Journal of Chromatography, 187 (1980) 264–270
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CHROM. 12,286

Note

High-performance liquid chromatographic analysis of amrinone and its N-acetyl derivative in plasma

Pharmacokinetics of amrinone in the dog

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(Received August 6th, 1979)

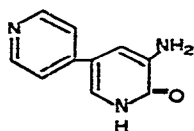
Amrinone (Inocor, Winthrop Labs., New York, N.Y., U.S.A.), 5-amino-3,4'-bipyridin-6(lH)-one, is a novel cardiotonic agent which has demonstrated inotropic activity in both laboratory animals[1,2] and man[3–5]. The drug exerts its activity after either oral or intravenous administration, by a mechanism that does not appear to involve the receptors responsible for the activity of either the catecholamines or the cardiac glycosides[1,2]. This potentially useful therapeutic agent is currently undergoing extensive clinical trials.

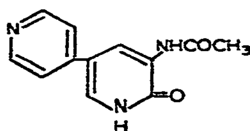
This report describes a high-performance liquid chromatographic (HPLC) method for the quantitation of both amrinone and its N-acetyl derivative, N-(1,6-dihydro-5-oxo-[3,4'-bipyridin]-5-yl)acetamide (I), in plasma. The assay was employed to quantitate amrinone and I in the plasma of dogs who had received amrinone by intravenous administration. The plasma concentration data was used to calculate pharmacokinetic parameters for amrinone.

EXPERIMENTAL

Materials

Amrinone, its N-acetyl derivative (I), and the internal standard, 5-amino-1-ethyl-[3,4'-bipyridine]-6(lH)-one, (Fig. 1) were synthesized at Sterling-Winthrop Research Institute. The HPLC column was purchased from Waters Assoc. (Milford, Mass., U.S.A.). The acetonitrile was redistilled in an all-glass distillation apparatus. Other chemicals were obtained commercially (reagent grade) and used without further purification.





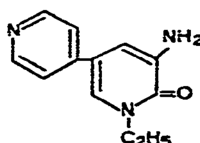


Fig. 1. Structural formulae.

Assay tube preparation

All assay tubes used in this procedure were silanized by allowing them to stand filled with a 5% solution of dichlorodimethylsilane (Applied Science Labs., State College, Pa., U.S.A.) in toluene for approximately 30 min. The tubes were then rinsed twice with toluene and once with methanol. After draining, the tubes were dried in an oven set for approximately 110°.

Preparation of plasma standards

Standard solution of amrinone and I were prepared in 0.01 *N* HCl. Appropriate volumes of these solutions were pipetted into 1 ml of control normal human plasma (sodium oxalate as anticoagulant) to produce plasma concentrations 0 and 0.10 to 5.0 μg/ml of both amrinone and I. Standards were freshly prepared, extracted and analyzed along with each set of samples.

Preparation of validation samples

Plasma samples (1.0 ml) were prepared in coded tubes such that there were two sets of triplicate samples at each of six concentrations. Each sample contained both amrinone and I. One set of triplicate samples was assayed upon preparation, the other was stored in a laboratory freezer before analysis. The validation samples were analyzed under single-blind conditions.

Assay procedure

To 1 ml of plasma in a silanized tube, were added 50 μl of a 0.01 *N* HCl solution of the internal standard, (40 μg/ml) approximately 1.5 g of $(NH_4)_2SO_4$, and 10 ml of ethyl acetate (Burdick & Jackson, Muskegon, Mich., U.S.A.). The tube was placed on a vortex mixer 15–20 sec, shaken vigorously for 15 min on a mechanical shaker and centrifuged. The ethyl acetate was transferred into a clean silanized tube and 300 μg of 1 *N* HCl were added. The stoppered tube was placed on a vortex mixer for 15–20 sec and centrifuged. The ethyl acetate was discarded. The tube was placed in a water bath (about 10°) for about 5 min and any remaining ethyl acetate was evaporated with the aid of a stream of nitrogen. The aqueous solution was neutralized to a pH of 7–9 (as indicated by wide-range pH paper) with 40–50 μl of concentrated NH_4OH. A 50-μl aliquot of the sample was introduced onto the HPLC system described below.

