILITY OF I IN PLASMA AND URINE QUALITY CONTROL SAMPLES

I added (ng/ml)	I found					
	Plasma (n = 14)			Urine (n = 3)		
	ng/ml	Percent theoretical	R.S.D. (%)	ng/ml	Percent theoretical	R.S.D. (%)
300	302.7	100.9	2.5	293.6	97.9	8.0
500	502.0	100.4	2.1	517.8	103.6	2.6
700	702.0	100.3	1.3	719.7	102.8	2.5

For the plasma samples, mean values obtained were within 1% of theoretical values with an R.S.D. less than 3%. The urine quality control samples were within 4% of the theoretical values with an R.S.D. less than 8%. These results indicated excellent drug stability in frozen plasma and urine.

The limit of assay quantitation was defined as the concentration of I per ml of plasma or urine at which replicate analyses have an R.S.D. of 15%. Analysis of 0.5-ml aliquots of 40 and 80 ng/ml spiked plasma samples (n = 6) yielded an R.S.D. of 15.5% and 9.0%, respectively. The reported limit of quantitation was 40 ng/ml. Variability at the lower concentrations was largely derived from plasma preparation since HPLC system reproducibility was essentially the same for replicate injections of 40 and 400 ng/ml standards (1.6% and 1.2% R.S.D. respectively, n = 6). The limit of quantitation can be extended to 20 ng/ml by analyzing 1.0-ml aliquots of plasma, deproteinizing with 4 ml of 10% TCA, and alkalinizing with 1.4 ml of 2 mol/l sodium hydroxide (R.S.D. = 6.0%, n = 6). For urine samples, the reported limit of quantitation was 100 ng/ml.

The described methods have been successfully applied to pharmacokinetic and dose ranging studies in laboratory animals following single 0.5 mg/kg intravenous and oral doses [2, 3]. The resulting plasma concentrations ranged from 40 to 500 ng/ml and showed that I was readily absorbed. Urinary excretion of unchanged I accounted for less than 1% of the dose.

In summary, the described methods are selective, reproducible, and sufficiently sensitive for single-dose pharmacokinetic studies.

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