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Note

Analysis of milrinone in plasma using solid-phase extraction and high-performance liquid chromatography

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Milrinone [1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile, WIN 47,203, Fig. 1] is currently under investigation in humans as a non-glycoside, non-catechol, inotropic agent [1—5]. It was developed as an analogue of amrinone [5-amino-1,6-dihydro-6-oxo-(3,4′-bipyridine), Sterling-Winthrop Research Institute, Rensselaer, NY, U.S.A.] in order to improve potency and reduce side-effects [6]. Amrinone has been shown to be an effective inotropic agent in terms of haemodynamic effect and maintenance of ventricular performance [7—10].

Milrinone Internal standard

Fig. 1. Structural formulae of milrinone and the internal standard for the assay.

Conventional methodology for many biological compounds and drugs in plasma and urine involves a solvent extraction step prior to a further separative, often chromatographic, step and quantitation. Introduction of high-performance liquid chromatogaphic (HPLC) techniques has greatly improved the separative step, particularly with the use of bonded-phase packing materials. The use of bonded-phase packing materials has also been applied to initial extraction

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procedures with resultant improvements in assay time, simplicity and recovery [11, 12].

This paper describes the use of C_{18} (octadecylsilyl) bonded silica gel for the extraction of milrinone from plasma. Established HPLC techniques were then used for subsequent separation and quantitation [13]. The resultant assay is simpler and more rapid than the published assay for milrinone using solvent extraction [13] and it maintains the precision, accuracy, recovery and selectivity of the latter technique.

EXPERIMENTAL

Reagents

Milrinone and the compound used as internal standard [1,6-dihydro-2-ethyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile, WIN 47,306, Fig. 1] were generously supplied by Sterling-Winthrop Research Institute. Stock solutions (1 mg/ml) were prepared in 0.1 *M* hydrochloric acid and stored at 4°C. Dilutions of the stock solutions were prepared in 0.01 *M* hydrochloric acid for each assay run.

HPLC-grade acetonitrile and tetrahydrofuran were obtained from Waters Assoc. (Milford, MA, U.S.A.). All other reagents were of analytical grade.

The 1- or 2-ml Bond-Elut™ columns packed with C_{18} bonded silica gel (Analytichem International, Harbor City, CA, U.S.A.) were activated prior to use by passage of 2 vols. methanol and 2 vols. distilled water. The columns were regenerated after use by passage of 1 vol. dimethylformamide, 2 vols. methanol and 2 vols. distilled water.

Plasma samples

Venous blood was collected from human subjects into tubes containing potassium oxalate as anticoagulant, on ice. Following centrifugation at 1000 *g* and 4°C, plasma was separated and frozen at −20°C until assayed.

Extraction procedure

Depending on the expected milrinone concentration, 1 or 2 ml plasma were assayed. To each sample were added 500 ng internal standard and 10 ml of 1 *M* ammonium acetate, pH 7. The resultant mixture was passed through prepared C_{18} Bond-Elut columns using gentle suction. The columns were washed three times with distilled water. Milrinone and the internal standard were eluted from the column with 1 or 2 ml ethanol (for 1- or 2-ml sized Bond-Elut columns, respectively). The ethanolic extracts were dried at 50°C in vacuo, then reconstituted in 200 μl of 0.2 *M* perchloric acid. Aliquots of 50—100 μl were injected for HPLC.

A calibration curve comprising 1000, 500, 250, 125 and 0 ng milrinone in 1 or 2 ml of drug-free plasma (control plasma from the subject being studied) was similarly assayed with each sample run.

Chromatography

HPLC was carried out using a Model M-45 pump (Waters Assoc.) with a 100-μl universal loop injector (Valco Instruments, Houston, TX, U.S.A.) and a

Spherisorb 5-μm ODS column (25 cm × 4 mm I.D.) (Phase Separations, Norwalk, CT, U.S.A.). The mobile phase was 0.1 *M* phosphate buffer, pH 6—tetrahydrofuran—acetonitrile (73:2:254). Flow-rate was 1 ml/min. Detection was by ultraviolet absorption spectrometry at 340 nm using an 8-μl flow cell (Model 220A spectrometer, Hitachi, Tokyo, Japan). Back-pressure (4 atm) was applied by a restrictor (Varian, Sunnyvale, CA, U.S.A.) to avoid the formation of bubbles in the detector. Retention times for milrinone and the internal standard were 4.3 and 5.5 min, respectively.

Human studies

The haemodynamic responses to stepped intravenous doses of milrinone (as the lactate salt) were studied in normal male volunteers and congestive heart failure (CHF) patients. The study protocol was approved by the Alfred Hospital Clinical Investigations (Ethics) Committee. All subjects were accepted into the study after giving their informed consent.

TABLE I

SEQUENTIAL BOLUS INJECTIONS AND INFUSION RATES OF MILRINONE TO GIVE FOUR SEQUENTIAL, STEADY-STATE PLASMA CONCENTRATIONS OF MILRINONE IN NORMAL SUBJECTS

Each infusion was given for 10 min.

Dose	Calculated concentration (ng/ml)	Bolus injection (μg/kg)	Infusion rate (μg/kg/min)
1	59.9	6.25	0.73
2	122	6.25	1.46
3	249	12.5	2.92
4	505	25.0	5.84

The dose regimen was calculated to give four sequential, steady-state, plasma concentrations of milrinone equivalent to 6.25, 12.5, 25 and 50 μg/kg. These concentrations were obtained by a combination of intravenous bolus injections followed by constant infusion for 10 min (Table I). The calculations used the pharmacokinetic data determined by Stroshane and co-workers for normal subjects [14] and cardiac heart failure patients [15] and fitted them to the biexponential equation $C = Ae^{-\alpha t} + Be^{-\beta t}$ where C equals the plasma concentration of milrinone at time t, A and B are the ordinate intercepts and α and β are the first-order rate constants for the fast- and slow-elimination phases. From these parameters, the four bolus injections and infusion rates were calculated using the equations of Shand et al. [16].

