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Note

High-performance liquid chromatographic analysis of milrinone in plasma and urine

Intravenous pharmacokinetics in the dog

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Milrinone, 1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile, is a new cardiotonic agent which has demonstrated inotropic activity in both laboratory animals and man. This novel drug is the subject of intensive clinical trials.

This report describes a high-performance liquid chromatographic (HPLC) method for the determination of milrinone in human plasma and human urine. The assay was used for the quantitation of milrinone in the plasma of dogs that had received milrinone by intravenous administration. The plasma concentration data were used to estimate pharmacokinetic parameters for milrinone in the dog.

EXPERIMENTAL

Chemicals

Milrinone and the internal standard for the assay, 1,6-dihydro-2-ethyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile, were synthesized at Sterling-Winthrop Research Institute. The acetonitrile and ethyl acetate were chromatographic grade. Other chemicals were obtained commercially (reagent grade) and used without further purification.

Preparation of plasma and urine standards and samples

Plasma and urine standards were prepared by supplementing 1.0 ml of control human plasma (or urine) with 50-μl aliquots of stock solutions of

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milrinone in 0.01 *N* hydrochloric acid. The final concentrations of the standards were 0.0, 10, 20, 40, 60, 80, 100, 150 and 200 ng/ml of plasma and 0.00, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10 μg/ml of urine. Duplicate standards at each concentration were prepared.

Four sets of randomized and coded samples, to be analyzed under single-blind conditions, were prepared as described above. Each plasma set contained triplicate samples at final concentrations of 0, 16, 32, 48, 120 and 180 ng/ml of plasma. Each urine set contained triplicate samples at final concentrations of 0, 0.06, 0.20, 2.40 and 9.0 μg/ml of urine. One set of plasma (or urine) samples was analyzed upon preparation. The other set was stored in the laboratory freezer for at least seven days before analysis.

Extraction procedure

To a tube containing 1.0 ml of sample (plasma containing potassium oxalate as the anticoagulant, or urine) were added 50 μl of internal standard solution (10 μg/ml for the plasma assay or 40 μg/ml for the urine assay). To the urine samples, 0.5 ml of 0.5 *M* phosphate buffer, pH 7.5, and 10 ml of ethyl acetate were added. To the plasma samples, 1.8 g of ammonium sulfate and 5 ml of ethyl acetate were added. Each sample was shaken for 10 min on a rotary shaker. The ethyl acetate layer was transferred to a clean 15-ml conical tube.

In the case of the plasma samples, it was necessary to repeat the extraction with a second 2.0-ml addition of ethyl acetate; the organic phases were combined. The combined extract was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 250 μl of ethyl acetate.

For both plasma and urine, 0.1 *N* hydrochloric acid (0.1 ml for plasma and 0.4 ml for urine) was added to the ethyl acetate. After vigorous shaking, the organic layer was discarded. Residual ethyl acetate was evaporated by heating in a 55°C water bath, under a stream of nitrogen, for several minutes. Failure to eliminate traces of ethyl acetate can adversely affect the chromatography.

A neutralizing solution was prepared by adding 6 ml of 10 *N* sodium hydroxide to 94 ml of 0.5 *M* phosphate buffer, pH 7.0. For the urine assay, 50 μl of neutralizing solution were added to the residual acid phase. For the plasma assay, 15 μl of neutralizing solution were added. A 50-μl aliquot of the neutralized sample was injected into the chromatograph for analysis.

Chromatography

The HPLC system was operated isocratically at ambient temperature. The system consisted of an automatic injector, a pump (Milton Roy, Riviera Beach, FL, U.S.A.), a Partisil 10/25 ODS-3 column (10 μm particle size; Whatman, Clifton, NJ, U.S.A.) with a Waters Assoc. (Milford, MA, U.S.A.) 37—50 μm particle size Corasil C-18 precolumn, and a UV detector with a 340-nm filter. The mobile phase was a ternary mixture, tetrahydrofuran—acetonitrile—0.1 *M* phosphate buffer, pH 6.0 (28:260:1000). The flow-rates were 1.2 ml/min for the plasma and 1.5 ml/min for the urine assay.

Data processing

The output of the detector was interfaced with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3356 LAS computer system which calculated peak-

height ratios (milrinone:internal standard) for each standard and sample. A least-squares regression analysis was performed on the standards and used to determine the concentration of milrinone in the samples by inverse prediction [1]. The minimum quantifiable level of the assay was estimated as the concentration whose lower 80% confidence interval just encompassed zero [2].

The observed concentrations for the prepared, spiked samples in each biological media were expressed as percent differences from the nominal value. The range of these percent differences was used to define the accuracy of each assay. Precision was estimated from the standard deviation of the mean square error term derived from a two-way analysis of variance on the percent differences.

