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Short communication

High performance liquid chromatography using UV detection for the quantification of milrinone in plasma: Improved sensitivity for inhalation

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ABSTRACT

An improved analytical assay was developed and validated for the quantification of milrinone concentrations in patients undergoing cardiac surgery. A solid-phase extraction was optimized to isolate milrinone from a plasma matrix followed by HPLC using UV detection. Plasma samples (1 ml) were extracted using a C_{18} solid-phase cartridge. Milrinone was separated on a strong cation exchange analytical column maintained at 23.4 °C. The mobile phase consisted of a gradient (10:90 to 45:55), 0.05 M phosphate buffer (pH 3):acetonitrile. Calibration curves were linear in the concentration range of 1.25–320 ng/ml. Mean drug recovery and accuracy were respectively ≥96% and ≥92%. Intra- and inter-day precisions (CV%) were ≤6.7% and ≤7.9%, respectively. This method proved to be reliable, specific and accurate. Using different types of column for extraction and separation of milrinone proved to be necessary to achieve the sensitivity and specificity required when milrinone is given by inhalation.

1. Introduction

Intravenous milrinone is commonly used to improve ventricular function after cardiac surgery by reducing pulmonary hypertension but is associated with systemic hypotension and increased vasoactive drug requirements [1]. Nebulized vasodilators allow targeted drug delivery, high local concentrations and less systemic hypotension [2]. Indeed, administration of milrinone by inhalation after cardiopulmonary bypass proved to be an alternative mean of reducing pulmonary hypertension while avoiding systemic hypotension [3,4]. More recently, a retrospective analysis suggested a preventive effect when inhaled milrinone is administered before cardiopulmonary bypass [5]. The pharmacokinetics of inhaled milrinone have not been characterized yet and lower plasma concentrations are expected. Although the therapeutic window for intravenous milrinone ranges from 100 to 400 ng/ml [6], preliminary results in patients given inhaled milrinone before cardiac surgery indicate plasma concentrations below 10 ng/ml after weaning from cardiopulmonary bypass.

Analytical assays used for the determination of milrinone in human plasma have been published suggesting different extraction methods prior to HPLC analysis [6–8]. These assays included either a double liquid–liquid or solid-phase extraction (SPE) prior to HPLC using a C_{18} analytical column and UV detection. Pharmacokinetics studies on milrinone most often used the method of Edelson et al. [7] that combined double liquid–liquid extraction with a back extraction. Oddie et al. [8] used SPE while a direct precipitation prior to SPE was added as an additional step by Woolfrey et al. [6]. Finally, two other studies proposed a slight modification of Edelson et al.'s method [9,10]. Most assays report a lowest limit of quantification (LLOQ) of 5 ng/ml except two of them where a 1 ng/ml LLOQ is reported [9,10]. Only one report [10] included a detailed validation and when trying to reproduce it, using 1 ml instead of 0.1 ml human plasma, endogenous interferences were such that the lowest sensitivity achieved with our detector was 10 ng/ml.

The aim of the present work was to optimize existing methods in order to reach a higher sensitivity that would be required for the quantification of milrinone given by inhalation in cardiac surgical patients.

2. Experimental

2.1. Chemicals and reagents

Milrinone, 1,6-dihydro-2-methyl-6-oxo[3,4′-bipyridine]-5-carbonitrile, was kindly supplied by Sandoz (Boucherville, QC, CAN). Amrinone, the internal standard (I.S.), 5-amino-3,4′-bipyridyl-6(1H)-one, was purchased from Sigma (St. Louis, MO, USA). HPLC grade solvents and reagents (Fisher Scientific, Nepean, ON, CAN)

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were filtered through a 0.2-μm Type HVLP membrane (Millipore, Billerica, MA, USA) before use. HPLC grade buffer salts were obtained from American Chemical Ltd. (Saint-Laurent, QC, CAN). Ultrahigh purified water was obtained from Milli-Q water dispensing system by Millipore Corporation (Billerica, MA, USA).

2.2. Standard solutions and buffers

Stock solutions (1mg/ml) of pure milrinone standard were prepared in methanol and stored at −20 °C. Working solutions (10 μg/ml water) were prepared extemporaneously. A stock solution of amrinone (100 μg/ml) was prepared in methanol and stored at −70 °C. The working solution (2.5 μg/ml water) was prepared daily.

