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Amrinone and *N*-acetylamrinone assay in human plasma using solid-phase extraction and reversed-phase chromatography

SYLVIE LAGANIÈRE* and LYNNE GOERNERT

Bureau of Drug Research, Drugs Directorate, Health Protection Branch, Banting Building, 2 West, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2

Abstract: A simple and sensitive liquid chromatographic assay for simultaneous quantitation of amrinone and *N*-acetylamrinone in human plasma was developed. The method involves extraction of samples via activated solid-phase extraction Bond Elut C_{18} disposable columns, followed by chromatographic separation on a reversed-phase phenyl column using isocratic condition and UV detection. The assay can measure concentrations of both compounds over the range 0.075–10 μg ml^{-1}. The injection interval is 11 min. The inter-day relative standard deviation (RSD) for replicate analysis of spiked samples is less than 10% and the accuracy more than 94% for both compounds over the standard curve range. The assay has been successfully applied to pharmacokinetic studies in humans.

Keywords: *Amrinone; assay; sample handling; reversed-phase chromatography; validation.*

Introduction

Amrinone, a bipyridine derivative, is a non-catecholamine inotropic agent used in the short-term management of severe congestive heart failure and in perioperative cardiac surgery patients with myocardial depression [1]. Preliminary data in human volunteers [2] and patients [3] indicate that amrinone undergoes polymorphic acetylation. A slow metabolizer could be at increased risk from use of amrinone following surgery. Sensitive analytical procedures capable of measuring amrinone and its metabolite *N*-acetylamrinone in human plasma could be useful for determining acetylation phenotype of amrinone in patients. It appears that, only one group has published assays for monitoring amrinone and *N*-acetylamrinone in dog plasma [4] and in human urine [5] by HPLC after liquid–liquid extraction procedure. Their reported limits of quantitation were 0.2 μg ml^{-1} in plasma and 0.1 μg ml^{-1} in urine. Because the sensitivity of the published plasma assay was not sufficient to follow the full kinetic profile of these two analytes, this article describes a sensitive analytical procedure capable of simultaneously determining amrinone and *N*-acetylamrinone from human plasma following solid-phase extraction and reversed-phase liquid chromatography, and reports validation data for a biological assay application.

Experimental

Materials

Amrinone, 5-amino-3,4′-bipyridin-6(1H)-one (AMR), the *n*-acetyl derivative (NAA), *N*-(1,6-dihydro-5-oxo-(3,4′-bipyridin)-5-yl)-acetamide, and the internal standard (IS) 5-amino-1-ethyl-(3,4′-bipyridin)-6(1H)-one HCl were kindly provided by Sterling Drug Inc. (Aurora, Ontario, Canada; Fig. 1). All organic solvents (acetonitrile, tetrahydrofuran and methanol) were of high-purity HPLC grade (Baker or Omnisolv, Ontario, Canada). All other chemicals were of reagent grade. Water was deionized by reversed-phase osmosis and purified by a Milli-Q Waters purification system (Waters, Mississauga, Ontario, Canada).

Glassware preparation

All reusable glassware used for the preparation of drug solutions, were silanized with a freshly prepared 10% solution of Surfasil (Pierce Chemical Co., Rockford, IL, USA) in toluene (in glass distilled, Caledon, Georgetown, Ontario, Canada), rinsed in methanol, dried in the oven at 100°C for 60 min and stored in desiccators.

* Author to whom correspondence should be addressed.

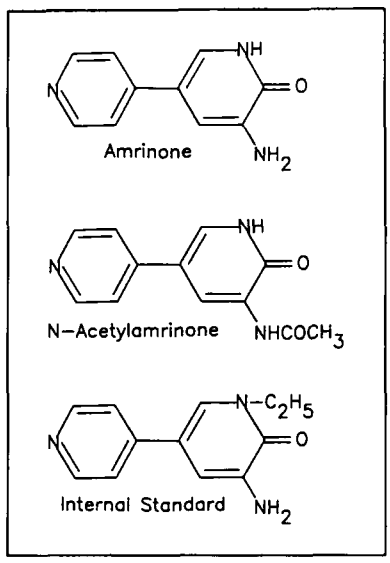

Figure 1
Structural formulae.