Extraction efficiency

The percent recovery of the extraction procedure for the internal standard was determined by comparing the peak heights of internal standard obtained from extracted samples with those obtained by injection of unextracted solutions. A similar procedure was used for milrinone over a range of concentrations; for plasma the concentrations were 20, 60 and 150 ng/ml and for urine the concentrations were 0.25, 2.5 and 10 μg/ml.

Animal study

Three female beagle hounds received single intravenous bolus doses of milrinone (5 mg/kg) via the saphenous vein. At appropriate intervals, blood samples were collected from the contralateral saphenous vein. Potassium oxalate was used as the anticoagulant. The blood samples were centrifuged and the plasma was transferred to clean tubes which were placed in the freezer (−20°C) until analyzed. Analysis was carried out as described above, with the exception that aliquots of plasma ranging from 0.25 to 1.0 ml were analyzed, depending on availability of sample and expected concentration. Those aliquots of less than 1.0 ml were supplemented with control dog plasma to 1.0 ml.

Pharmacokinetic calculations

The data obtained from the analysis of the dog plasma samples were fit to an open two-compartment body model by means of a nonlinear (NLIN) regression analysis using the Marquardt method [3]. The plasma concentrations were weighted as the squares of their reciprocals. The model is described by the equation:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where C is the concentration of milrinone in the plasma, α is the apparent first-order distribution rate constant, β is the apparent first-order disposition rate constant, t is time in hours, $A = D(\alpha - k_{21})/V_p(\alpha - \beta)$; $B = D(k_{21} - \beta)/V_p(\alpha - \beta)$; D is the administered dose in mg, V_p is the volume of the plasma compartment, and k_{21} is the apparent first-order rate constant from tissue to plasma.

The area under the curve of plasma concentration vs. time was estimated using the trapezoidal rule and extrapolated to zero concentration. The volume

of distribution at steady-state (V_{dss}) was calculated by a model-independent method [4] and by a regression-dependent method [5]:

$$V_{dss} = V_p\left(1 + \frac{k_{12}}{k_{21}}\right)$$

Other pharmacokinetic parameters were estimated by classical techniques [6].

RESULTS AND DISCUSSION

Analytical method

Representative chromatograms of an extracted plasma standard and an extracted plasma blank are shown in Fig. 1a and b, respectively. A plot of peak height ratios (milrinone:internal standard) vs. concentration of milrinone in the plasma standards was linear over the range of 0—200 ng/ml of plasma, as determined by linear regression analysis. The concentrations of the prepared plasma samples were estimated from the regression analysis by inverse prediction [1]. The accuracy of the assay, defined as the mean percentage difference from the nominal value, ranged from −8.2% to 1.7%. The mean (± S.E.M.) minimum quantifiable level (MQL) of the two sets was 3.7 ± 0.5 ng/ml using the full set of standards and 2.3 ± 0.05 ng/ml using a set of standards truncated to the range of 0–60 ng/ml. The estimated precision of the assay was ± 5.0%. Freezing and thawing of the plasma samples had no significant effect on the assay results.

The extraction efficiency studies in plasma indicated an apparent recovery of

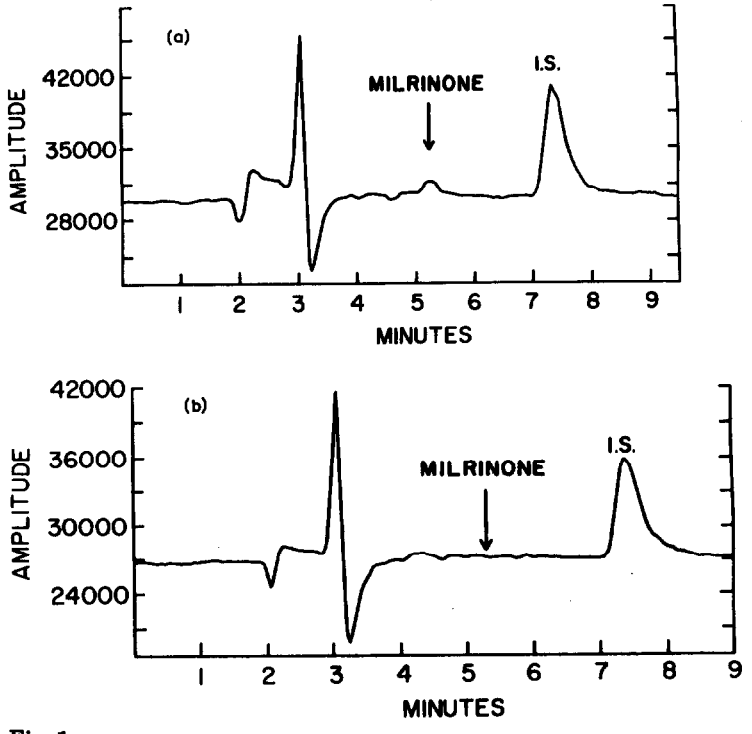

Fig. 1.