The neutralizing solution of 1 M ammonium acetate was prepared in water. The eluting solution of MeOH–HCl (0.02 mol/l) was prepared by adding a concentrated solution of HCl to pre-filtered methanol. Tetrahydrofuran (THF) was added to 0.05 M NaH_2PO_4 buffer (pH 3) immediately before HPLC analysis to obtain the final THF–NaH_2PO_4 buffer (21:916, v/v).

2.3. Equipment and analytical method

HPLC analysis was performed on a Hewlett Packard 1100 series HPLC system (Wilmington, DE, USA) equipped with a multiple solvent delivery system and a variable wavelength UV/visible detector. Chromatographic separations were carried out on a Spherisorb strong cation exchange (SCX) column (15.0 cm × 4.6 mm, 5 μm; HiChrom Ltd., Reading, Berkshire, UK) protected by a security cartridge system (Upchurch Scientific Inc., Oak Harbor, WA, USA). Mobile phase consisted of a mixture of ACN:THF–NaH_2PO_4 buffer delivered at 1 ml/min using a linear gradient from 10:90 to 45:55 for the first 10 min and maintained at 45:55 up to 13 min.The column was kept at 23.4 °C and run time was set at 13 min with 2 min post-run time. Injection volume was 100 μl and UV detection at 340 nm. Chromatographic peaks were integrated by the ChemStation software (Version A.09.01, Agilent Technologies, Santa-Clara, CA, USA).

2.4. Plasma calibration curves

Nine concentrations of milrinone ranging from 320 to 1.25 ng/ml (LLOQ) were used to establish plasma calibration curves. Calibration samples were prepared extemporaneously by serial 1:1 dilutions with previously screened blank plasma.

Quality control (QC) samples were prepared by spiking blank plasma with milrinone stock solutions to obtain final concentrations of 7.5 and 100 ng/ml.

Calibration curves were generated from the nine plasma calibration samples by plotting analyte/I.S. peak–height ratio against milrinone concentration. Linearity was assessed using a weighted least square regression ($1/x^2_{\text{nominal}}$).

2.5. Plasma samples preparation

Varian Bond Elut® C_{18} reversed-phase sorbent (3 cm^3/100 mg) SPE cartridges (Lake Forest, IL, USA) were used to isolate milrinone from plasma samples. To 1 ml of plasma standard/sample, 50 μl of I.S. (125 ng) was added and 1 ml of 1 M ammonium acetate was added to neutralize pH. The solid-phase extraction cartridges were pre-conditioned with methanol (2 × 1 ml), water (2 × 1 ml) and 1 M ammonium acetate (2 × 1 ml). Then, plasma mixture was deposited and slowly aspirated through sorbent bed. Cartridges were then washed with water (3 × 1 ml) before recovery of the analyte into a glass tube using 1 ml of eluting solution. Extracts were dried at 50 °C under a gentle nitrogen stream and reconstituted with 0.2 ml of mobile phase. Each tube was vortexed for 30 s before transfer of its content into clean, capped 1.5 ml disposable conical polypropylene tubes (Ultident Scientific, Saint-Laurent, QC, CAN). Samples were then centrifuged at 15 850 × *g* for 5 min to remove any particulate matter before injection onto the HPLC column.

2.6. Clinical application

Milrinone plasma concentration–time profile were determined in a few cardiac patients after a 5 mg dose (Primacor, Sanofi-Synthelabo Canada Inc., Markham, ON, CAN) inhaled over a 15 min period. Informed and written consent was obtained from subjects. Blood samples were obtained before starting inhalation (time zero), at 20, 25, 30 min thereafter, and immediately after cardiopulmonary bypass. Samples were kept in an ice-water bath for less than 10 min before centrifuging at 2000 × *g* for 5 min. Plasma was immediately flash-frozen on dry ice. Samples were then stored at −70 °C until analyzed.

2.7. Bioanalytical validation

2.7.1. Specificity

Blank plasma samples from six healthy volunteers were assayed to determine whether endogenous plasma components interfere with the analyte or I.S.

2.7.2. Linearity

Nine calibration standards (1.25–320 ng/ml) covering the expected clinical range were prepared in plasma.

2.7.3. Sensitivity

Milrinone at a concentration of 1.25 ng/ml in plasma was extracted and injected on five different days (inter-day). The limit of detection (LOD) of milrinone in the mobile phase was also determined.

2.7.4. Recovery

Six replicate sets of samples spiked at 7.5 and 100 ng/ml concentrations were prepared. Recovery was assessed by comparing the peak height of milrinone spiked prior and after extraction. Recovery of the I.S. was also determined by comparing the peak heights of six extracted samples with the 100% value determined using *in vitro* samples.