Preparation of standards

Amrinone (730 μg ml^{-1}) and *N*-acetylamrinone (460 μg ml^{-1}) stock solutions were prepared in 0.01 N HCl. Solutions were sonicated to allow complete dissolution. Six working standard solutions (range from 1.5 to 200 μg ml^{-1}) were prepared by diluting the stock solutions and adjusting the pH of the solution to 7.5 with concentrated NH_4OH before final adjustment to volume with diluted NH_4OH (pH 7.5). Standard solutions were stable for only 3 weeks at +4°C.

Calibrators were prepared by spiking 1 ml of blank human plasma with 50 μl of each standard solution containing amrinone and *N*-acetylamrinone to achieve final concentrations ranging from 0.075 to 10 μg ml^{-1}.

The IS is first prepared in 0.01 N HCl to dissolve the drug, then the pH is adjusted to 7.5 with concentrated NH_4OH, and diluted NH_4OH (pH 7.5) is added to reach final volume.

Solid-phase extraction procedure

A 1 ml aliquot of human plasma (patient's specimen or blank plasma) was placed in 13 × 100 mm glass tubes (Maple Leaf, John's Scientific, Toronto, Ontario, Canada). Outdated blank plasma was used for method validation and standard curves (Ottawa Civic Hospital or the Ottawa Red Cross). A 50 μl IS solution (5 μg) was added to all samples except the blank which received 50 μl of diluted NH_4OH (pH 7.5). A 50 μl aliquot of AMR and NAA standard solutions was added to standard curve and quality control samples. One millilitre of isotonic phosphate buffer solution (PBS; pH 7.4) was added and tubes vortexed.

Disposable solid-phase extraction columns were selected (Bond Elut C_{18}, 3cc, 500 mg from Varian, Mississauga, Ontario, Canada) for the extraction procedure. Prior to application, each column mounted on a Lida vacuum manifold apparatus (Wennick Sc., Ottawa, Ontario, Canada) was solvated with 2 × 3 ml of methanol and 2 × 3 ml of water under slight vacuum (5 cm Hg). The column was not allowed to dry. The plasma mixture was added to the column and aspirated through by gravity or slight vacuum. The column was washed with 1 ml of 10% methanol in water (v/v). Gentle vacuum (15 cmHg) is then used for 1 min to remove water. Compounds of interest were eluted with 2 × 500 μl of 1% NH_4OH in methanol solution (v/v, pH 9.5) using gravity or slight vacuum. Then, vacuum was applied (30 cm Hg) for 1 min. Samples were collected in conical silanized glass vials (2 ml; Kimax, Fisher Sc., Nepean, Ontario, Canada), evaporated to dryness under vacuum (2 mmHg; Speedvac, Savant Instruments Inc., Hicksville, NY, USA) at 43°C. Complete evaporation takes about 1.5 h. Residue was dissolved in 220 μl of dilute acetic acid, pH 3.4 (glacial acetic acid in water, 4:500, v/v), then the final pH was adjusted to 7.5 with 30 μl of a 0.5% ammonium hydroxide solution. The reconstituted solution was transferred to a 250 μl flat-bottom insert suitable for the autosampler vial and 50 μl was injected on the HPLC column.

Instrumentation and chromatographic conditions

The chromatographic system consisted of an SP8800 HPLC pump from Spectra Physics (Toronto, Ontario, Canada), an SP8780XR autosampler fitted with a 50 μl sample loop, an SP4290 integrator, and a Perkin–Elmer LC95 UV detector (Montreal, Quebec, Canada) set at 254 nm, 0.5 AUFS, 100 ms.

Chromatographic separation was achieved with a 10 μm μBondapack phenyl column (3.9 × 300 mm, Waters, Mississauga, Ontario, Canada) maintained at 35°C with a Waters Column heater. A precolumn μBondapack phenyl from Waters was used. The mobile phase consisted of phosphate buffer (0.04 M, pH 6)–acetonitrile–tetrahydrofuran–*N*-butylamine (84:13.7:2:0.3, v/v/v/v). The pH of the

mixed solvent system was adjusted to 7.0 with phosphoric acid solution (85%). The mobile phase flow-rate was set at 2 ml min^{-1} (1600 psi). Data were collected on an AT computer (Tatung TCS-7000) equipped with an SP ChromNet Data controller.