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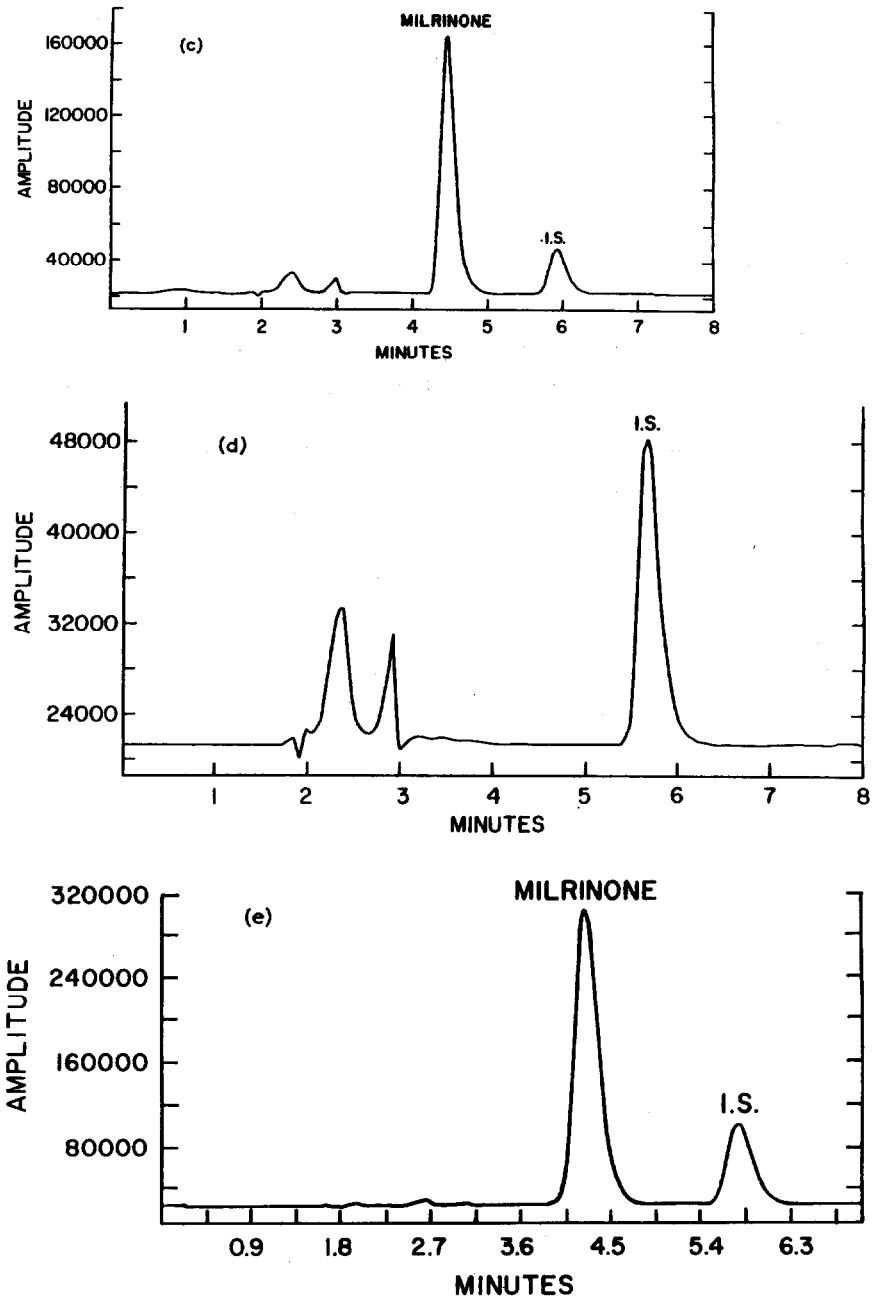

Fig. 1. Computer-reconstructed chromatograms of: (a) processed plasma standard containing 10 ng/ml of milrinone and 100 ng/ml of internal standard (I.S.); (b) processed plasma standard containing only the internal standard; (c) processed urine standard containing 10 μg/ml of milrinone and 2 μg/ml of internal standard; (d) processed urine standard containing only the internal standard; (e) processed plasma from a dog that had received a 5 mg/kg dose of milrinone, intravenously; specimen contains 6.2 μg of milrinone in 0.5 ml of plasma (5 min after medication).