2.7.5. Precision and accuracy

The intra-assay precision and accuracy were assessed in plasma as follows: 7.5 100 ng/ml QC concentrations were assayed in replicates of 6. All samples were assayed on the same day and their back-calculated concentrations determined from the calibration curve prepared the same day.

Inter-assay precision and accuracy were assessed as follows: 7.5 and 100 ng/ml QC concentrations were assayed in duplicate for each calibration curve. Five calibration curves with their respective QC samples were assayed over five different days.

Precision was expressed as the coefficient of variation (CV, %) and accuracy as the percent bias (%). Accuracy was determined by comparing the calculated concentration of the extracted milrinone plasma standard with the nominal concentration of milrinone.

2.7.6. Stability

Short-term stability. Bench stability was also verified in duplicates of plasma standards at 7.5 and 100 ng/ml thawed at room temperature and kept at this temperature for 24 h before analysis.

Freeze-thaw cycles. Two aliquots of 7.5 and 100 ng/ml were prepared and frozen at –70 °C for 24 h. The samples were thawed unassisted at room temperature and analyzed. The samples were

refrozen for 24 h under the same conditions. The freeze-thaw cycle was repeated one more time before reanalysis.

Processed samples stability. Stability of extracts reconstituted with mobile phase stored in the autosampler at room temperature for 24 h was tested. Results were compared with those obtained for the freshly prepared samples.

Long-term stability. Long-term stability of milrinone in plasma stored at −70 °C was studied at two plasma concentrations (7.5 and 100 ng/ml). Samples were assayed in duplicate on the day of preparation (day 0), one month and two months later. Long-term stability of aqueous standard solutions was also evaluated at regular intervals. The samples were analyzed every two weeks and were compared with freshly prepared standards.

3. Results and discussion

3.1. Optimization of the method

In preliminaries, milrinone was determined by HPLC using a C_{18} analytical column after direct precipitation of plasma, according to the method described by Lindsay et al. [9] In our conditions, sensitivity of the assay proved to be 10 ng/ml. After analysis of milrinone plasma concentrations in the first patient, it became obvious that this method was not sensitive enough for this route of administration and that plasma constituents often interfered. Several approaches were tried to overcome this problem.

First, Lindsay's procedure was further optimized by using solid-phase extraction on a Bond Elut C_{18} reversed phase sorbent cartridge [8] prior to HPLC instead of direct precipitation [9] for a better sample clean up. Prior neutralization of the plasma and cartridges at pH 7 was necessary to retain milrinone on the cartridge otherwise the extraction recovery was very low. Acidified methanol was used for elution. Therefore, the retention mechanism seems not to be only reverse-phase one but at least partially ionic one.

Then, variations of the mobile phase properties were tested. Retention times of milrinone and I.S. on the C_{18} column proved to depend highly on the pH and ionic strength of the mobile phase. Without any change in the sensitivity, faster elution was achieved by increasing the ionic strength from 0.005 to 0.05 M of NaH_2PO_4 in buffer and adjusting the pH from 6.5 to 3. Under the same HPLC conditions, better chromatographic separations were obtained by using a SCX column, and faster elution could be achieved by increasing the ionic strength and the pH of buffer. However, use of a gradient proved to be essential for an optimal elution time of both compounds while avoiding interfering peaks. The run time for this assay is longer than previous methods because interfering peaks were consistently before milrinone. Milrinone peak was monitored at different wavelengths and, in our conditions, 340 nm offered the best sensitivity.

Using these modifications, an excellent recovery and a higher sensitivity (1.25 ng/ml) were achieved.

3.2. Assay validation

3.2.1. Specificity

Chromatograms of (a) blank plasma and (b) milrinone spiked plasma used for the calibration curve, and (c) plasma sample obtained in a cardiac patient 5 min after the end of nebulization of 5 mg milrinone over 15 min are shown in Fig. 1. Variations in day-to-day elution times were the followings: Milrinone was generally eluted between 8.1 and 8.5 min and the I.S. was eluted between 11.1 and 11.5 min.

Blank plasma obtained from healthy volunteers (not necessarily in the fasted state) used for each calibration curve and plasma sampled for each patient undergoing cardiac surgery prior to milrinone administration were analyzed and shown to be free of co-extracted endogenous interference. Several drugs are administered for patient's comfort before surgery and during anesthesia induction (lorazepam, morphine, midazolam, sufentanil, pancuronium) as well as during surgical procedure in order to maintain patient's stability. Hence, those drugs are not taken by healthy volunteers, which can explain that disturbances in the patient sample are different from blank and spiked samples.