HPLC system

The HPLC system consisted of a Waters Assoc. Model 6000A pump; a Waters Assoc. μBondapak-phenyl column* (30 cm × 3.9 mm I.D.) with a 3 cm × 3.9 mm I.D. pre-column of phenyl Corasil; a Waters Assoc. Model U6K injector; and a Waters Associates Model 440 detector operated at 340 nm. The mobile phase was 86% pH 6.0, 0.4 *M* NaH_2PO_4–Na_2HPO_4 buffer and 14% redistilled CH_3CN (v/v); the flow-rate was 2.0 ml/min, 2000 p.s.i. Relative retention times were for amrinone, 4.0 min; for I, 6.0 min; and for the internal standard, 11.5 min. The temperature was ambient.

* Must be prepared by subjecting to the manufacturer's recommended clean-up procedures prior to use and when the regression analysis of the standards becomes non-linear.

Dog study

Two fasted female beagle hounds weighing between 7–8 kg were suspended in slings which allowed the dogs to remain standing but restrained. The dogs received a 5 mg/kg intravenous bolus dose of amrinone.

Blood samples (0–14 h) were collected through a B-D 21 gauge Longdwel catheter-needle with a Safedwel obturator. The catheter was inserted into the saphenous vein of a hindleg. Following the 14-h sample, the catheters were removed and the dogs were placed in metabolism cages. Subsequent blood samples were collected by venipuncture.

Blood samples were mixed in a tube containing 12.5 mg of potassium oxalate as the anticoagulant. Samples was placed in an ice bath until they were centrifuged. The separated plasma was pipetted into a clean glass tube and frozen at —40° until analyzed.

RESULTS AND DISCUSSION

Fig. 2 depicts representative chromatograms derived from extracted control plasma and from a plasma sample containing amrinone and I. Fig. 3 shows a typical plot of the peak height ratio of amrinone and I to the internal standard *versus* the concentration of amrinone or I added to plasma. A regression analysis of the peak height ratio of the title compounds to the internal standard *versus* the concentration added to plasma showed this relationship was linear for both compounds.

In Table I is presented the statistical summary and the data derived from analysis of the plasma validation samples for amrinone. The accuracy (mean percent-

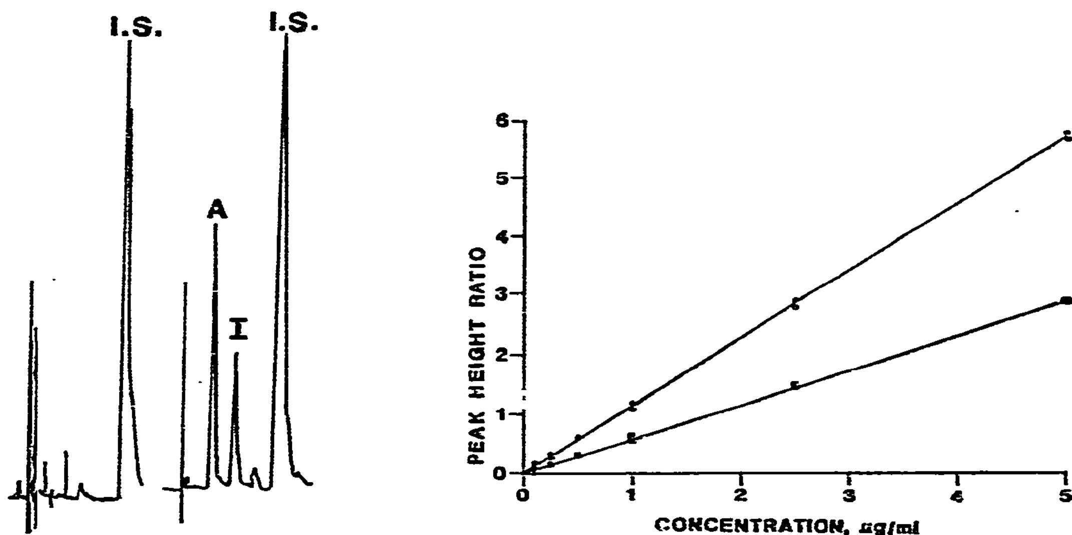


Fig. 2. Left, chromatogram of processed human plasma containing only the internal standard (I.S.). Right, the same sample containing 5 µg/ml of amrinone (A) and 5 µg/ml of the N-acetyl derivative (I).

Fig. 3. Standard curves for amrinone (circles) and the N-acetyl derivative (I, squares) extracted from human plasma.