Calibration

On each analytical day, a set ($n = 6$) of working standard solutions was analysed in duplicate as well as quality control samples at concentrations of 0.1, 1 and 10 μg ml^{-1} of plasma. Calibration plots were constructed by log–log linear least-squares regression of the peak area ratios of amrinone or *N*-acetylamrinone to that of the internal standard versus plasma concentrations. The intra-day precision and accuracy were evaluated at three plasma concentrations, namely 0.075, 1 and 10 μg ml^{-1} with replicate samples ($n = 9$). Inter-day assay variation was determined at concentrations of 0.1, 1 and 10 μg ml^{-1} with replicate samples ($n = 24$) obtained over a 2-month period.

Stability

The stability of amrinone and *N*-acetylamrinone in plasma was determined with samples spiked with these two analytes at concentrations of 0.1, 1 and 10 μg ml^{-1}. Stability of analytes was tested for (1) long term storage of plasma samples at −80°C over an 8-month period; (2) for unprocessed plasma samples left at ambient temperature (20°C) for 24 h; (3) for run-time stability of processed samples, in reconstituted solutions after extraction, left at ambient temperature (20°C) for 24 h prior to injection on the HPLC; and (4) for two freeze–thaw cycles of spiked plasma samples.

The stability assessment was based on data from duplicates of test samples spiked at three different concentrations compared with duplicates of plasma samples freshly spiked with the analytes. Amrinone and *N*-acetylamrinone were considered stable over time if the 90% confidence limits around the geometric mean difference, expressed relative to the control samples fell within the interval from −10 to 10%.

Results and Discussion

Assay validation

The extraction recovery was determined by comparing peak areas for plasma extracts ($n = 9$) with those from directly injected solutions ($n = 5$). The recovery was 87.0 ± 6.1 and 93.6 ± 0.8% for AMR, 96.9 ± 5.6 and 93.9 ± 1.1% for NAA at 0.075 and 10 μg ml^{-1}, respectively, and 99.3 ± 1.1% for the IS at 5 μg ml^{-1}.

Calibration curves for amrinone were linear over the concentration range 0.075–10 μg ml^{-1} (mean r^2 of 0.9996, root mean square error for four curves was 0.0396, using the log–log linear regression analysis, the slope being not significantly different from 1). The mean intra-day accuracy defined as mean per cent deviation from nominal value and the mean RSD for precision are listed in Table 1. The inter-day accuracy and mean RSD for precision obtained over a 2-month period are presented in Table 2. The limit of quantitation determined from four standard curves was 0.079 μg ml^{-1} based on the 95% two-sided upper prediction interval for a predicted Y ($\hat{Y}_i$) at a particular concentration (X_i)

Calibration curves for *N*-acetylamrinone were linear over the concentration range of 0.075–10 μg ml^{-1} (mean r^2 of 0.999, root mean square error for four curves was 0.065), using the log–log linear regression analysis. Validation data on the intra- and inter-day accuracy and precision can be found in Tables 1 and 2. The limit of quantitation determined from four standard curves was 0.085 μg ml^{-1}.

Specificity

Blank plasma was free to endogenous contaminants at the retention times of 4.67, 6.49 and 10.40 min corresponding to amrinone, *N*-acetylamrinone and the IS, respectively. Figure 2 shows typical chromatograms of extracts of blank human plasma, of a plasma sample spiked with amrinone and *N*-acetylamrinone, and of a patient's sample containing 1.4 μg of amrinone and 1.1 μg of *N*-acetylamrinone per ml of plasma.

Drugs that are likely to be administered concurrently with amrinone, either before, during the surgery or to stabilize the patient's condition following surgery were subjected to the entire analytical procedure to evaluate potential interferences. These drugs are: acetaminophen, allopurinol, captopril, cephalothin, diazepam, digoxin, dimenhydrate, dopamine, enalapril, lorazepam, morphine, nifedipine, ranitidine, lasix and warfarin. None was found to interfere with the

Table 1
Intra-day assay precision and accuracy

Actual conc. ($\mu g\ ml^{-1}$)	Precision: Mean calculated conc. (n = 9)	Precision: RSD (%)	Accuracy: Mean deviation from nominal (%)
Amrinone			
0.075	0.069	6.4	−7.7
1.00	1.063	1.0	+6.3
10.00	9.461	1.0	−5.4
N-acetylamrinone			
0.075	0.076	6.7	+0.9
1.00	1.072	1.9	+7.2
10.00	9.084	1.0	−11.0

Table 2
Inter-day assay precision and accuracy*

Actual conc. ($\mu g\ ml^{-1}$)	Precision: Mean calculated conc. (n = 24)	Precision: RSD (%)	Accuracy: Mean deviation from nominal (%)
Amrinone			
0.10	0.096	5.4	+4.2
1.00	0.98	4.7	−1.6
10.00	9.46	3.6	−5.4
N-acetylamrinone			
0.10	0.095	9.1	+4.9
1.00	0.99	3.5	−0.8
10.00	9.66	4.3	−3.4

*Obtained over 2 months.