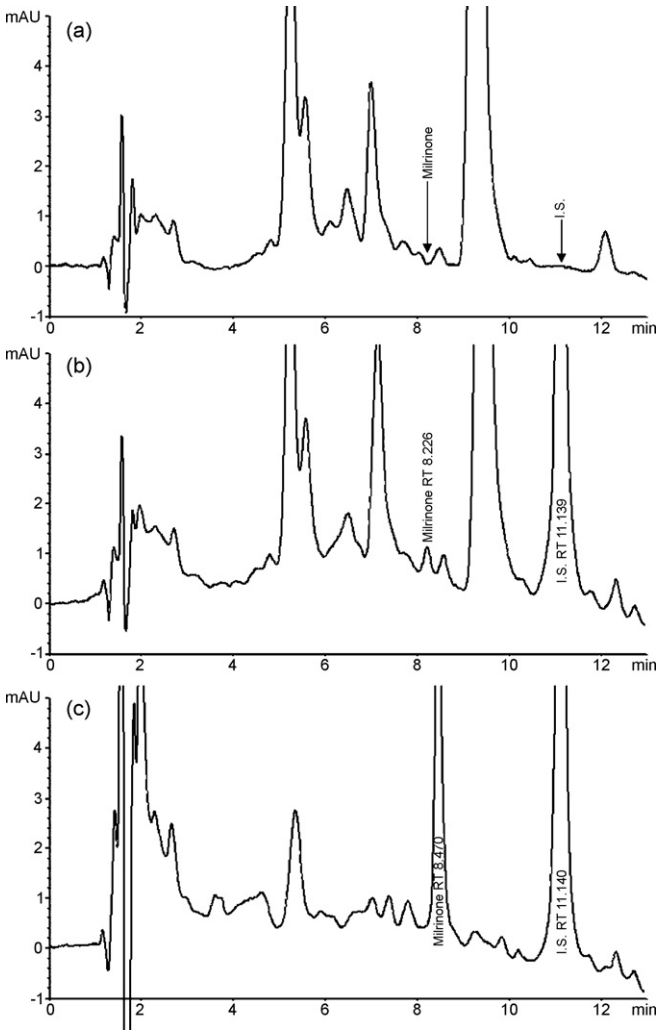

Fig. 1. Typical HPLC chromatograms after extraction of (a) blank plasma, (b) plasma sample spiked with 2.5 ng/ml of milrinone and I.S., and (c) plasma measured with 42.7 ng/ml of milrinone obtained in a cardiac patient 5 min after the end of 15 min inhalation of 5 mg of milrinone.

3.2.2. Linearity

Calibration curves of milrinone in plasma were linear from 1.25 to 320 ng/ml with a mean average correlation coefficient (r^2) of 0.9938 ($n = 5$; range: 0.9908–0.9974).

3.2.3. Sensitivity

Mean inter-assay precision and accuracy for the LLOQ (1.25 ng/ml) were 6% and 98%, respectively ($n = 5$). Intra-assay precision was not evaluated at LLOQ. The LOD in aqueous solution was 0.6 ng/ml.

3.2.4. Recovery

Mean recoveries of milrinone for the 7.5 and 100 ng/ml concentrations were 96 ± 3% and 104 ± 9% ($n = 6$), respectively. The mean recovery of I.S. was 100% (CV < 13%, $n = 6$).

Table 1
Intra-assay and inter-assay precision and accuracy.

	Concentration (ng/ml)			Precision C.V. (%)	Accuracy bias (%)
	n	Actual	Observed mean ± S.D.		
Intra-assay	6	7.5	6.9 ± 0.5	6.7	−8.1
	6	100	98.8 ± 6.3	6.4	−1.2
Inter-assay	5	1.25	1.23 ± 0.07	5.7	−1.6
	5	7.5	7.5 ± 0.6	7.9	0.1
	5	100	102.0 ± 5.0	4.9	2.0