TABLE I

DATA FROM ANALYSIS OF AMRINONE PLASMA VALIDATION SAMPLES

Concentration level (μg/ml)	*Assayed level (μg/ml)*	
	*Set 1**	*Set 2***
0	<MQL***	<MQL§
	<MQL	<MQL
	<MQL	<MQL
Mean	—	—
S.E. %§§	—	—
Mean % Diff.§§§	—	—
0.20	0.28	0.34
	0.29	0.32
	0.31	0.34
Mean	0.29	0.33
S.E. %	3.0	2.0
Mean % Diff.	47†	67†
0.45	0.49	0.48
	0.46	0.50
	0.49	0.47
Mean	0.48	0.48
S.E. %	2.1	1.8
Mean % Diff.	6.7	7.4
0.80	0.77	0.82
	0.84	0.86
	0.87	0.84
Mean	0.83	0.84
S.E. %	3.6	1.4
Mean % Diff.	3.3	5.0
1.25	1.27	1.22
	1.40	1.22
	1.25	1.29
Mean	1.31	1.24
S.E. %	3.6	1.9
Mean % Diff.	4.5	−0.5
4.00	4.34	4.01
	4.06	4.00
	4.17	4.07
Mean	4.19	4.03
S.E. %	1.9	0.0
Mean % Diff.	4.08	0.6

* Samples analyzed upon preparation.

** Samples analyzed after storage in freezer.

*** MQL = 0.024 μg/ml.

§ MQL = 0.027 μg/ml.

§§ $\text{S.E. \%} = \frac{\text{S.E.M.}}{\text{M}} \times 100.$

§§§ $\text{Mean \% Diff.} = \left(\frac{\text{Assayed mean}}{\text{Concentration level}} - 1\right) 100.$

† These values were high due to 3–4% hydrolysis of a 4 μg/ml concentration of I present in the validation samples (see text).

TABLE II

DATA FROM ANALYSIS OF I IN PLASMA VALIDATION SAMPLES

Concentration level (μg/ml)	*Assayed level (μg/ml)*	
	*Set I**	*Set 2***
0	<MQL***	<MQL§
	<MQL	<MQL
	<MQL	<MQL
Mean	—	—
S.E. %‖	—	—
Mean % Diff.¶	—	—
0.20	0.17	0.19
	0.19	0.20
	0.17	0.19
Mean	0.18	0.19
S.E. %	3.8	1.7
Mean % Diff.	−11.7	−3.3
0.45	0.47	0.46
	0.47	0.46
	0.47	0.46
Mean	0.47	0.46
S.E. %	0.0	0.0
Mean % Diff.	4.4	2.2
0.80	0.88	0.88
	0.88	0.86
	0.88	0.88
Mean	0.88	0.87
S.E. %	0.0	0.8
Mean % Diff.	10.0	9.2
1.25	1.37	1.36
	1.33	1.36
	1.37	1.31
Mean	1.36	1.34
S.E. %	0.1	1.2
Mean % Diff.	8.5	7.5
4.00	4.18	3.88
	4.35	4.38
	4.29	4.40
Mean	4.27	4.22
S.E. %	1.2	5.5
Mean % Diff.	6.8	4.3

* Samples analyzed upon preparation.

** Samples analyzed after storage at −4° for 5 days.

*** MQL = 0.024 μg/ml.

§ MQL = 0.022 μg/ml.

‖ $\text{S.E. \%} = \frac{\text{S.E.M.}}{\text{M}} \times 100.$

¶ $\text{Mean \% Diff.} = \left(\frac{\text{Assayed mean}}{\text{Concentration level}} - 1\right) 100.$

age difference from the expected value) ranged from 0.5% low to 7.4% high for the validation samples between 0.4 to 4 μg/ml. The 0.2 μg/ml samples in the fresh and frozen sets were 47 and 67%, high respectively. The reason for this greater than expected value for amrinone was that I was present in these samples at a concentration of 4 μg/ml. Independant studies have shown that approximately 3–4% of the I is hydrolyzed to amrinone during the extraction process. Two studies were performed to confirm this: first, extraction of plasma samples containing just I produced a linearly, concentration dependent peak for amrinone which represented 3–4% of the amount of I in the samples; second, when samples of amrinone in plasma (0.15 μg/ml) without I present were analyzed in triplicate, the three concentrations determined were 0.19, 0.17 and 0.18 μg/ml. Since the analysis of human and animal samples have shown that concentrations of I are not high with respect to the levels of amrinone, the small amount of hydrolysis of I during the extraction procedure will not affect the quantitation of amrinone. The minimum quantifiable level (MQL) was determined by inverse prediction[6] as that concentration whose lower 80% confidence limit just encompasses zero[7]. The mean MQL for the two experimental runs was 25 ng/ml. The standard deviation (precision) of the amrinone assay was equal to $\pm$ 4.2%, for samples excluding the lower 0.2 μg/ml samples.