104—105% for milrinone and the internal standard. After correction for the volume loss incurred during the evaporation of residual ethyl acetate, the recovery for both compounds was 93%.

Fig. 1c and d are representative chromatograms of processed urine standard and an extracted urine blank. A plot of the peak height ratios against concentration was linear over the range of 0 and 0.05—10 μg/ml.

The accuracy of the urine assay, defined as the mean percentage difference from the nominal value, ranged from −2.6% to 2.2%. The average MQL of the two sets of urine standards was 0.003 ± 0.001 μg/ml, using the set of standards which had been truncated to cover the range 0—0.5 μg/ml. The estimated precision of the assay was 2.6%. Freezing and thawing of the urine samples had no significant effect on the analytical results. The extraction efficiency for milrinone was 89%, and 99% for the internal standard.

Animal study

The milrinone levels in plasma, collected from three beagle hounds at various times after administration, are plotted against time in Fig. 2. Fig. 1e shows a chromatogram of plasma taken from a dog and processed as described above. The plasma data were fitted to an open two-compartment body model of intravenous administration, and the pharmacokinetic parameters were calculated (Table I). The predicted curves are represented as lines in Fig. 2. The mean half-lives of the distribution phase (α-phase) and the disposition phase (β-phase) were 0.48 and 3.6 h, respectively. The mean (± S.E.M.) regression-dependent

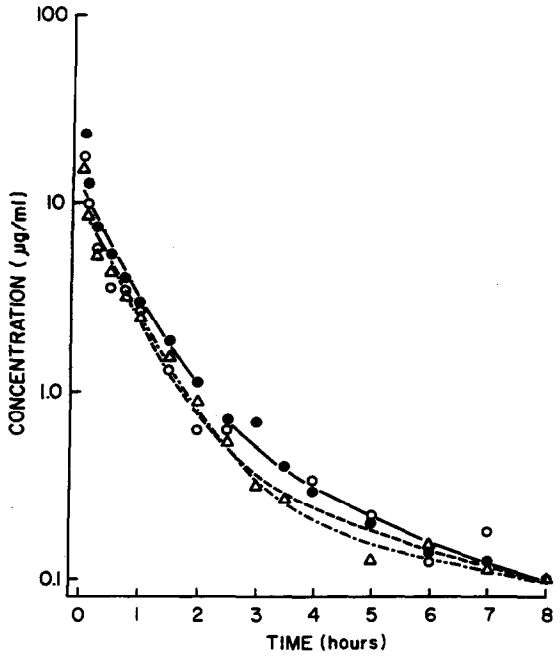

Fig. 2. Plasma concentration of milrinone in three dogs that had received a 5 mg/kg dose of milrinone, intravenously. Lines are those predicted by non-linear regression, see text. ○———○, dog 51793; ●———●, dog 52484; △ – ∘ — △, dog ED26.

TABLE I

PHARMACOKINETIC PARAMETERS DERIVED FROM INTRAVENOUS PLASMA LEVEL DATA IN DOGS FOLLOWING A 5 mg/kg BOLUS DOSE OF [^{14}C]MILRINONE

Parameter	Dog 51793	Dog 52484	Dog ED26	Mean ± S.E.M
Weight (kg)	8.0	10.2	8.4	8.9 ± 0.7
Dose (mg)	39.7	51.3	41.3	44.1 ± 3.6
V_p (l/kg)	0.52	0.42	0.55	0.50 ± 0.04
V_{dss} (l/kg)				
(1) Model-dependent	1.13	0.69	2.42	1.41 ± 0.52
(2) Model-independent	1.00	0.66	2.33	1.33 ± 0.51
k_{12} (h^{-1})	0.34	0.26	0.33	0.31 ± 0.03
k_{21} (h^{-1})	0.29	0.40	0.097	0.26 ± 0.09
k_{10} (h^{-1})	1.15	1.13	0.93	1.07 ± 0.07
α (h^{-1})	1.57	1.49	1.29	1.45 ± 0.08
β (h^{-1})	0.21	0.30	0.07	0.19 ± 0.07
A (μg/ml)	8.95	11.1	8.67	9.57 ± 0.77
B (μg/ml)	0.53	0.94	0.20	0.56 ± 0.21

volume of distribution was 1.41 (± 0.52) l/kg; the mean regression-independent volume of distribution was 1.33 (± 0.51) l/kg.

In summary, an accurate, sensitive and reproducible assay has been developed for the HPLC determination of milrinone in plasma and urine. This technique has been successfully applied to estimate pharmacokinetic parameters in dogs that had received an intravenous dose of milrinone.

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