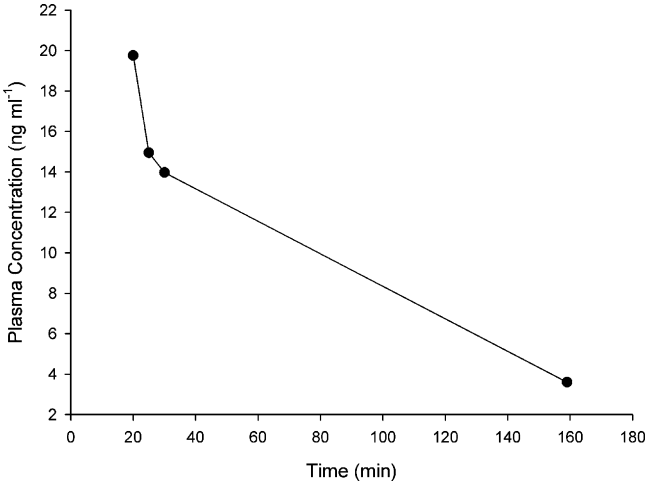

Fig. 2. Milrinone plasma concentration–time profile in a cardiac patient after inhalation of a 5 mg dose over 15 min. No blood samples were drawn during cardiopulmonary bypass (44–159 min).

3.2.5. Intra-assay and inter-assay precision and accuracy

Intra-assay precision study revealed a CV < 7% ($n = 6$) for milrinone concentrations and accuracy ranged from 92 to 99% (Table 1).

Inter-assay precision study revealed a CV < 8% ($n = 5$) for milrinone concentrations and accuracy ranged from 100 to 102% (Table 1).

3.2.6. Robustness

Minor variations in the method parameters did not have any effect on experimental results.

3.2.7. Stability

Short-term stability. Accuracy ranged from 108 to 117%.

Freeze and thaw cycles. Accuracy ranged from 92 to 113% after two freeze thaw cycles.

Processed samples stability. Accuracy following storage of reconstituted extracts in the autosampler varied between 91 and 109%.

Long-term stability. Milrinone in plasma stored at −70 °C was shown to be stable for at least two months. Standard solutions were found stable at least 5 months when stored at −20 °C and accuracy was 99.9% (w/w).

4. Application of the method

The level of sensitivity obtained from this analytical method (1.25 ng/ml) proved to be adequate for the characterization of the concentration–time profile of inhaled milrinone in a few cardiac patients after weaning from cardiopulmonary bypass. Pre-dose plasma sample was free of endogenous or drug interferences under the anesthetic procedure. Plasma concentration–time profile of milrinone for this patient was comprised between 19.8 ng/ml (20 min) and 3.6 ng/ml (160 min) (Fig. 2).

5. Conclusion

A highly selective HPLC assay with UV detection has been optimized for determination of milrinone in human plasma. This assay provides a specific and reproducible alternative to currently available methods and, to our best knowledge, offers the level of sensitivity required to study the pharmacokinetics of inhaled milrinone.

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References

[1] B.E. Jaski, M.A. Fifer, R.F. Wright, E. Braunwald, W.S. Colucci, J. Clin. Invest. 75 (1985) 643.
[2] S.L. Katz, I. Adatia, E. Louca, K. Leung, T. Humpl, J.T. Reyes, A.L. Coates, Pediatr. Pulmonol. 41 (2006) 666.
[3] A. Haraldsson, N. Kieler-Jensen, U. Nathorst-Westfelt, C.H. Bergh, S.E. Ricksten, Chest 114 (1998) 780.
[4] A. Haraldsson, N. Kieler-Jensen, S.E. Ricksten, Anesth. Analg. 93 (2001) 1439.
[5] Y. Lamarche, L.P. Perrault, S. Maltais, K. Tetreault, J. Lambert, A.Y. Denault, Eur. J. Cardiothorac. Surg. 31 (2007) 1081.
[6] S.G. Woolfrey, J. Hegbrant, H. Thysell, P.A. Fox, D.W. Lendrem, G.F. Lockwood, K. Lasher, J. Rogers, D. Greenslade, J. Pharm. Pharmacol. 47 (1995) 651.
[7] D.S. Baim, A.V. McDowell, J. Cherniles, E.S. Monrad, J.A. Parker, J. Edelson, E. Braunwald, W. Grossman, N. Engl. J. Med. 309 (1983) 748.
[8] C.J. Oddie, G.P. Jackman, A. Bobik, J. Chromatogr. 374 (1986) 209.
[9] C.A. Lindsay, P. Barton, S. Lawless, L. Kitchen, A. Zorka, J. Garcia, A. Kouatli, B. Giroir, J. Pediatr. 132 (1998) 329.
[10] D.R. Brocks, T.J. Spencer, A. Shayeganpour, J. Pharm. Pharm. Sci. 8 (2005) 124.