Statistical methods

All data are presented as mean ± standard deviation (S.D.) or standard error of the mean (S.E.M.).

RESULTS AND DISCUSSION

The wide applicability of the C_{18} (ODS) bonded phase in HPLC provides an

indication of its potential as an initial separative technique. When applied to the extraction of the bipyridine compound, milrinone, from plasma, improved methodology is the result. The method described herein shows simplification and a shorter assay time when compared to a solvent extraction technique [13]. At the same time, it shows similar recovery, accuracy and precision to the solvent method.

Sample chromatograms for standard and extracted control plasma, plasma sample and standard are shown in Fig. 2. Milrinone and the internal standard were well resolved from contaminating peaks in the normal subject and the cardiac heart failure patient plasmas. The following drugs, when administered to CHF patients, did not interfere in the assay: frusemide, cyclopenthiazide, captopril, mexiletine hydrochloride, glyceryl trinitrate, quinine sulphate, heparin and diazepam.

Recoveries of milrinone from 1 or 2 ml of plasma were similar and averaged 87%. Recovery of the internal standard similarly was 91%.

Calibration curves were linear and reproducible over the range 0–1000 ng/ml with mean ± S.E.M. values for the constants in the regression equation of $y = (0.0025 \pm 0.0009)x + (0.0064 \pm 0.0065)$ and with correlation coefficient of 0.999 ($n = 12$).

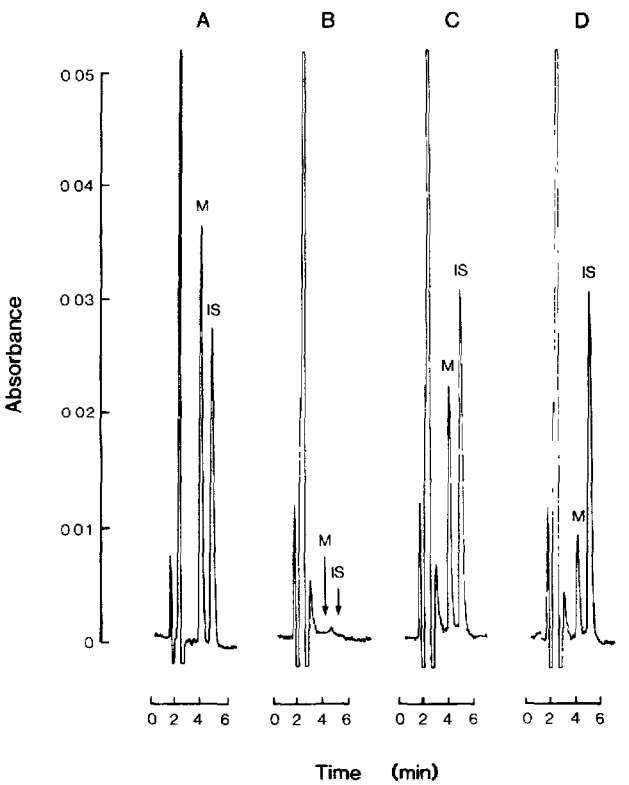

Fig. 2. Chromatograms of milrinone (M) and internal standard (IS). (A) Standards; (B) extract of normal subject control plasma; (C) extract of control plasma containing 250 ng milrinone standard; (D) extract of subject plasma following the 6.25 μg/kg dose of milrinone and equal to a plasma concentration of 28.2 ng/ml.

TABLE II

ACCURACY AND PRECISION OF THE ASSAY

Added concentration (ng/ml)	Obtained concentration (mean ± S.D.) (ng/ml)	*n*	Coefficient of variation (%)
1000	1001 ± 22	6	2.2
100	100 ± 1.3	6	1.3
10	9.76 ± 0.28	5	2.8

TABLE III

PLASMA MILRINONE CONCENTRATIONS IN FIVE NORMAL SUBJECTS FOLLOWING FOUR STEPPED, INTRAVENOUS DOSES OF MILRINONE

Dose	Calculated concentration (ng/ml)	Obtained concentration (ng/ml)						
		1	2	3	4	5	Mean	S.E.M.
1	59.9	77.7	50.2	55.4	28.2	41.7	50.6	8.2
2	122	167	106	123	104	100	120	12.4
3	249	305	197	307	227	210	249	23.7
4	505	642	555	698	466	483	569	44.8

Intra-assay precision and accuracy were determined by replicate analysis of control plasma to which 1000, 100 or 10 ng/ml milrinone was added. The results are shown in Table II.

The lower limit of sensitivity of the assay was 5 ng/ml using 2 ml of plasma. For increased sensitivity, 4 ml of plasma could be extracted without adverse effect on assay parameters.

Analytical results for the human study in which milrinone was administered to normal subjects are shown in Table III. Results for the CHF patients will be the subject of a later communication.

The close correlation between the calculated plasma milrinone concentrations derived using the pharmacokinetic parameters determined using the solvent extraction assay [13] and those obtained in our study (r^2 = 0.998, Table III), verifies the selectivity of the solid-phase extraction methodology.

In summary, use of C_{18} bonded silica gel, solid-phase columns has been successfully applied to the initial extraction step of the new drug, milrinone, from plasma. The C_{18} columns simplified the assay and reduced assay time, whilst at the same time maintaining the accuracy, precision and recovery of a conventional solvent extraction technique.

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