Table II summarizes the plasma analysis data for I. The accuracy ranged from 12% low to 9.2% high and the estimated precision of the assay was $\pm$ 4.12%. The average MQL for the two runs was 23 ng/ml. The average ($\pm$ S.E.M.) recovery of amrinone and I from plasma, based on a comparison of peak heights of extracted *versus* direct standards, was 75 $\pm$ 3 and 87 $\pm$ 4%, respectively.

In the dog study, no detectable levels of the N-acetyl derivative were observed

TABLE III

AMRINONE PLASMA CONCENTRATIONS IN DOGS FOLLOWING 5 MG/KG BOLUS INTRAVENOUS ADMINISTRATION

Time (min)	*Amrinone (μg/ml)*		*Time (h)*	*Amrinone (μg/ml)*	
	Dog 1	*Dog 2*		*Dog 1*	*Dog 2*
0	<MQL*	<MQL	1.5	2.5	2.5
1	6.0	11.3	2	2.6	2.3
2	5.1	5.8	3	2.0	1.8
3	4.3	5.5	4	1.3	1.3
4	4.2	4.9	5	1.1	1.0
5	4.5**	4.9	6	0.81	0.82
7.5	4.2	4.2	7	0.44	0.48
10	3.3	4.1	8	0.29	0.42
12.5	4.1	3.5	9	0.21	0.27
15	3.9	3.3	10	0.16	0.22
20	3.6	3.2	11	0.12	0.14
30	3.5	2.6	12	0.11	0.13
45	3.2	2.8	13	0.08	0.11
60	3.6	2.9	14	0.09	0.11
			15	<MQL	0.10
			24	<MQL	<MQL
			28	<MQL	<MQL

* Minimum quantifiable level.

** Data point omitted from pharmacokinetic analyses.

at any time studied. The plasma concentration data for amrinone (Table III) was computer-fitted by a modification of a program[8] for a two-compartment open body model of intravenous drug administration. The kinetic parameters resulting from the analysis are presented in Table IV. The microscopic rate constants, K_{12}, K_{21} and K_{10} suggest that the compound is rapidly distributed into the tissues, and that this process is much faster than the elimination of the amrinone into the urine (K_{10}). The volume of distribution at steady state indicates that the compound is well distributed in the body. In the two dogs the plasma clearance rates were 43 and 47 ml/min and the disposition half-lives during the β-phase were 2.2 and 2.5 h. These values are in agreement with an average plasma elimination half-life of 1.8 ± 0.3 h ($\pm$ S.E.M.) which determined from amrinone plasma concentration data from 12 dogs following termination of a 2.25, 9 or 36 mg/kg 3-h infusion of amrinone.

TABLE IV

PHARMACOKINETIC PARAMETERS DERIVED FROM A TWO-COMPARTMENT OPEN MODEL FOLLOWING i.v. ADMINISTRATION OF AMRINONE TO DOGS

Parameter	*Unit*	*Dog 1*	*Dog 2*
K_{12} Microscopic rate constant, plasma to tissue	h^{-1}	35.5713	10.4984
K_{21} Microscopic rate constant, tissue to plasma	h^{-1}	24.4641	6.8049
K_{10} Elimination rate constant	h^{-1}	0.7761	0.7126
α Distribution rate constant	h^{-1}	60.497	17.74
$t_{\alpha 1/2}$ Half-life, distribution phase	h	0.0115	0.039
Volume of central compartment	ml	3633	3580
Volume of tissue compartment	ml	5283	5524
Volume of distribution, steady state	ml	8917	9105
Plasma clearance	ml/h	2821	2552
β Disposition rate constant	h^{-1}	0.314	0.2733
$T_{1/2\beta}$ Half-life, disposition phase	h	2.208	2.535
A-Constant for α-term		6.5909	6.119
B-Constant for β-term		4.4173	3.6546

In conclusion, an accurate, reproducible and sensitive HPLC assay has been developed for the measurement of the concentration of amrinone and its N-acetyl derivative in plasma. This method was successfully applied to estimate pharmacokinetic parameters in dogs that received intravenous amrinone.

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