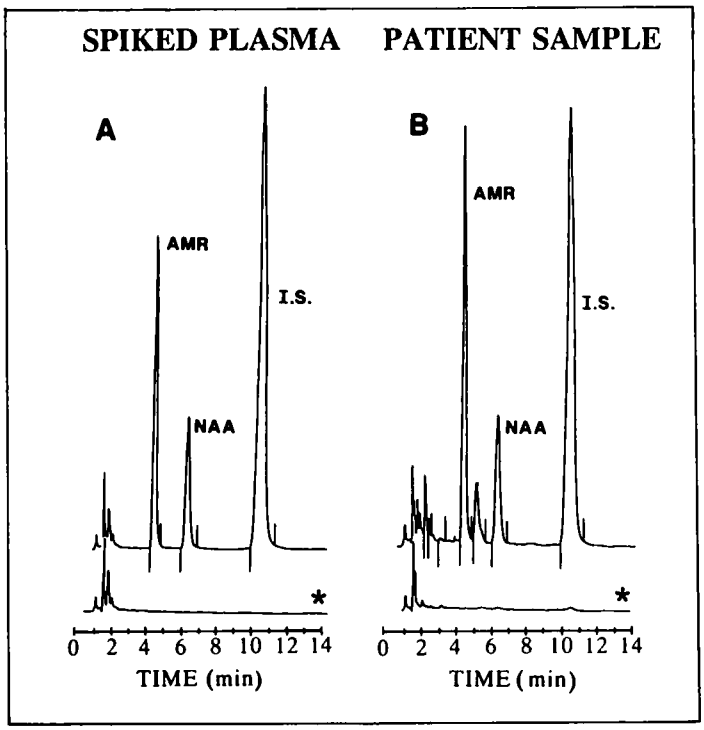

Figure 2
Chromatograms of human plasma extracts. (A, upper) plasma spiked with 1 $\mu g\ ml^{-1}$ of amrinone (AMR) and *N*-acetylamrinone (NAA). (B, upper) patient's sample containing 1.4 and 1.1 $\mu g\ ml^{-1}$ of amrinone and *N*-acetylamrinone after amrinone intravenous administration, and blank plasma samples (*, A and B lower). The amount of internal standard (IS) added to the sample was 5 μg. Experimental conditions as stated in the text.

retention times of our compounds of interest.

Stability

There was no significant difference in the measurements of amrinone and *N*-acetylamrinone when plasma samples were stored frozen in glass compared with those from polypropylene tubes. No significant degradation of the analytes was observed for spiked human plasma samples stored at −80°C over an 8-month period. Amrinone and *N*-acetylamrinone were considered stable over time (ANOVA, respective P of 0.33 and 0.45) with overall mean RSD for observed concentrations of 6.6 and 6.4%. The 90% confidence limits around the geometric mean difference, expressed relative to the control samples fell within the confidence interval from −10 to 10%. In addition to the long-term stability study, the analytes were also found to be stable under all the conditions listed above with an overall mean RSD for observed concentrations under fresh and study conditions being less than 7%.

Biological application

The method has been applied successfully to the analysis of several hundred specimens of human plasma obtained during and after discontinuation of intravenous amrinone given by bolus (1.5 mg kg^{-1}) followed by infusion of 10 μg kg^{-1} min^{-1} for 24 h, in post-cardiac surgery patients with poor ventricular function. A typical chromatogram of patient plasma extract is shown in Fig. 2. The sensitivity of this assay allows monitoring of drug concentrations over the therapeutic range (2–4 μg ml^{-1}), monitoring of the metabolite *N*-acetylamrinone, determination of the amrinone acetylation phenotype based on the ratio of amrinone to *N*-acetylamrinone and allows, in fast and slow metabolizers, their respective pharmacokinetic disposition to be followed over several half-lives.

Conclusion

The analytical method reported is specific, very sensitive and reproducible. The low limit of quantitation of 0.079 μg ml^{-1} for amrinone and of 0.085 μg ml^{-1} for *N*-acetylamrinone has allowed us to apply the method to clinical pharmacokinetic studies.

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